Universitat Autònoma de Barcelona

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PhD Thesis

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Bellaterra, 2019

IRTA RESEARCH & TECHNOLOGY FOOD & AGRICULTURE

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Universitat Autònoma de Barcelona

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Tesis doctoral presentada por **Cristina Santamaría Domínguez** para acceder al grado de Doctor en el marco del programa de Doctorado en Medicina y Sanitat Animal de la Facultat de Veterinaria de la Universitat Autònoma de Barcelona, bajo la dirección del **Dr. Nonito Pagès Martínez** y de la **Dra. Núria Busquets Martí** y la tutoría del **Dr. Joaquim Segalés Coma.**

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El **Dr. Nonito Pagès Martínez** y la **Dra. Núria Busquets Martí**, investigadores del *Centre de Recerca en Sanitat Animal* y del *Institut de Recerca i Tecnologia Agroalimentàries* (CReSA-IRTA) como directores de esta tesis, y el **Dr. Joaquim Segalés Coma**, Profesor Titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona e investigador adscrito al CReSA-IRTA, y tutor de esta tesis.

Certifican:

Que la memoria titulada "*Aedes albopictus* as a potential vector of arboviruses in Catalonia", presentada por **Cristina Santamaría Domínguez** para la obtención del grado de Doctor en Medicina y Sanitat Animal, se ha realizado bajo su dirección y supervisión.

Y para que así conste a los efectos oportunos, firman el presente certificado en Bellaterra (Barcelona), el 17 de Septiembre del 2019.

Director — Directora

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Tutor — Doctoranda

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A mis padres y hermanos.

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LIST OF ABBREVIATIONS

Abbreviation	Term
arthropod-borne virus	arbovirus
BSL-3	Biosafety Level 3
CNS	Central Nervous System
Ct	threshold cycle
CHIKV	Chikungunya virus
DDT	Dichloro-Diphenil-Trichloroethane
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DIR	Dissemination infection rate
DMEM	Dulbecco's modified Eagle's medium
dpe	Days post-exposure
dpi	Days post-inoculation
DSS	Dengue shock syndrome
ECSA	East/Central/South African
EIP	Extrinsic incubation period
ENSO	El Niño–Southern Oscillation
ER	Emergence rate
FBS	Fetal bovine serum
FEFs	Fully engorged females
FFU	Focus-forming units
FTA™	Flinders Technical Associates
GEC	Genome-equivalent copies
H&E	Hematoxylin-eosin
i.p.	Intraperitoneally
IFN-α/α R-/-	Alpha/beta interferon receptor-deficient
IR	Infection rate
MBDs	Mosquito-borne diseases
MEB	Midgut escape barrier
MEM	Modified Eagle's medium
MIB	Midgut infection barrier
PBS	Phosphate-buffered saline
PEFs	Partially-engorged females

LIST OF ABBREVIATIONS

Abbreviation	Term
PFU/mL	Plaque-forming units/mL
PR	Pupation rate
RH	Relative humidity
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RT-nPCR	Reverse transcription-nested polymerase chain reaction
RVFV	Rift Valley fever virus
s.c.	Subcutaneously
SGEB	Salivary gland escape barrier
SGIB	Salivary gland infection barrier
SPF	Specific pathogen-free
TCID50/mL	Median Tissue Culture Infectious Dose /mL
TE	Transmission efficiency
TR	Transmission rate
VBDs	Vector-borne diseases
VC	Vector competence
WNL	Within normal limits

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ABSTRACT

The Asian tiger mosquito, *Aedes albopictus*, is widely established in tropical, subtropical, and temperate areas, where is considered a vector for arboviruses like chikungunya and dengue. In the last ten years, autochthonous outbreaks of chikungunya and dengue in Europe have highlighted the risk of arbovirus transmission in areas where *Ae. albopictus* mosquitoes have become established. To estimate the risk of transmission and spread of arboviruses in Catalonia, we investigate whether local mosquitoes are competent to Chikungunya and Dengue viruses (CHIKV and DENV).

In this study, we assessed the susceptibility of a Spanish strain of *Ae. albopictus* for two CHIKV strains (S27 and ITA) at two viral doses (high and low) in environmental conditions mimicking the summer and autumn seasons in Catalonia. These results were compared with previously published data from other European *Ae. albopictus* populations (Chapter III). The pathogenesis of CHIKV was also investigated in mice deficient in the alpha/beta interferon receptor (IFN-α/β R-/-). In order to study CHIKV infection, the mice were injected subcutaneously with three different doses (low, medium and high) of two CHIKV strains (S27 and ITA) (Chapter IV). To evaluate CHIKV transmission, we employed a mouse model (IFN-α/β R-/-), and an *in vitro* assay (Chapter V). To evaluate the survival of CHIKV in nature in temperate areas, we assessed the susceptibility of larvae mosquitoes to CHIKV through viral suspension and infected carcasses mosquitoes. The potential contribution of this mechanism to the persistence of CHIKV in an epidemic settle was discussed (Chapter VI). Finally, we assessed the vector competence of a Spanish *Ae. albopictus* strain for two different DENV strains (serotype 1 and 2). Infection, dissemination, and transmission rates were assessed and compared with previous vector competence studies for DENV in European *Ae. albopictus* populations (Chapter VII).

The present study found that environmental conditions had a significant effect on development time, larval and adult survival, biting rate and quantity of CHIKV ingested. As shown in previous studies of vector competence for CHIKV, cooler temperatures in the larval and adult stage enhanced CHIKV infection rate relative to warmer temperatures. We found that *Ae. albopictus* was capable of transmitting the emergent ITA strain (5 %). We also observed that mice lacking in the alpha/beta interferon (IFN-α/β -/-) were highly susceptible to CHIKV infection, in congruence with previously published studies. By contrast, we could not demonstrate transstadial

transmission (passage of a pathogen from one instar to the next) of CHIKV. We found that the strain of *Ae. albopictus* tested was susceptible to oral infection with both DENV strains, although was able only to transmit DENV-1 (4.6%).

In conclusion, the results of this dissertation demonstrated that the Spanish strain of *Ae. albopictus* tested was competent for CHIKV and DENV. This study confirms the potential of *Ae. albopictus* to start local transmission cycles in the Mediterranean region. We expect the findings of this study can improve our general understanding of vector competence of *Ae. albopictus* in Spain.

RESUMEN

El mosquito tigre, *Aedes albopictus*, se encuentra ampliamente establecido en regiones tropicales, subtropicales y templadas, donde es un vector para arbovirus como chikungunya y dengue. Los brotes que se han producido de ambos virus en Europa durante la última década, han puesto de manifiesto el riego de transmisión de arbovirus en zonas donde estén establecidos los mosquitos *Ae. albopictus*. Para determinar el riesgo de que se produzca un brote en Cataluña, investigamos la competencia vectorial de una población española de mosquitos *Ae. albopictus* para ambos virus en experimentos de laboratorio.

En este estudio, se evaluó la susceptibilidad del mosquito *Ae. albopictus* al virus chikungunya usando diferentes variables: dos dosis virales de diferente concentración (alta y baja), dos cepas (S27, ITA) simulando las condiciones ambientales de las estaciones de verano y otoño de Cataluña. Se compararon los resultados obtenidos con la información existente sobre otras poblaciones europeas de *Ae. albopictus* (Capítulo III). Se evaluó la patogénesis del chikungunya en ratones con deficiencia en el receptor de interferón alfa/beta (IFN-α/β R-/-). Para estudiar la infección del chikungunya, a los ratones se les inoculó subcutáneamente con tres dosis (baja, media y alta) de dos cepas (S27, ITA) (Capítulo IV). Para evaluar la transmisión del chikungunya, empleamos un modelo de ratón (IFN-α/β R-/-) y un ensayo *in vitro* (Capítulo V). Para determinar la persistencia del virus chikungunya en la naturaleza en regiones templadas, se evaluó la susceptibilidad de las larvas de mosquito al virus a través de una suspensión viral y de cadáveres de mosquitos infectados. Asimismo, se argumentó la contribución que tendría este mecanismo en la persistencia de dicho virus durante una epidemia (Capítulo VI). Finalmente, se evaluó la competencia vectorial del mosquito *Ae. albopictus* frente al virus del dengue usando dos cepas diferentes (serotipos 1 y 2). Se analizaron las tasas infección, diseminación y transmisión del virus y se compararon dichos resultados con los estudios existentes de competencia vectorial para el virus del dengue en poblaciones europeas de *Ae. albopictus* (Capítulo VII).

Los resultados de este estudio mostraron que las condiciones ambientales ejercieron una influencia importante en el tiempo de desarrollo del mosquito, la supervivencia de las larvas y adultos, así como la cantidad de virus chikungunya ingerido. Se encontraron mayores tasas de infección y diseminación en la estación de otoño respecto a la de verano, en acuerdo con otros estudios sobre competencia vectorial para el chikungunya. Encontramos que *Ae. albopictus* fue capaz de

transmitir la cepa emergente ITA (5%). Asimismo, se observó que los ratones con deficiencia en el receptor de interferon alfa/beta fueron altamente susceptibles a la infección por el virus chikungunya, lo que es congruente con los estudios ya existentes. Sin embargo, no se pudo demostrar la transmisión transestadial de dicho virus Se encontró que la población analizada de *Ae. albopictus* fue susceptible a la infección oral con ambas cepas del virus del dengue, aunque sólo fue capaz de transmitir la cepa de serotipo 1 (4,6%).

En resumen, los resultados de esta tesis demostraron que la población de *Ae. albopictus* en Cataluña es competente para ambos virus, chikungunya y dengue. Este estudio confirma el potencial que tiene como vector el mosquito *Ae. albopictus* para iniciar ciclos de transmisión local de los virus del chikungunya y del dengue en la región Mediterránea.

Esperamos que nuestras aportaciones puedan ayudar a tener un conocimiento más profundo de la competencia vectorial del mosquito *Ae. albopictus* en España.

RESUM

El mosquit tigre, *Ae. albopictus*, es troba àmpliament establert en regions tropicals, subtropicals i templa des, on són un vector per l'arbovirus com chikungunya i dengue. Els brots que s'han produït dels dos virus a Europa durant l'última dècada, han posat de manifest el risc de transmissió d'arbovirus en zones on estan establerts els mosquits *Ae. albopictus*. Per determinar el risc que es produeixi un brot a Catalunya, investiguem la competència vectorial d'una població espanyola de mosquits *Ae. albopictus* per a tots dos virus en experiments de laboratori.

En aquest estudi, es va avaluar la susceptibilitat del mosquit *Ae. albopictus* al virus chikungunya utilitzant diferents variables: dues dosis virals de diferents concentracions (alta i baixa), dues soques (S27 i ITA) simulant les condicions ambientals de les estacions d'estiu i tardor de Catalunya. Es van comparar els resultats obtinguts amb la informació existent sobre altres poblacions europees d' *Ae. albopictus* (Capítol III). Es va avaluar la patogènesi del chikungunya en ratolins amb deficiència en el receptor d'interferó alfa/beta (IFN-α/β R-/-) Per estudiar la infecció del chikungunya, als ratolins se'ls va inocular subcutàniament amb tres dosis (baixa, mitja i alta) de dues soques (S27, ITA) (Capítol IV). Per avaluar la transmissió del chikungunya, emprem un model de ratolí (IFN-α/β R-/-) i un assaig *in vitro* (Capítol V). Per determinar la persistència del virus chikungunya en la natura en les regions temperades, es va avaluar la susceptibilitat de les larves de mosquit al virus mitjançant una suspensió viral i de cadàvers de mosquits infectats. Així mateix, es va argumentar la contribució que tindria aquest mecanisme en la persistència del virus chikungunya durant una epidèmia (Capítol VI). Finalment, es va avaluar la competència vectorial dels mosquits *Ae. albopictus* enfront del virus del dengue utilitzant dues soques diferents (serotip 1 i 2). Es van analitzar les taxes d'infecció, disseminació i transmissió del virus i es van comparar aquests resultats amb els estudis existents de competència vectorial per al virus del dengue en poblacions europees de *Ae. albopictus* (Capítol VII).

Els resultats d'aquest estudi van mostrar que les condicions ambientals van exercir una influència important en el temps de desenvolupament del mosquit, la supervivència de les larves i adults, així com la quantitat de virus chikungunya ingerit. Es trobaren majors taxes d'infecció i disseminació en l'estació de tardor respecte a la d'estiu, en consonància amb altres estudis sobre competència vectorial per al chikungunya.
Trobem que *Ae. albopictus* va ser capaç de transmetre la soca emergent ITA (5%). Així mateix, es

va observar que els ratolins amb deficiència en el receptor d'interferó alfa/beta van ser altament susceptibles a la infecció pel virus chikungunya, el que es congruent amb els estudis existents. No obstant això, no es va poder demostrar la transmissió transestadial del anomenat virus. Es va trobar que la població analitzada d' *Ae. albopictus* va ser susceptible a la infecció oral amb les dues soques del virus del dengue, encara que només va ser capaç de transmetre la soca de serotip 1 (4,6%).

En resum, els resultats d'aquesta tesi demostren que la població d' *Ae. albopictus* a Catalunya és competent per als dos virus chikungunya i dengue. Aquest estudi confirma el potencial que té com vector el mosquit *Ae. albopictus* per iniciar cicles de transmissió local dels virus del chikungunya i del dengue en la regió Mediterrània.
Desitgem que les nostres aportacions puguin ajudar a tindre un coneixement més profund de la competència vectorial del mosquit *Ae. albopictus* a Espanya.

PUBLICATIONS

The results presented in this thesis have been published or submitted for publication in international scientific peer-reviewed journals:

• **Santamaría C., Talavera S., Brustolin M., Napp S., Rivas R., Pujol N., Valle M., Verdún M., Sánchez-Seco MP., Lucientes J., Busquets N.,* Pagès N*.,** Chikungunya virus infection in *Aedes albopictu*s is modulated by blood meal viraemia, viral strain and seasonal temperature.

** These authors contributed equally to this work.*

• **Brustolin M., * Santamaría C.,* Napp S., Verdún M., Rivas R., Pujol N., Talavera S.,* Busquets N.,***. Experimental study of the susceptibility of a European *Aedes albopictus* strain to dengue virus under a simulated Mediterranean temperature regime. Med Vet Entomol. (2018) 32, 393-398.doi:10.1111/mve.12325

** These authors contributed equally to this work.*

Chapter 1
INTRODUCTION

"La recherche est un processsus sans fin dont on ne peut jamais dire comment il évoluera. L'imprévisible est dans la natura même de la science".

François Jacob

1. VECTOR-BORNE DISEASES

Vector-borne diseases (VBDs) are illnesses caused by pathogens in human, animal or plant hosts transmitted by vectors [1]. Vector-borne plant and animal diseases reduce agricultural productivity, affect ecosystem dynamics and have devastating outcomes for health livestock [2]. The majority of animal VBDs (61%) are zoonoses, that is to say, diseases transmitted from animal reservoirs to humans (and *vice versa*), causing an increasing public health problem [3]. According to statistics provided by the World Health Organization (WHO), VBDs represents around 17% of all human infectious diseases, causing more than 700,000 million deaths annually [4]. However, these figures are inaccurate and underreported due to several reasons. First, most VBDs are found in tropical and subtropical areas, where precise diagnostic tools are scarce, and the surveillance is insufficient. [5]. Second, most human infections are asymptomatic infections. The incidence data only reflect symptomatic cases of infection [6]. Third, notification of VBDs varies according to the country. The burden of these diseases is highest in tropical and subtropical countries where VBDs are often neglected [7]. Therefore, the true incidence, prevalence, morbility and mortality of VBDs are underestimated [7]. VBDs of major public health importance, from a veterinary and medical point of view, are diseases transmitted by mosquitoes. According to the latest report of the WHO, mosquito-borne diseases (MBDs) have the greatest burden in number of cases, mortality and disability-adjusted life years of all the known VBDs [8].

Mosquitoes have a complex life cycle with four main life stages: embryonic stage (eggs), larva, pupa and imago stage (adult) (Figure 1.1). The eggs, larvae an pupa are aquatic, whereas the imago mosquito has a terrestrial habitat. Larvae emerge from the mosquito eggs, when the water covers the eggs in the breeding sites. After moulting three times (from the first to fourth instars), the larva undergoes metamorphosis into a pupa. The pupa develops into an adult mosquito. The newly emerged adult mosquitoes include male and female (usually 1:1 rate male/female) [9].

Mosquitoes at their larval-stage are aquatic and generally feed on microorganisms, small invertebrates and particulate organic detritus [10], while at adult-stage, they usually feed on sugar sources from plants [11].

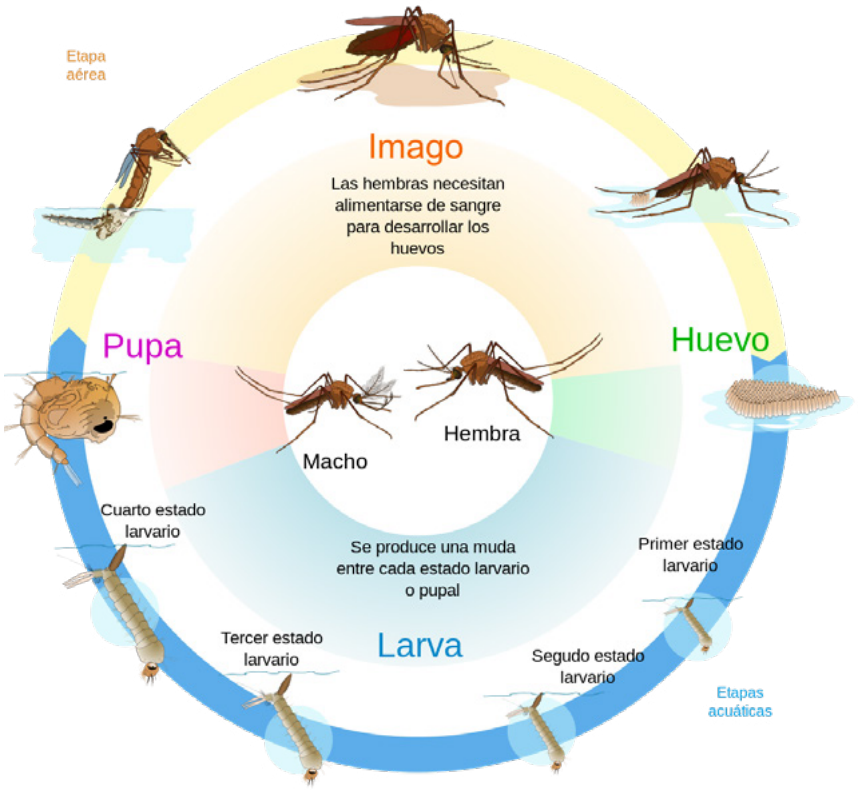

Figure 1.1. Life cycle: the mosquito goes through four separate and distinct stages of its life cycle: egg, larva, pupa, and imago. Source: LadyofHats, vi Wikimedia Commons.

Females of most mosquito species are anautogenous, that is to say, they require a blood meal to mature their eggs [11]. The time from blood feeding, egg maturation and oviposition to subsequent blood feeding is known as the gonotrophic cycle. Due to their blood feeding behaviour, female mosquitoes can acquire and transmit pathogens (bacteria, parasites and viruses). Viruses that circulate in nature between mosquitoes and other hematophagous arthropods and vertebrate hosts are named arbovirus (**ar**thropod-**bor**ne **virus**) [12]. Most arboviruses are maintained in nature in cycles involving a vertebrate animal as main amplifying hosts and an arthropod vector [13]. Usually, humans do not develop a sufficient level of viraemia to infect arthropods, thus, they are considered dead-end-hosts since do not contribute to the transmission cycle [14]. Currently, there are 3,567 species of mosquitoes (order Diptera, family Culicidae) recognised worldwide (Harbach 2013, Mosquito Taxonomic Inventory, http://mosquito-taxonomic-inventory.info, accessed 15 September 2019). Of them, there are only 300 known species of mosquitoes that can be vectors of human and animal arboviruses [15]. There are over 535 different arboviruses distributed around the world, mainly in the tropics and in temperate regions [12]. Nearly all of them are included in eight different taxonomic families (Figure 1.2). Approximately 135 viruses cause human diseases [12], and 40 viruses cause animal diseases [16].

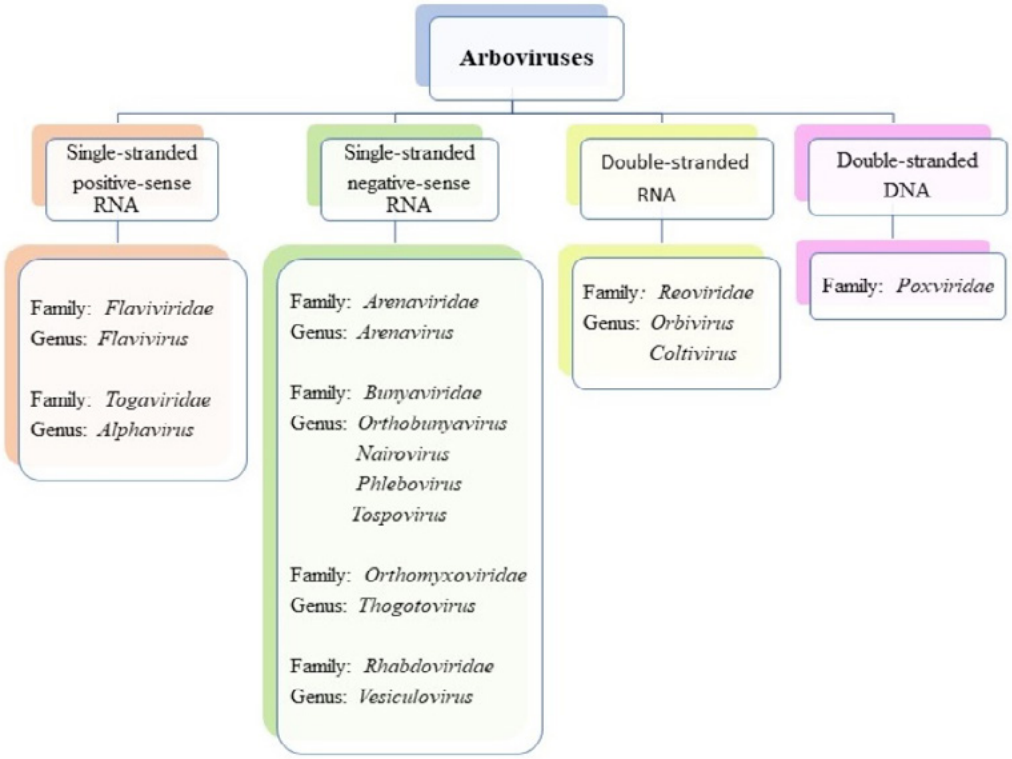

Figure.1.2. Classification of arboviruses. Data obtained from Valisakis and Gubler, 2016. Arboviruses: Molecular Biology, Evolution and Control.

Most known arboviruses were first isolated in tropical regions in Africa, South America and Asia [17] due to the geographic distribution of both arthropod vectors and vertebrate hosts [13]. However, arboviral diseases have expanded dramatically across the world since the 1970s [18]. Possible factors explaining this emergence/re-emergence of arboviral diseases are global trade and travel, demographic switches, unplanned urbanization, changes in public health policies, lack of effective mosquito control, agricultural changes, geographical spread of mosquitoes, viral genetic variation and environmental changes such as climate change [12].

The concept that weather (local daily events in the atmosphere) and climate (average weather pattern at a specific location) are linked to the incidence and spread of human infectious diseases is recognized since Hippocrates era [19]. The climate tends to affect the geographic distribution of MBDs, while variations in weather such as temperature, rainfall and humidity influence the transmission dynamics of MBDs [20]. Climatological research over the past two decades makes clear that Earth's climate is changing and may probably alter the range and transmission potential of MBDs [21]. According to the United Nations Intergovernmental Panel on Climate Change (IPCC), climate change is 'a statistically significant variation in either the mean state of the

climate or in its variability, persisting for an extended period (typically decades or longer)'. This climate change may be due to natural internal processes such as *El Niño*–Southern Oscillation, or persistent anthropogenic changes in the composition of the atmosphere or changes in land use [22]. Although climate change may result from natural climate variability, the IPCC states that 'most of the warming observed over the last 50 years is likely (at least 95% certainty) to be attributable to human activities' [23]. Future global warming projections indicate that average global land and sea surface temperature may increase by 1.5 to 4.5 ºC over the period 1990 to 2100 [24]. The effect of climate change on the future frequency and intensity of *El Niño* is uncertain [25], but there are concerns that *El Niño* events might become more frequent or more intense [26]. Beside, several studies point to an impact of climate change on the hydrological cycle, which can led to more intense rainstorms, air dry, causing extreme events like flooding and intense drought [27, 28]. The temperature of the environment is one of the most important abiotic factors affecting distribution and transmission of MBDs [29, 30] since mosquitoes are poikilotherms thats is, their body temperature is not constant, and it is regulated by the external environmental conditions [31]. In theory, a climate change resulting in a rise in temperatures, will affect the mosquitoes' distribution, which will cause expansions of the current geographic range of many MBDs to naïve areas. However, there is an ongoing debate over the influence climate factors have on MBDs occurrence. Recent works highlight that global warming trends will lead to a higher incidence and broader geographic range of MBDs [32-35]. For example, the transmission of many MBDs is associated with *El Niño* events in some historical data sets [36, 37]. These studies have documented a strong association of some MBDs with the *El Niño*–Southern Oscillation cycle, such as malaria outbreaks in South America [38], and in the Asia-Pacific region [39], Dengue fever in Southeast Asia [40], Ross River fever in Australia [37], and Rift Valley fever in eastern Africa [41]. However, the importance of climate change in the emergence of MBDs is controversial, and some studies hold that the current evidence is insufficient to clearly attribute local resurgences or such geographic spread to regional changes in climate [21, 42, 43]. The studies mentioned above considered that the risk of MBDs is highly variable geographically and non-climatic factors including epidemiological, environmental, demographic, socio-economic, host immunity, public health infrastructure and vector control interventions must be considered. In Europe, climate change has already impacted the transmission of some MBDs [44]. The data from the IPCC confirmed that most of Europe has warmed by a mean of 0.8 °C over the last 100 years, especially in mountainous areas and the Mediterranean region. Since 1950, high-temperature extremes (hot days, tropical nights, and heatwaves) have become more frequent, while low-temperature extremes (cold spells, frost days) have become less frequent [45].

Although the consequences of these changes are difficult to predict, this could result in increased vulnerability within Europe for the re-introduction of MBDs [46]. The eradication of dengue and malaria in Europe by the 1950s, let us consider that MBDs were limited to the tropics. However, at the beginning of the 21st century, the landscape is different, and an increasing number of autochthonous cases of MBDs, have occurred in Europe [47]. These MBDs were caused by mosquito species established in Europe transmitting both imported and circulating pathogens, such as Chikungunya virus [48], Dengue virus [49], Malaria parasite [50], Sindbis virus [51], Toscana virus [52], Usutu virus [53], and West Nile virus [54]. The (re)-emergence of MBDs in Europe has been associated with changes in ecosystems, human behaviour all of them climate and facilitated by globalisation [44, 55].

2. EUROPEAN MOSQUITOES

In recent years, European autochthonous mosquito species have been implicated in the return of malaria (mosquitoes of the genus Anopheles) [56], the presence of Usutu virus (*Culex pipiens*) [57], endemic transmission of West Nile virus (*Culex pipiens*) [58], as well as the ongoing transmission of Sindbis virus (Culex mosquitoes) [47].

In Europe, there is a growing interest to control the establishment and spread of invasive mosquitoes, especially the incursion of *Aedes albopictus* (Skuse, 1894) and the re-introduction of *Aedes aegypti* (Linnaeus, 1762) [59]. More recently, four other non-European aedine mosquito species, *Aedes atropalpus* (Coquillett, 1902), *Aedes japonicus* (Theobald, 1901), *Aedes koreicus* (Edwards, 1917) and *Aedes triseriatus* (Say, 1823) have been established locally and are spreading [59]. In June 2019, another exotic species, *Aedes flavopictus* (Yamada, 1921), has been detected in Netherland, outside its area of origin in north-east Asia [60]. These new invasive mosquitoes are "container-breeding" species [59], this is, mosquitoes that breed in small water containers in domestic settings, increasing contact with humans and the pathogens that they could carry. It is currently estimated that 45% of the total human population of Europe is exposed to invasive mosquito species and eventually, the pathogens that they could transmit [61].

2.1. *Aedes* (Stegomyia) *aegypti*

Aedes aegypti (Linnaeus, 1762), known as yellow fever mosquito, was endemic from West Africa where its ancestral form was a zoophilic tree-hole mosquito named *Aedes aegypti* formosus [62]. It was most likely spread throughout the rest of the world with the slave trade and other trade routes during the 17th to 19th centuries [49]. *Aedes aegypti* has been responsible for large outbreaks of yellow fever and dengue in the Mediterranean region from the late 18th to the mid-20th century [63]. It disappeared from the Mediterranean, the Black Sea and Macaronesian biogeographical region (Canary Islands, Madeira and the Azores) following the Second World War [64], probably attributable to malaria eradication efforts and the widespread use of the insecticide DDT [65]. This species was re-introduced in Madeira [66] and parts of the Black Sea coast (Russia, Abkhazia, Georgia) in 2004 and 2008, respectively [67]. The resurgence of *Ae. aegypti* in some areas of Europe raised concern for an eventual re-colonization of the Mediterranean basin and for the resurgence of the pathogens that it can be transmitted [64]. Recently, *Ae. aegypti* was reported in Netherlands in 2010 [68], in Germany in 2016 [69], and

in the Canary Islands in 2017 [63], but they has not yet become established. Cold temperatures limit *Ae. aegypti* distribution in Europe to areas with an average winter isotherm of 10 ºC in the northern and southern hemispheres. *Aedes aegypti* is a predominantly urban vector, has anthropophilic behaviour and preferentially feeds on humans, even in the presence of alternative hosts [70]. Unlike numerous other mosquito species, *Ae. aegypti* takes multiple blood-meals during each gonotrophic cycle, and thus increases the risk of disease transmission (reviewed in[71]).

2.2. *Aedes* (Stegomyia) *albopictus*

Aedes albopictus (Skuse, 1894), known as the Asian tiger mosquito, is native of the forest of south-east Asia, where it breeds in three-holes [72]. Although *Ae. albopictus* mosquitoes have a short flight range (200 to 600 m) [73], it has been introduced worldwide by the transportation of eggs in used tires or lucky bamboo plants [74]. The first report of the species in Europe was in 1979 in Albania [75], and since then, it has colonized much of the Mediterranean area, being detected in Spain in 2004 [76]. Its invasiveness is linked to: i) its physiological plasticity, which allows surviving in both tropical and temperate conditions, [74] and has the ability to overwinter as an egg or an adult (reviewed in [71]) ii) its ecological plasticity which allows to exploit a wide range of habitats, natural (e.g., bamboo stubs and tree holes) and man-made (e.g., tires and pots) breeding sites [77], iii) its oviposition behaviour, which increases the chance of survival of their progeny [78], and iv) its competitiveness for breeding sites, being able to displace other mosquito species such as *Ae. aegypti* [79]. *Aedes albopictus* has long been considered mainly zoophilic, but there is growing evidence that prefers to feed on humans in urban environments [79]. The establishment of *Ae. albopictus* in Europe raises public health concerns because it is a vector of CHIKV, DENV and Zika virus, and it considered a competent vector of at least 22 other arboviruses in the laboratory including yellow fever virus, Rift Valley fever virus, West Nile virus [80].

Aedes aegypti and *Aedes albopictus* are important vectors for emerging diseases caused by arboviruses such as Chikungunya and Dengue. Both mosquito species are considered the main vectors of Chikungunya and Dengue viruses in both tropical and temperate climatic areas.

3. CHIKUNGUNYA VIRUS

Chikungunya virus (CHIKV) is a member of the *Alphavirus* genus of the family *Togaviridae*. Chikungunya fever usually develops 2 to 6 days after the mosquito bites and transmits CHIKV [81]. Silent infections (infections without clinical signs and symptoms) only occurred in around 15 % of cases [82]. This contrasts with many other arboviral infections, such as Dengue, West Nile and Zika, where most infections (approximately 80%) are asymptomatic [83-85]. Signs and symptoms of chikungunya fever often resemble those of Dengue fever (Table 1.1).

Table 1.1. Frequency of signs and symptoms reported in acute illness cases of Chikungunya fever compared with Dengue fever.

CLINICAL SIGNS AND SYMPTOMS	Frequency of clinical signs and symptoms (%) of Chikungunya fever and Dengue fever in symptomatic patients.	
	Chikungunya fever	Dengue fever
Range of symptomatic infections	75-97%	20-25%
Duration of infection	4 to 7 days	3 to 7 days
Viraemia (acute phase)[1][1]	10^9 copies/ml	10^6 copies/ml*
Fever	75-100%	80-100%
Type of fever	high fever ›38º C	above 38º C
Polyarthralgia (joint pain)[2]	70-100%	30-56%
Myalgia (muscle pain)	70-96%	50-80%
Headache	62-73%	75-80%
Rash	40-80%	10%
Polyarthritis[3]	14-50%	Rare
Vomiting	4-59%	20-55%

Data compiled from several studies [81, 83-94]

1. Samples from European travellers returning from tropical areas in the first days after the onset of symptoms.
* values for serotypes 1 and 2 of DENV, the most frequently imported serotypes within Europe.

2 Polyarthralgia involves the peripheral joints, primarily wrists, knees, ankles and small joints of the extremities.

3 Results (14-50%) from a systematic review and meta-analysis realised in 2015 [95]. In the most conservative scenario approximately 25% of CHIK cases would develop chronic inflammatory rheumatism. Although arthralgia is a common feature of dengue, true arthritis is rare in dengue fever.

The disease is characterised by abrupt onset of high fever, skin rash, myalgia, and incapacitating arthralgia. The latter distinguishes chikungunya from dengue fever [99], together with the lack of retro-orbital pain [100] (Table 1.1). Most infections completely resolve within weeks, but there are reported cases of chronic incapacitating arthralgia lasting for months, or even for years, in the form of recurrent or persistent episodes [101]. These more severe outcomes often occur in patients more than 65 years of age, and in those with underlying medical conditions [90]. Currently, there are no licensed antivirals or vaccines available for chikungunya fever, but numerous candidate vaccines are under development [102].

Recent studies suggest that chikungunya could have been misdiagnosed as dengue, and underreported in dengue-endemic regions since the early nineteenth century [103.] This is due to the fact that CHIKV and DENV are transmitted sympatrically in urban areas by the same mosquito vectors [104] and both infections have similar clinical features during the acute phase [105]. Although CHIKV-affected areas often overlap with DENV endemic areas, simultaneous outbreaks are rare or undetected [106]. However, cases of co-infection with CHIKV and DENV have been documented since 1967 [107]. Recently, a simultaneous outbreak of CHIKV/DENV led by *Ae. albopictus* was detected in 2007 in Gabon [108]. Further studies are needed to determine how co-infections affect both vector and human hosts.

3. 1. History and spread of Chikungunya virus

CHIKV was first isolated in 1953 from the serum of a febrile human [109] in Tanganyika Territory (now in Tanzania) during an epidemic of dengue-like illness [100]. The virus and the disease were called chikungunya, which in the Bantu language of the Makonde (an ethnic group from Tanzania and Mozambique) means, "to become contorted". This description refers to the stooped posture of the patient due to debilitating joint pain [110]. Retrospective case reviews have suggested that the term "dengue" was originally applied to a clinical syndrome closely resembling that now it is associated with chikungunya virus infection. The first evidence of CHIKV epidemics occurred in 1779 in Batavia [103]. In 1823 an epidemic of CHIKV was described in Zanzibar (an island of Tanzania), and later, in 1827, it arrived in the Caribbean and spread to North and South America [111]. CHIKV outbreaks have cyclically emerged across African and Asian continents with intervals of 7 to 20 years between consecutive epidemics [112]. Strains from Africa and Asia are reported to differ biologically, indicating that distinct lineages

may exist [105]. Phylogenetic analyses of numerous CHIKV sequences (based on E1 gene) have identified three genotypes termed West African, East/Central/South African (ECSA) and Asian [113]. A large-scale epidemic of CHIKV emerged in Kenya in 2004 and spread to several Indian Ocean islands. The outbreak strain belonged to the ECSA genotype [114]. In March 2005, the first cases of chikungunya occurred in *La Réunion* Island (a French overseas territory in the Indian Ocean), where this virus had never been detected before [115]. This epidemic was associated with the emergence of a novel viral strain adapted to an alternative vector: *Ae. albopictus*. The new strain presented an amino acid mutation from alanine (A) to valine (V) at position 226 of the E1 glycoprotein (E1-A226V) [112]. Laboratory investigations confirmed that this mutation increase (50-100 fold) its infectivity in *Ae. albopictus* when compared to its infectivity in *Ae. aegypti* [116].

During the first period of the *La Réunion* outbreak (from March to June 2005), E1-226A was the only genotype observed. However, since the beginning of September 2005 the emergent genotype E1-226V [112] appeared driving an explosive epidemic peak 3 months later, in mid-December 2005 indicating a genotype switch during the winter season [117]. Since December 2005, more than 90% of the strains incorporated such mutation [118].

Between 2005 and 2007, the adaptive mutation (A226V) occurred independently via convergent evolution several times [119]. This mutation was detected in isolates from India, Gabon and all the Indian Ocean islands where *Ae. albopictus* was present [120]. In 2007, for the first time, CHIKV reached the temperate climate when a viraemic traveller introduced the new viral strain (E1-226V) in Italy, which was propagated by local populations of *Ae. albopictus* [121]. In 2010 and 2014, CHIK autochthonous cases associated with the ECSA lineage (with and without the E1-A226V mutation) were reported in France and *Ae. albopictus* was pointed out as the responsible vector [122, 123]. In 2013, CHIKV re-emerged in the Caribbean after two centuries without known reports and rapidly spread to other parts of the American continent. The strain introduced belonged to the Asian genotype and did not have the E1-A226V mutation [124]. More recently, chikungunya autochthonous cases of the ECSA lineage have been reported in Italy (E1-226A) and France (E1-226V) in 2017 [125, 126].

3. 2. Transmission cycles of Chikungunya virus

CHIKV circulates in two ecologically distinct transmission cycles, sylvatic and urban. The sylvatic transmission cycle is confined within Africa, involving wild non-human primates, small mammals (e.g. bats, rodents) and forest-dwelling *Aedes* spp. mosquitoes [127]. The mosquito species involved in transmission vary geographically [128, 129]. In rural regions of Africa, outbreaks usually affect small villages and are associated with rainy periods that increase sylvatic mosquito densities [130]. Outside Africa, the sylvatic transmission has been investigated in very few studies [131]. One experimental study has demonstrated that two local sylvatic Neotropical mosquito species, *Haemagogus leucocelaenus* (Dyar &Shannon, 1924), and *Aedes terrens* (Walker, 1856) from the American continent could initiate a sylvatic cycle in the tropical Americas [132].

In contrast, urban cycles are primarily maintained in Asia [133] and, in some parts of Africa [134]. The virus circulates between humans and mosquitoes, resulting in urban epidemics with *Ae. aegypti* or, most recently, *Ae. albopictus* as the main vectors [135]. There is limited evidence for sylvatic CHIKV transmission in Asia, and the absence of an animal reservoir is currently accepted.

Human beings serve as the Chikungunya virus reservoir during epidemic periods [99], whereas outside these periods, African green monkeys (*Chlorocebus sabaeus*), monkeys (*Erythrocebus patas*), Guinea baboons (*Papio papio*), rodents, birds, and other unidentified vertebrates are the main reservoirs [136]. Beyond their role as vectors, mosquitoes might act as CHIKV reservoir between epidemics. During unfavourable periods, dry season in tropical areas, or cold season in temperate regions, desiccated or eggs in diapause may provide a means of viral survival [137]. Vertical transmission in mosquitoes (transmission of a pathogen from parent to the offspring) has been demonstrated in the field [138, 139], and the laboratory for *Ae. albopictus* and *Ae. aegypti* [140, 141].

4. DENGUE VIRUS

Dengue virus (DENV) is a member of the family *Flaviviridae* that belong to genus *Flavivirus*. *Flavivirus*es are derived from the Latin word *flavus*, that meaning yellow, taking their name from Yellow fever virus (YFV) [142].

There are five distinct serotypes, called DEN-1, DEN-2, DEN-3, DEN-4, DEN-5 and several genotypes within each serotype [143]. The fifth serotype (DENV-5) was isolated in 2013 during a screening of viral samples in 2007 in India [144]. The public health implications of this fifth serotype are unknown because it remains unclear if this virus is capable of sustained transmission between humans [145]. Infection with one dengue serotype provides lifelong immunity to that virus, but there is no cross-protective immunity to the other serotypes [18].

DENV infection in humans can cause a spectrum of illness ranging from unapparent or self-limiting febrile illness termed dengue fever, to the severe forms of the disease, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [18]. It has been found that 50-90% of all DENV infections are asymptomatic, whereas 10-50% of infections are symptomatic [146]. Dengue fever is a self-limited disease characterised by a sudden onset of fever, arthralgia, myalgia, anorexia, rash, and retro-orbital pain [143]. The most severe form of the disease, DHF and DSS, are approximately 1-2% of human infections [147]. There is no specific treatment for dengue/ severe dengue, but early detection and access to proper medical care reduce fatality rates below 1% of cases [148]. Currently, there is available a licensed DENV vaccine, Dengvaxia® (CYD-TDV) for human use in some endemic countries [149]. According to WHO recommendations based on data from clinical trials, this vaccine is only available for patients with seropositive serostatus [150].

4. 1. History and spread of Dengue virus

Dengue disease is mainly asymptomatic; therefore, it has been difficult to determine when DENV first appeared in human populations. The earliest record of illnesses compatible with dengue fever was recorded in a Chinese medical encyclopaedia in 992 [17], although the major epidemics of well-documented cases occurred on three continents, Asia, Africa, and North America at the end of the 18th century [18]. The initial geographic expansion of dengue was

related to the global expansion of the shipping industry through the 17th and 18th centuries [151]. Slave trade between West Africa and America was responsible for the introduction and the widespread geographic distribution of *Ae. aegypti* in the New World [17]. Because mosquitoes and viruses were spreading by sailing ship, the disease pattern associated with dengue (from 1780 to 1940) was characterized by sporadic epidemics with long intervals between them (10-40 years) [17]. During and after World War II, that disease pattern of the disease was disrupted by the emergence of DHF in Southeast Asia [152]. It was hypothesized that troop movements, along with environmental destruction and rapid urbanization in Southeast Asia, contributed to increased transmission of viruses and the co-circulation of multiple DENV serotypes (i.e., hyperendemicity) [146]. It was during this period that the first known epidemic of DHF occurred in Southeast Asia in 1953 [153]. Since then, this region remained hyperendemic to all DENV serotypes (DENV 1-4) [146]. In the Americas, the decline and re-emergence of epidemic dengue since the 1980s has been linked to the presence of *Ae. aegypti* (Figure 1.3) [146].

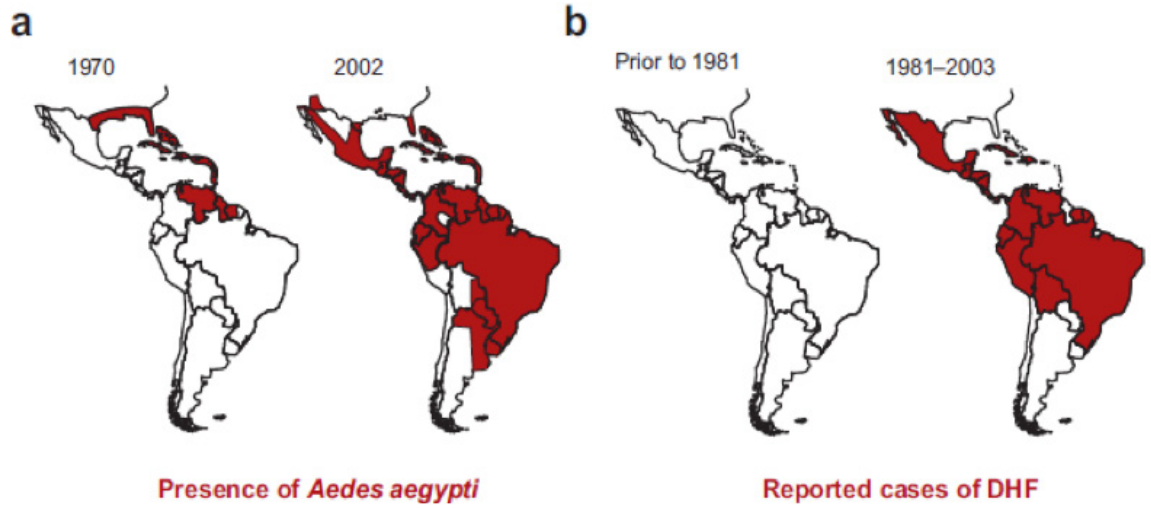

Figure 1.3. Spread of Ae. aegypti and DHF in the Americas. (a) The shaded areas represent the regions in the Americas where *Ae. aegypti* was present in 1970 (left) and in 2002 (right). (b) Shaded areas represent the countries that reported cases of DHF prior to 1981 (left) and between 1981 and 2003 (right). The increased distribution of DHF mirrors the dissemination of *Ae. aegypti*. Source: Reproduced from [146].

During the 1950s and 1960s, epidemic dengue was controlled in the Americas because of the primary vector, *Ae. aegypti,* was eradicated from 23 countries [153]. The eradication program was discontinued in the early 1970s, and the species reinvaded those countries from which it was previously eradicated (Fig.1.3). By the 1980s, the American continent was experiencing significant dengue epidemics in countries that were free of the disease for 35–130 years [17].

In Europe, several epidemics occurred during the 18th and 19th centuries, in ports of the eastern Mediterranean region [49]. The largest and almost last dengue outbreak occurred in Greece in 1927–28. After 55 years of absence in the Mediterranean region, dengue re-emerged in France and Croatia in 2010, propagated by *Ae. albopictus* [49]. During 2012-13, a massive outbreak was reported in the Portuguese island of Madeira driven by *Ae. aegypti* [154]. Others autochthonous cases were also reported in France in 2013, 2014, 2015 and 2018 [155-158] and Spain in 2018 [159]. Strains belonging to two different serotypes have been identified during these outbreaks: i) DENV-1 in France (2010, 2014, 2015 and 2018), Croatia (2010), Madeira (2012) and Spain (2018); ii) DENV-2 in France (2013, 2014 and 2018).

4. 2. Transmission cycles of Dengue virus

Dengue virus can be maintained in nature via two transmission cycles: 1) a sylvatic cycle between non-human primates and tree-hole mosquitoes of the genus *Aedes*, in the rain forests of Asia and Africa, and 2) an urban epidemic/endemic cycle between humans and the peridomestic mosquitoes *Ae. aegypti* and *Ae. albopictus* [160]. The most relevant transmission cycle from a public health standpoint is the urban endemic/epidemic cycle in large urban centres of the tropics, where multiple serotypes co-circulate and provide ideal conditions for the emergence of epidemic DHF [18]. Human beings serve as DENV reservoirs during epidemic periods in urban cycles. Several monkey species may act as host reservoirs for the sylvatic cycle in West Africa and South-eastern Asia [161]. Mosquitoes may act as reservoirs of DENV during adverse conditions for vector activity [162]. Vertical transmission of DENV has been reported in nature by the detection of DENVs in field-collected eggs [163], larvae [164], and adult male mosquitoes in *Ae. aegypti* and *Ae. albopictus* [165].

5. VECTORIAL CAPACITY FOR MOSQUITO-BORNE VIRUSES

Local transmission of CHIK and DEN viruses require the simultaneous presence of the virus, competent mosquitoes and susceptible hosts. However, the presence of these items does not necessarily result in the transmission of the virus. In addition, environmental conditions may be determinant to facilitate the emergence and persistence of the virus.

A simple approach for assessing such risk is to evaluate the vectorial capacity, an entomological analogue of the pathogen's basic reproductive rate (R_0). Vectorial capacity (C) is a measure of the mosquito population's capacity to transmit an infectious agent to a susceptible host population [166]. Vectorial capacity is given by the modified equation of Ross-McDonald [167].

$$C = \frac{m \cdot a^2 \cdot p^n \cdot b}{-Ln\ p}$$

- *m:* is the density of vectors (in relation) to host
- *a:* is the daily probability of the vector feeding on a host (a vector has to bite twice (a^2) to acquire and transmit a pathogen)
- *p:* is the probability of daily survival
- *n:* is the number of days between infection of the vector and the time it becomes capable of infecting a new host (i.e., the extrinsic incubation period (EIP))
- *b:* is the vector competence (VC), measured as Transmission Efficiency (proportion of vectors ingesting an infective meal that are later able to transmit the infection)
- *1/ (-Ln p):* is the duration of the vector's life after surviving the EIP

Vectorial capacity represents the average daily number of secondary cases generated by one primary case introduced into a fully susceptible population [168]. As a result, quantification of vector capacity is useful to determine the intensity of arbovirus transmission generated by local vector populations [169], to predict the risk of transmission of pathogens [79], and for assessing the effectiveness of intervention campaigns [170]. The parameters of the vectorial capacity equation are influenced by both, intrinsic (e.g., vector and viral genetics, vector life-history traits, host susceptibility) and extrinsic (e.g., temperature, rainfall, humidity and human land use) factors [166]. Thus, vectorial capacity changes from vector to vector, among geographic locations and between transmission seasons.

Vector competence, a component of the vectorial capacity equation, refers to the ability of the mosquito to become infected with and transmit a pathogen following an infectious blood meal [171]. Although the presence of highly competent vectors is thought to be essential to trigger arbovirus transmission, a relatively low competent vector may be involved in an epidemic. For example, a low competent population of *Ae. aegypti* supported an epidemic of Yellow fever virus in Nigeria in 1987. This epidemic was likely due to the presence of high population density of *Ae. aegypti* [172]. The parameters of vectorial capacity vary spatially and temporally according to environmental conditions, overall the temperature. The temperature can influence entomological parameters in complex and antagonistic ways. For example, warm temperatures may enhance the vector competence (b) and decrease the extrinsic incubation period EIP (n) but shorten the longevity of the vector (p) [173]. Therefore, understanding how temperature affects the likelihood of virus transmission is essential to evaluate the risk of arbovirus transmission.

Due to the difficulty in obtaining accurate estimates of some entomological parameters of vectorial capacity, such as biting rate (a) and vector lifespan (p) [79], the parameter most frequently used to assess the risk of MBDs is the vector competence.

6. VECTOR COMPETENCE OF MOSQUITOES FOR ARBOVIRUSES

The ability and likelihood that a mosquito transmits an arbovirus to human hosts depends on several factors. One of them is the vector competence, that is, the intrinsic ability of a mosquito vector to acquire, maintain and transmit an arbovirus.

Following infection of a viraemic blood meal from a vertebrate host, virus particles reaches the midgut, the initial site of infection (Figure 1.4). The mosquito midgut consists of a single layer of epithelial cells surrounded by a matrix termed basal lamina.

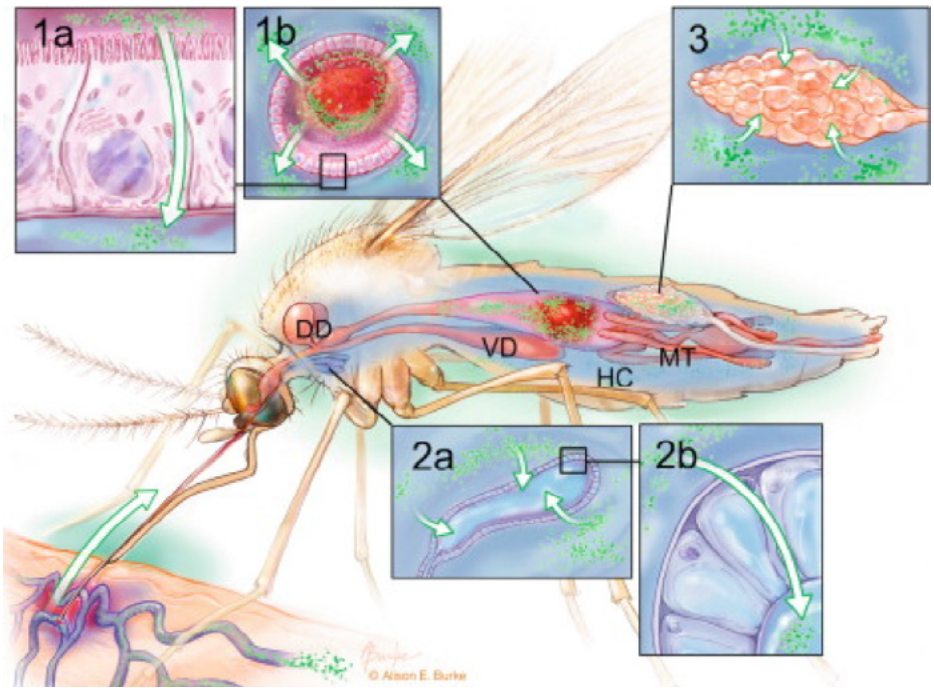

Figure 1.4. The key steps for a productive arbovirus infection of a mosquito include (1) initiation of infection in the midgut (panel 1a); (2) productive viral propagation within the midgut epithelium (panel 1b); (3) dissemination of virus from midgut epithelial cells to secondary tissues in the haemocoel and secondary amplification; (4) infection of salivary glandular acinar cells (panel 2a); and (5) release of the virus into salivary ducts for horizontal transmission to an uninfected vertebrate host (panel 2b). Abbreviations: DD, dorsal diverticulum; HC, haemocoel; MT, Malpighian tubules; VD, ventral diverticulum. Source: Reproduced from [171].

A minimum threshold level of virus is required to trigger an infection in the mosquito; this threshold varies according to virus-mosquito species combinations [175]. The first potential barrier that a virus encounter in the midgut is the peritrophic membrane, a sac that forms a matrix surrounding the blood meal [176]. The virus particles need to infect the midgut epithelial cells before the development of the peritrophic matrix, since the pore size (20–30 nm) of the

peritrophic membrane are smaller than the diameter of arboviruses (i.e., 50-60 nm of CHIKV [177]). Once the virus has overcome the peritrophic matrix, virions penetrate the epithelium cells and viral replication takes place (Figure 1.4, step 1, panel 1a). After infection and replication in the midgut cells (Figure 1.4, step 1, panel 1b), viral particles pass through the basal lamina of the midgut epithelium to enter the haemocoel (body cavity where the haemolymph circulates). The ability of virions to reach the haemolymph (mosquito blood) depends on the thickness of the basal lamina, which differs between different mosquito species [176]. There are two alternative ways of reaching the haemolymph, one is via infection of the tracheae and the other is through the phenomenon commonly referred to as 'leaky midgut'. The leaky midgut is 'the direct passage of virions into the haemolymph, immediately after an infectious blood meal, without a replication cycle in the midgut epithelial cells' [176]. Following escape from the midgut epithelial cells, virions disseminate to secondary tissues, such as the fat body, haemocytes (blood cells), muscles, and nerve tissue [178] (Figure 1.4, step 3). Finally, the virus spread to the salivary glands. The salivary gland lobes are surrounded by the salivary gland basal lamina, which forms a physical barrier to virus infection of the salivary gland epithelial cells. When the virus overcomes the salivary gland barrier, viral replication takes place (Figure 1.4, step 4, panel 2a). Subsequently, the virus is deposited into the apical cavities of acinar cells, which can lead to inoculation into the host during the subsequent bloodmeal [178] (Figure 1.4, step 5, panel 2b). The times it takes for an arbovirus to complete all this process, between the ingestion of the virus and the earliest time at which virus is released in the saliva, is referred as extrinsic incubation period [179]. The length of the EIP can vary depending on mosquito strain [180], virus strain [181], viral dose [182], and temperature [183], but it generally ranges from 7–14 days [184]. After this period, the mosquito is able to transmit the virus to a new vertebrate host during a subsequent bloodmeal.

Not all mosquitoes become infected after ingestion of an infectious blood meal. In some cases, the virus cross the midgut successfully through the haemocoel, and therefore the midgut is considered permissive to infection (Figure 1.5). By contrast, if the virus fails to bind, enter, and/or replicate within the midgut epithelial cells, the mosquito presents a midgut infection barrier (MIB) (Figure 1.5) and therefore it is refractory to infection. Several hypothesis have been proposed to explain it (reviewed in [178]): i) diversion of the blood and virus into the ventral diverticulum (a sac used as carbohydrate storage); ii) filtration of virus by the peritrophic matrix; iii) inactivation of virions by digestive enzymes; iv) failure of the virus to attach to a cell receptor; and v) the absence of appropriate receptors on the epithelial cells. If the virus infected

and replicated within the midgut epithelial cells, but was unable to disseminate to other organs, the mosquito exhibits a midgut escape barrier (MEB) (Figure 1.5) [175]. This MEB prevents viruses from escaping into the haemocoel. The MEB was found to be dose-dependent, and may occurred when low doses of virus had been ingested [178].

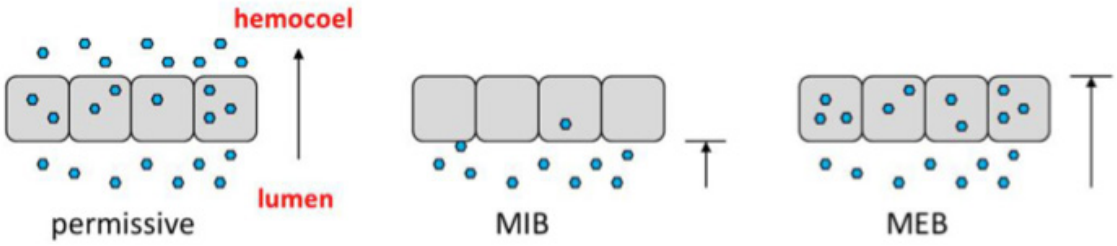

Figure 1.5. Schematic representation of a permissive midgut infection, midgut infection barrier (MIB), and midgut escape barrier (MEB). Grey squares represent midgut epithelial cells, and blue hexagons represent virions. Source: Reproduced from [175].

Once the virus overcame the MEB, the virus spread to the salivary glands. The invasion of the mosquito's salivary glands is essential for the mosquito to be competent for virus transmission and it is likely receptor-mediated. When the virus disseminated from the midgut is unable to enter the salivary gland tissue or to establish a productive infection in acinar cells, the mosquito exhibits a salivary gland infection barrier (SGIB) [178]. This SGIB prevents the entry of the virus or if the virus cross the barrier, it can restrict virus replication within the acinar cells by antiviral immune pathways. Finally, a salivary gland escape barrier (SGEB) prevents virions escaping from the salivary gland cells [174]. Release of virus-containing saliva might require the induction of apoptosis of the acinar cells of the glands [176, 178].

In summary, the virus must be able to overcome various barriers within the mosquito body: the peritrophic membrane, the midgut barriers (MIB and MEB), and the salivary gland barriers (SGIB, SGEB). These barriers can limit virus infection both mechanically and through antiviral immune responses [176], and thereby determining the VC of the mosquito. Once the virus overcomes successfully all these tissue barriers, the infection is persistent, that it, the mosquito will remain infected with the virus for its entire life [185].

6.1. Factors affecting Vector Competence of mosquitoes for arboviruses

It is known that VC is shaped by intrinsic (genetic, innate immune response) and extrinsic factors (environmental conditions, viral dose). Along with these, the vector mosquito microbiota play a dual role as an intrinsic factors to the mosquito (innate immunity) and as an extrinsic factor shaped by environmental conditions. The VC of mosquito populations varies across different mosquito species, within the mosquito species an even at individual level [186]. Knowing how and why varies the VC is key in developing appropriate vector control measures, adapted to each place.

This thesis focuses overall on extrinsic factors that influence VC, such as environmental conditions.

6.1.1. Temperature

The temperature may influence mosquito VC for an arbovirus in different ways: directly via effects on the virus replication and indirectly by inducing changes in mosquito physiology and immunology.

Virus and mosquito life-history traits exhibit non-linear relationships with temperature, and the optimal temperature values for virus and mosquito may not be the same. The net effect on mosquito VC depends on the relative thermal sensitivity of both mosquito and virus traits [187]. Previous studies have shown that temperature influences vector development rates [188], mortality, feeding behaviour [189], and viral replication within the mosquito [190]. Moreover, water temperature is a factor that can influence the diversity, density and activity of other aquatic microorganisms (such as microbiota or other pathogens), and food resources [184].

6.1.2. Precipitation and drought

Precipitation changes are known to affect the reproduction, development, behaviour, and population dynamics of mosquitoes and their pathogens [191], and therefore mosquito VC for arboviruses.

Rainfall is essential to create and maintain potential habitats and breeding sites for the aquatic stages of mosquitoes. However, container-breeding mosquitoes, such as *Ae. albopictus* and *Ae. aegypti*, can be mostly independent of rainfall patterns in urban areas [192]. The effects

of rainfall on vector breeding sites and vector densities may have opposite effects. Increased precipitation could either increase or reduce the mosquito abundance by creating breeding sites or flushing container-breeding mosquitoes [193]. In case of drought, several studies have found an association between drought and incidence of MBDs, such as West Nile virus [62], CHIKV [194] and DENV [195]. For example, in wetlands, drought can cause a decrease in the mosquito breeding sites as well as mosquito predators and competitors [196]. In those habitats where the predator populations are lost, drought and subsequent re-wetting was shown to increase mosquito population [62]. Furthermore, droughts can increase mosquito populations by increasing stagnant water in streams [197]. The possible impact of the precipitation and drought on the incidence of MBDs is complex and require further investigation Additionally, temperature interacts with precipitation also affecting the availability of water habitats [198].

6.1.3. Humidity

Relative humidity (RH) is one of the factors together with temperature, rainfall, mosquito density, genetic factors and food availability, which influences mosquito survival [199], and blood-feeding activity [200]. Low levels of RH during dry-season conditions are known to decrease the lifespan of mosquitoes. By contrast, the dehydration increases blood feeding in mosquitoes [201], and thus, it might prompt higher arbovirus transmission. A high RH favours the survival and proliferation of mosquitoes and stimulates the metabolic processes of vectors [202]. This climatic condition may accelerate the virus multiplication within the mosquito and therefore the risk of arbovirus transmission risk is higher.

6.1.4. Seasonality

Seasonality is a 'periodic surge in disease incidence corresponding to seasons or other stereotyped calendar periods', that characterize many MBDs [203]. In temperate regions, mosquitoes developed strategies to survive the winter, as did the pathogens they transmit. In the tropics, comparable adaptations are necessary for surviving in unfavourable dry periods. In both cases, such adaptations impose seasonality on transmission. For example, the incidence of chikungunya and dengue outbreaks in the Mediterranean region of Europe shows seasonality (as seen in Tables 1.2 and 1.3), with outbreaks in summer and autumn seasons. According to European Environment Agency [204], Europe has experienced several extreme heat waves since the year 2000 (2003, 2006, 2007, 2010, 2014, 2015, 2017 and 2018) and, we can speculate that summer temperature anomalies could facilitate the transmission of CHIKV and DENV in Europe.

Table 1.2. Autochthonous transmission of Chikungunya virus in the Mediterranean Europe

Local cases	Country	Location	Latitude [1]	Year	Period	Season	Origin of primary case	Virus strain [2]	Reported cases [3]		Ref *	Meteoro-logical conditions	Ref *
									Confirmed	Probable			
CHIKV	Italy	Region of Emilia Romagna [4]	44° 50' 36" N	2007	jul-sep	summer	India	E1-266V	281	53	[121]	Extreme heat wave	[204]
	France	Frejús	45° 40' 32" N	2010	sept	summer	India	E1-266A	2	0	[123]	Extreme hot summer	[204] [205]
	France	Montpellier	43° 36' 39" N	2014	sep-oct	autumn	Cameroon	E1-266V	12	0	[122]	Extreme rainfall event	[206]
	France	Le Cannet-des-Maures and Taradeu[5]	43° 23' 33" N 43° 27' 16" N	2017	jul- sep	summer	Central Africa	E1-266V	17	3	[207] [208]	Hot and dry summer	[204]
	Italy	Anzio Latina Rome and Guardavalle Marina [6]	41° 29' 5" N 41° 27' 58" N 41° 53' 30" N 38° 28' 46" N	2017	aug-nov	summer	Asia (India-Pakistan)	E1-266A	Latium region 297 Calabria region 132	Unknown Unknown	[209] [210]	Hot and dry summer	[204] [211]

[1] data obtained from www. Latitude.to. 2 all strains of CHIKV outbreaks belonged to the ECSA genotype; 3 laboratory-confirmed cases based on CHIKV genome detection by RT-PCR or presence of virus-specific antibodies. Probable cases are based on the clinical and epidemiological criteria of the patient;

* Ref: reference; 5 it was reported 16 cases distributed in two clusters: ten cases in Les Cannet de-Maures and six cases in Taradeau. Both locations are ten kilometres apart; 6 two clusters: one in the Lazio Region (Anzio, Rome and Latina) and other in the Calabria región (Guadarvalle Marina).

1.3 Table. Autochthonous transmission of Dengue virus in the Mediterranean Europe

Local cases	Country	Location	Latitude [1]	Year	Period	Season	Origin of primary case	Virus srain	Reported cases [2]		Ref *	Meteorological conditions	Ref *
									Confir-med	Probable			
DENV	Croatia	Pelješac	42° 54' 36" N	2010	aug-oct	summer	Unknown[3]	DENV-1	15 (1+)	13	[212] [213]	Extreme hot summer	[204] [205]
	France	Nice	43° 40' 32" N	2010	aug-sept	summer	Martinique	DENV-1	2	0	[214] [215]	Extreme hot summer	[204] [205]
	France	Bouches-du Rhône	43° 29' 59" N	2013	sep-oct	autumn	Guadaloupe	DENV-2	1	0	[156]	No data	-
	France	Augbane	43° 17' 33" N	2014	aug-sep	summer	Thailand	DENV-2	2	19	[155] [215]	Extreme rainfall event	[204]
	France	Toulon	43° 07 ' 0" N	2014	jul-aug	summer	Unknown [4]	DENV-1	1	50	[155]	Extreme rainfall event	[204]
	France	Toulon	43° 07 ' 0" N	2014	aug-sep	summer	Unknown [5]	DENV-2	1	50	[155]	Extreme rainfall event	[204]
	France	Nîmes	43° 49' 59" N	2015	jul-sep	summer	French Polynesia	DENV-1	6	1	[157]	Flooding	[205]
	France	St. Laurent du Var	43° 40' 24" N	2018	sep-oct	autumn	Unknown	DENV-2	5	0	[158] [216]	Heatwave	[204]
	France	Clapiers	43° 36' 39" N	2018	sep-oct	autumn	Unknown	DENV-1	2	0	[158] [216]	Heatwave	[204]
	France	Nîmes	43° 49' 59" N	2018	sep-oct	autumn	French Polynesia	DENV-1	1	0	[215]	Heatwave	[204]
	Spain	Murcia	37° 59' 13" N	2018	aug-sep	summer	Unknown	DENV-1	5	0	[158] [217]	Heatwave	[204]
	Spain	Barcelona	41° 23' 20" N	2018	oct	autumn	Unknown	DENV-1	1	0	[158] [217]	Heatwave	[204]

1 data obtained from www. Latitude.to; 2 laboratory-confirmed cases based on DENV genome detection by RT-PCR or presence of virus-specific antibodies. Probable cases are based on the clinical and epidemiological criteria of patient; * Ref: reference; + one case of dengue acquired in Croatia was reported in Germany; 3 phylogenetic sequence analyses showed that DENV-1 of the case was close to the isoales from the Indian subcontinent; 4 phylogenetic sequence analyses showed that DENV-1 of case 1 was close to isolates found in America (especially Venezuela and Nicaragua); 5 phylogenetic analyses showed that sequence of DENV-2 was close to isolates from Asia (China, Vietnam, Singapore).

6.1.5. Mosquito immunity

Mosquitoes are exposed to a wide variety of microorganisms in their aquatic habitats and during feeding processes. The mosquito's innate immune system and the microbiote play an essential role in modulating arbovirus infection in the mosquito [218, 219].

Mosquitoes have an innate immune response against arbovirus infection to prevent mortality [220]. Mosquitoes have evolved several antiviral responses: i) the RNA interference (RNAi), ii) Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), iii) Toll, and iv) apoptosis pathways [221]. Of these antiviral mechanisms, the RNAi pathway is the primary antiviral mechanism, leading to the viral RNA degradation, thereby inhibiting viral replication and promoting viral clearance [218].

Mosquito resistance to infection is not a static phenotype comprised solely of immune genes involved in standard immune responses. Therefore environmental factors (overall temperature) can have complex effects on mosquito immunity and mosquito-arbovirus interaction [222]. For example, the RNAi pathway that inhibits viral replication becomes more active at high temperatures [223], whereas several immune responses (melanization, phagocytosis and expression of the antimicrobial peptide defensin) have been reported being more robust at low temperatures [224].

Mosquito host microbial communities in their digestive tract consist primarily of bacteria [225]. This microbiota colonizes other organs as well such as salivary glands, haemocoel, ovaries and Malpighian tubules (renal excretory tissues of mosquitoes). The relationship between these microbes and insects is complex and can range from pathogenesis to commensalism or mutualism [226]. The composition of bacterial microbiota varies intra- and interspecies [225], and depends on the development stage [10], the sex of the mosquito [227], and ecological niche [228]. Although larval mosquitoes expel many bacteria during moulting and metamorphosis [229], some of them are transstadially transmitted to the adult gut [230]. The microbiota plays essential roles in vector physiology, such as nutrition and digestion [227], mosquito survival [225], reproduction [231], and immunity at the adult stage [232].

The midgut is the first site of viral proliferation; therefore, gut microbiota may play a crucial role in antiviral resistance and therefore, on mosquito VC. The bacteria *Wolbachia pipientis,* an obligate intracellular bacterium, has been shown to modulate pathogen infection and transmission in

mosquitoes [230]. It has been detected in several genera of *Aedes* [233] however; it has never been detected in a natural population of the species *Ae. aegypti*, a vector of CHIKV and DENV. The effect of *Wolbachia* on mosquito VC depends on the combination of the *Wolbachia* strain and mosquito species. This impact ranges from reduced virus proliferation and transmission [234] to enhanced virus infection rates [228].

Positive *Wolbachia*-mediated effects strains have been used for dengue vector control programmes (http://www.eliminatedengue.com/program).

7. LABORATORY VECTOR COMPETENCE STUDIES

The first laboratory VC study found that the principal factors governing mosquito infection with the virus were 1) the characteristics of the virus strain, 2) the characteristics of the mosquito, 3) the virus dosage ingested, and 4) the environmental conditions [235]. The first report considering the influence of environmental factors, in particular, temperature, on the VC of mosquitoes was described by Davis in 1932 [236]. These first experiments revealed that environmental temperature at which *Ae. aegypti* mosquitoes were maintained, affected the time interval between ingestion of Yellow fever virus and subsequent transmission by the mosquito (i.e., extrinsic incubation period, EIP) [237]. Despite this early discovery about the influence of environmental temperature on the mosquito VC, most of the VC studies were focused on genetic factors of the mosquito strain and viral strain, keeping the environmental factor constant. In these studies, mosquitoes have been reared and maintained at constant temperatures of 27-28 °C [238], that approximates the mean temperature in tropical areas where most of MBDs are endemic. There are relatively few studies that explored vector-virus interactions using environmental regimens more realistic. Such studies support the notion that fluctuating temperatures may alter estimates of both life-history traits and VC of mosquitoes in tropical regions [239, 240]. In addition, these studies have evaluated the effect of environmental temperature in the adult stage rather than during the whole life-cycle [241]. This approach is logical, as it is only the adult female mosquitoes that transmit the pathogen and larval development is not a direct component of VC. However, it has been demonstrated that the environmental conditions experienced by the immature stages of mosquitoes may have latent effects that continue to adulthood and alter VC indirectly [242].

8. RISKS OF INTRODUCTION OF ARBOVIRUSES IN CATALONIA

Catalonia is an autonomous community located in the northeast of Spain. The human population as of January 2019 is around 7.5 million inhabitants, with the majority (74%) of the popula-tion concentrated in the capital city, Barcelona (https://www.idescat.cat) and metropolitan area. The risk of arbovirus introduction via travellers into Catalonia is the most likely considering that Barcelona is one of the world's most popular tourist destination, with the seventh busiest airport in Europe [243]. It is receiving every year travellers from areas with active arbovirus transmission. The overall incidence of DENV and CHIKV was 0.19 imported cases/10,000 inhabitants-year [244].

In Spain, chikungunya and dengue are included in the Mandatory Notification Disease [245]. This epidemiological surveillance is useful for early detection of local outbreaks. To date, locally transmitted cases of chikungunya have not been reported, although imported cases are regu-larly detected. By contrast, two small clusters of dengue fever were detected in 2018 in Spain, in Andalusia and Catalonia. Moreover, a high flow of travellers returning from endemic areas might introduce such viruses in a silent way (asymptomatic and misdiagnosed cases). Catalonia is probably one of the Spanish regions with the highest risk for arbovirus introduction and spreading due to:

1. The wide presence of a competent vector, *Ae. albopictus*. In Spain, *Ae. albopictus* was first detected in Sant Cugat del Vallès [76], a residential area in the vicinity of Barcelona, in 2004. Since then, *Ae. albopictus* has been established along the Mediterranean coast and gradually spread to the south of the country, the Balearic Islands and the Basque Country [246].

2. Presence of a naïve and susceptible human population. Catalan population has not been exposed to arboviruses and consequently lacks herd immunity. Last arboviral outbreak in Barcelona city dates from 1870 for Yellow fever virus [247].

3. Regular arbovirus importation events. Arbovirus importation is regularly reported by the introduction of viraemic passengers travelling from endemic countries. Other likely routes of introduction might be: i) importation of infected mosquitoes aboard aircraft or ships, and ii) viraemic non-human hosts (such as viraemic migratory birds). The entry of infected mosquitoes is less likely than viraemic travellers, however, cases of malaria

of malaria contracted in airports and ports from infected mosquitoes have been reported [248]. Surveys at international airports have found adult mosquitoes of *Ae. aegypti*, Culex quinquefasciatus (Say, 1823) and Anopheles gambiae (Meigen, 1818), aboard aircraft arriving from countries where MBDs are endemic [249]. In addition, Catalonia has one of the most important Mediterranean wetlands (Delta del Ebro), which serve as major rallying points for bird migration between Africa and Europe [250]. These routes have the potential of dispersing pathogens that can be dangerous for public and animal health [250].

4. Environmental conditions. The Mediterranean climate offers suitable conditions for rapid population growth during the summer season and enables successful overwintering of European populations of *Ae. albopictus* in mild and wet winters [251]. Several studies have previously highlighted the increasing climatic suitability for *Ae. albopictus* in Europe [252, 253], and projected the re-establishment of *Ae. aegypti* in the coastal zones of Europe in 2080 because of climate change [254, 255].

5. The proximity to Africa. The continent with the highest MBDs burden [256]. Because of the short geographical distance between southern Spanish coasts and northern Africa, the high rate of immigration has increased the likelihood of introduction of arboviruses into Spain and Catalonia in the north.

Chapter 2

PROJECT SUMMARY AND OBJECTIVES

" El objetivo es importantes pero no tanto como el camino hacia él".
Buda

PROJECT SUMMARY AND SIGNIFICANCE

Autochthonous arbovirus transmission in Spain requires virus introduction into areas where competent vectors are established. The most likely scenario is the introduction of the virus through infected travellers returning to regions of Spain where *Ae. albopictus* mosquitoes are present and active. Taking into account the genetic variation in the ability of the species *Ae. albopictus* to transmit arboviruses, the potential of Spanish *Ae. albopictus* mosquitoes to act as vectors should be demonstrated in laboratory experiments. Therefore, the first objective of this thesis is to determine the competence of the invasive mosquito *Ae. albopictus,* present in Catalonia, for two significant arboviruses, CHIKV and DENV. The vector competence experiments allow us to understand the factors that determine if a vector can become infected, spread the infection, and then transmit the virus to a susceptible host. To better evaluate the potential of *Ae. albopictus* as a vector, we investigate its competence under realistic environmental conditions mimicking the Mediterranean climate recorded in Catalonia in summer and autumn seasons.

In recent years, there have been many outbreaks of CHIKV worldwide, characterized by rapid spread, and a high proportion of symptomatic people in geographically naïve areas. The unprecedented size of these CHIKV outbreaks, as well as the severe morbidity in the affected populations, led to the National Institutes of Health of the United States (US) to declare CHIKV a high priority pathogen. To investigate the pathophysiological properties of these emerging CHIKV strains, the use of experimental animal models, such as mice and non-human primates, is required. For this reason, the second aim of this thesis was to study CHIKV infection and disease in a mouse model.

Understanding the transmission cycle of CHIKV is critical to evaluate how it might establish and persist in areas where it is not endemic yet, such as in the Mediterranean region. Currently, there is little information about CHIKV transstadial transmission (i.e., the passage of a pathogen from one life stage to the next) in *Ae. albopictus*. Therefore, the third aim of this thesis was studying whether CHIKV may persist through immature life-stages of a Spanish strain of *Ae. albopictus* mosquitoes.

To achieve the general goals, the following five objectives were formulated:

OBJECTIVES

• Objective 1

To assess how seasonal patterns (summer and autumn) of Mediterranean climate affect life cycle history traits in *Aedes albopictus* (Chapter III).

• Objective 2

To evaluate how seasonality, viraemia and viral strains modulate susceptibility of infection of *Aedes albopictus* for Chikungunya virus (Chapter III).

• Objective 3

To assess the virulence and pathogenesis of two Chikungunya virus strains in a mouse model (Chapter IV).

• Objective 4

To measure Chikungunya virus transmission by *Aedes albopictus* (Chapter V).

• Objective 5

To assess the susceptibility of *Aedes albopictus* mosquito larvae to Chikungunya virus (Chapter VI).

• Objective 6

To determine the vector competence of *Aedes albopictus* for Dengue virus in the summer season (Chapter VII).

Chapter 3

Chikungunya virus infection in *Aedes albopictus* is modulated by blood meal viraemia, viral strain and seasonal temperature.

"Now let us consider the seasons and the way we can predict whether it is going to be a healthy or an unhealthy year"

Hippocrates

1.ABSTRACT

Background

Chikungunya virus (CHIKV) has emerged in Europe, particularly in the Mediterranean Basin where the vector *Aedes albopictus* is established. Viraemic travellers regularly introduced the virus into areas where environmental conditions are favourable for transmission. Few studies are investigating the vector competence of *Ae. albopictus* to assess the risk of CHIKV outbreaks in temperate zones. The present study assesses the effect of three variables on the vector competence of *Ae. albopictus*; the level of viraemia, the viral strain, and the local environmental conditions.

Principal findings

A Spanish strain of *Ae. albopictus* was susceptible to CHIKV infection under simulation of summer and autumn environmental conditions. We found that the level of viraemia influenced the rates of infection (IR) and disseminated infection (DIR) of CHIKV. The equivalent of a residual viraemia in humans was able to trigger an infection pattern equivalent to that of mosquito females exposed to a high viral dose that developed a high-level infection called "strongly susceptible" ($\geq 6 \log_{10}$ GEC), which are expected to transmit the virus. The IRs in *Ae. albopictus* mosquitoes were significantly higher when the CHIKV mutated (E1-226V) strain was tested, and when autumn conditions were simulated. Especially, mosquitoes reared in autumn conditions and with a lower initial virus dose resulted in higher IRs and DIRs for the CHIKV mutated strain (E1-226V) compared to the summer profile rates. Furthermore, we found that *Ae. albopictus* showed two distinct profiles of infection for any of the CHIKV strains (with and without the E1-A226V mutation): either females strongly susceptible or females weakly susceptible to infection.

Conclusions

The results of the present study suggest that *Ae. albopictus* could become infected and develop a disseminated infection, a prerequisite for transmission, in summer and autumn conditions, even from human residual viraemia. Additional studies are needed to evaluate the viral transmission by this mosquito strain further.

Author summary

Incidence of mosquito-borne neglected tropical diseases is becoming more common in Europe due to the increase of international travels, the immigration from endemic areas, and the expanding geographic range of vectors. Autochthonous transmission of Chikungunya virus (CHIKV) during the past decade has highlighted the vulnerability of Europe to arboviruses transmitted by Aedes mosquitoes, especially in temperate regions where *Ae. albopictus* is established. In this study, we proved the susceptibility to CHIKV infection of a Spanish strain of *Ae. albopictus* while studying the effect of three factors: level of viraemia, viral strain and seasonal environmental conditions. Our findings suggest that *Ae. albopictus* could become infected with CHIKV and develop a disseminated infection in summer and autumn seasons in temperate regions. This information should be considered to assess more accurately the risk of CHIKV transmission.

2. INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus (Alphavirus genus, *Togaviridae* family) transmitted to humans by the bite of Aedes mosquitoes, primarily *Aedes aegypti* (Linnaeus, 1762) and *Aedes albopictus* (Skuse, 1894) [135]. Since it was first isolated in 1953 in Tanzania [100], CHIKV outbreaks have cyclically emerged across the African and Asian continents [257]. According to the virus geographical distribution, three major phylogenetic groups have been described: West African, East/Central/South African (ECSA), and Asian lineages [105]. In 2005, ECSA CHIKV genotype emerged in the Indian Ocean islands. This epidemic was associated with the emergence of a novel viral strain adapted to an alternative vector: *Ae. albopictus*. The new strain presented an amino acid mutation from alanine (A) to valine (V) at position 226 of the E1 glycoprotein (E1-A226V) that increased viral replication in *Ae. albopictus* [116]. This adaptation to *Ae. albopictus* boosted the global expansion of CHIKV to new naïve areas through viraemic travellers [258]. Unexpectedly, the autochthonous transmission of CHIKV was reported in Mediterranean areas of Europe where the vector *Ae. albopictus* have been established. The first European epidemic of CHIKV was confirmed in 2007 in Italy. This outbreak was associated with the introduction of the new viral CHIKV strain (E1-226V) by a viraemic traveller returning to Italy from India [121]. Later, two new CHIKV outbreaks were reported in France during 2010 and 2014. The first local episode was due to the wild-type virus, characterized by the presence of an alanine (E1-226A) [123], whereas the second showed the alanine to valine substitution (E1-A226V) [122]. Recently, in 2017, clusters of autochthonous chikungunya cases were reported in Italy, where the isolates did not carry the E1-A226V mutation [125], and in France where the isolates did contain the substitution (E1-226V) [126]. This ongoing CHIKV transmission in the Euro-Mediterranean area highlights the vulnerability of the region to new local CHIKV events. In Spain, *Ae. albopictus* was first detected in Catalonia in 2004 [76], and since then, it has been established on the Mediterranean coast [246]. Cases of traveller-imported CHIKV are regularly reported [259] although there is no evidence of autochthonous transmission to date. To assess the risk of local transmission events, it is essential to evaluate the vector competence (VC) of local mosquito populations. Most VC studies for CHIKV have been performed at constant (28 ºC) tropical temperatures (reviewed in [260]), thus approximating the mean temperature in tropical countries where CHIKV has been circulating. Only a few studies have evaluated the VC of European *Ae. albopictus* mosquitoes for CHIKV using climatic field conditions [261-263]. There are still gaps in the knowledge about the risk posed by the arrival of a viraemic traveller exposed to *Ae. albopictus* according to the level of viraemia, the strain infecting the

traveller and the season. Moreover, the likelihood of CHIKV transmission in a scenario of low or residual host viraemia has not been tested in Europe. Therefore, we performed a set of experiments mimicking field conditions to evaluate the risk of CHIKV transmission in Spain. The specific aims of this study were to assess the effect of three factors on the VC for CHIKV: the level of viraemia, the viral strain and the environmental conditions. To reproduce the effect of field conditions in the laboratory, the experiments were carried out with: i) a strain of *Ae. albopictus* derived from local mosquito populations in Catalonia (NE Spain), ii) the use of the environmental conditions of interest during the whole mosquito life cycle (egg, larva, pupa and imago), representing two climatic seasons (summer and autumn) of the Mediterranean-type climate, and iii) infectious blood meals reproducing the peak of viraemia corresponding to the acute phase of CHIKV infection in humans, and the estimated viral load during the transient-residual viraemia of the recovery phase.

3.MATERIAL AND METHODS

3.1. Meteorological data

Two environmental profiles (summer and autumn) were defined using three variables: temperature, photoperiod and relative humidity (RH). The profiles simulate the field conditions in which *Ae. albopictus* imagoes can be found in Catalonia (NE Spain) during summer and autumn. Climatic data were obtained from the Catalan Meteorological Service (http://www.meteo.cat).

Day and night mean temperature, RH and photoperiod were calculated for each seasonal profile. Summer profile was inferred from July average temperatures, with a temperature gradient from 26 ºC (day) to 22 ºC (night), a photoperiod of 14h: 10h (light: dark), and 86% RH. Autumn profile dataset was inferred from October average temperatures, with a temperature gradient from 18 ºC (day) to 15 ºC (night), a photoperiod of 12h: 12h (light: dark), and 76% RH. July and October were the representative months for CHIKV outbreaks in Europe during the summer and autumn seasons, respectively [264].

3.2. Mosquitoes

Mosquito strains

Aedes albopictus strain was collected in Sant Cugat del Vallès (Catalonia, Spain) in 2009 and reared since then as a laboratory colony. *Aedes aegypti* strain was kindly provided from Bayer Bioagents (Germany) where it was kept as a regular laboratory colony. Both mosquito strains were screened for alphavirus and *flavivirus* by reverse transcription nested polymerase chain reaction (RT-nPCR) [265, 266] and confirmed to be non-infected. The laboratory colony of *Ae. aegypti* was included to provide a comparison with the results of *Ae. albopictus*.

Mosquito rearing

Aedes albopictus and *Ae. aegypti* strains were reared under standard laboratory conditions (25 ºC, 80% RH, and 14h: 10h (light: dark)) before the experiments. Eggs with embryos of the mosquito generation to be used in the experiments were incubated in water inside climatic cabinets with the specific environmental conditions (summer/autumn). After egg hatching, 200 first-instar larvae (L1) were transferred into a new tray containing 1 litre of dechlorinated water

and fed with pellets (Tetra Min®). Dead larvae were removed daily, and mortality recorded, including missing larvae due to cannibalism (replicates of trays per environmental condition ranged between 25 and 32). Date of the first pupation was recorded and pupae transferred inside a 30×30×30 cm BugDorm cage® (Megaview Ltd., Taiwan) for adult emergence. Date of first imago emergence was recorded using a group of approximately 1,600 pupae for the summer season and 300 pupae for the autumn season. Adult mosquitoes were fed with sucrose (10%) *ad libitum*. Groups of 65 ± 16 female 7-15- day-old mosquitoes were transferred inside plastic cages and starved for 24h prior to blood feeding. Immature stages and adults were reared with the same environmental profile in a climatic cabinet.

3.3. Viruses

Viral strains

Two CHIKV strains of the ECSA lineage were used: S27 Petersfield and ITA1_TAM_E1, hereafter named S27 and ITA, respectively. The amino-acid sequences of these strains differ by three changes in the E1 glycoprotein. The main difference is the change at the position 226: S27 harbours an alanine (E1-226A), whereas ITA harbours a valine (E1-226V). Other two changes have been described [267] at E1-269 (methionine for S27 and valine for ITA) and E1-284 (aspartic acid for S27 and glutamic acid for ITA). The strain S27, (GenBank AF345888), was isolated from a febrile patient during the 1953 Tanzania outbreak. It was kindly provided by the Department of Arboviruses and Imported Viral Diseases of the National Centre for Microbiology, Institute of Health Carlos III (CNM-ISCIII), Madrid, Spain. The emergent ITA strain (GenBank EU188924) was isolated in Italy from an imported case returning from Mauritius in 2006. It was kindly provided by the Lazzaro Spallanzani National Institute for Infectious Diseases (INMI), Rome, Italy. Viral stocks were produced in Vero cells (African green monkey kidney cell line), kindly provided by Dr. Joan Pujols (*Institut de Recerca i Tecnologia Agroalimentàries- Centre de Recerca en Sanitat Animal (IRTA-CReSA)*) and were stored at -80 ºC until used for mosquito experimental infection assays.

Viral dose

Infectious blood meals for mosquitoes were adjusted to two viral loads. The higher viral load was representative of viraemia levels observed in patients during the acute phase [268] and was adjusted to 6.5 $\log_{10}$ $TCID_{50}$ /mL. The lower viral load simulated the residual viraemia

[92] and was adjusted to 3.5 $\log_{10}$ $TCID_{50}$ / mL. Blood meals were prepared by mixing heparinized bovine blood with a virus suspension and supplemented with 5×10^{-3} M of ATP (Sigma-Aldrich Corp., U.S.A.) as a phagostimulant.

3.4. Experimental infections

Design

Experimental infections were performed to evaluate the influence of CHIKV strain (S27 and ITA), viral load (high and low) and environmental condition (summer and autumn profiles) on *Ae. albopictus* and *Ae. aegypti* infection.

Procedure

Female mosquitoes were allowed to feed on infectious blood meals through the membrane using a Hemotek feeding system (Discovery Workshops, UK) with 1-day-old SPF chicken skin as a membrane. Fully engorged females (FEFs) were selected under CO2 anaesthesia. For each experimental condition, 5-10% of FEFs were randomly collected and killed as day 0 control samples. The remaining FEFs were transferred either individually in cardboard cages (Watkins & Doncaster, U.K.) or inside plastic cages in groups of 5-10 mosquitoes with sucrose (10%) *ad libitum*. Females were kept inside the climatic cabinet for an extrinsic incubation period (EIP) of 9 days after which alive mosquitoes were killed by freezing at −80 ºC. Experimental infections were performed at IRTA-CReSA Biosafety Level 3 (BSL-3) facilities.

3.5. Samples

Mosquitoes sampled at day 0 were individually transferred into tubes with 0.5 mL Dulbecco's modified Eagle's medium (DMEM) and frozen at -80 ºC to evaluate the initial viral dose ingested. Each mosquito sampled at 9 days post-exposure (dpe) was dissected by separating legs from the rest of the body. Both samples (legs and body) were transferred into tubes with 0.5 mL DMEM. All mosquito samples were homogenized at 30 Hz for 1 min using TissueLyser II (Qiagen GmbH, Germany) and stored at −80 ºC until tested for CHIKV. Infection rate (IR) was calculated as the number of CHIKV-positive bodies divided by the number of engorged mosquitoes. Disseminated infection rate (DIR) was calculated as the number of the CHIKV-

positive leg samples divided by the number of mosquitoes with CHIKV-positive bodies.

3.6. Viral detection

Viral RNA was extracted using NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. The presence of CHIKV RNA was detected by an in-house quantitative real-time RT-PCR (RT-qPCR) used in the routine diagnostic of CHIKV infection by the National Centre for Microbiology, Institute of Health Carlos III (CNM-ISCIII). A standard curve was generated using duplicates of 10-fold serial dilutions of DNA plasmid containing a partial region of the CHIKV genome. Quantification of viral RNA was performed by comparison of the threshold cycle (Ct) values of the samples to the standards according to the Ct analysis and expressed as genome-equivalent copies (GEC). Limit of detection was 5.37 GEC of plasmid per reaction.

3.7. Ethics statement

This study was carried out following the European Directive 2010/63/EU, which aims primarily at guaranteeing the protection of animals used in research. In this study, bovine blood samples using to feed mosquitoes were collected from the jugular vein of a cow at the *Universitat Autònoma de Barcelona*. One-day-old specific pathogen-free (SPF) chicken skins were obtained from a parallel experiment, in which variation in egg fertility forced rationalized excess in the number of incubated individuals. SPF eggs were purchased from VALO BioMedia GmbH (Osterholz-Scharmbeck, Germany) and euthanasia of 1-day-old chickens was performed by cervical dislocation by trained personnel to ensure that the procedure was performed safely and correctly. All procedures were approved by the Comissió d'Experimentació Animal i Humana de la *Universitat Autònoma de Barcelona* and the *Comissió d'Experimentació Animal de la Generalitat de Catalunya* with protocol number 4239.

3.8. Statistical Analyses

We compared the duration of the larval and pupal development periods in *Ae. albopictus* and *Ae. aegypti*, both in summer and autumn, using either the t-test or the non-parametric Mann-Whitney test, depending on the normality of the data. Normality of data was assessed using the Shapiro-Wilk test. The percentages of mortality during larval and pupal development in summer and autumn were compared using Pearson's Chi-squared test. Differences in the mean $\log_{10}$ viral loads ingested at day 0 between species (*Ae. albopictus* and *Ae. aegypti*) and seasons (summer and autumn) were evaluated using a linear regression model. Only the mosquitos fed with a high viral dose (6.5 $\log_{10}$ $TCID_{50}$ /mL) were used for this analysis. The effect of mosquito species (*Ae. albopictus* and *Ae. aegypti*), environmental conditions (summer and autumn profiles), viral dose (high: $\log_{10}$ $TCID_{50}$ /mL and low: 3.5 $\log_{10}$ $TCID_{50}$ /mL) and viral strain (S27 and ITA), on the mosquito infection (IR) and dissemination (DIR) rates for CHIKV were evaluated using a logistic regression model. Furthermore, in order to assess whether CHIKV strains (S27 versus ITA) differ in their abilities to infect and disseminate in *Ae. albopictus* mosquitoes infected at high dose (6.5 $\log_{10}$ $TCID_{50}$ /mL) in a particular season (summer vs autumn), IRs and DIRs of *Ae. albopictus* were compared using Pearson's Chi-squared test with Yates' continuity correction. For small sample sizes (less than 5 individuals in one of the categories), Fisher's Exact Test was employed. Finally, to estimate the mean differences in $\log_{10}$ viral loads between the body and the legs for the different combinations of species (*Ae. albopictus* and *Ae. aegypti*), season (summer and autumn) and strain (S27 and ITA), viral loads were evaluated using either the t-test or the non-parametric Mann–Whitney tests, depending on the normality of the data. Based on those plots, a standard cut-off value of 6 for the $\log_{10}$ viral load was selected. Besides, the correlation in the $\log_{10}$ viral loads detected in the body and the legs of mosquitoes were also evaluated. In the case of normally distributed data, the Pearson correlation test was used, while for non-normally distributed data, the non-parametric Spearman rank-correlation test was employed. Normality of $\log_{10}$ viral loads was assessed using the Shapiro-Wilk test. All statistical analyses were carried out using R statistical software (http://cran.r-project.org/), and figures were created with GraphPad Prism version 7.04 (GraphPad Software, La Jolla CA, USA www.graphpad.com).

4. RESULTS

4.1. Mosquito development and survival

The mean development time, from egg hatching to female emergence was estimated in summer and autumn season, in both *Ae. albopictus* and *Ae. aegypti* mosquitoes. An inverse correlation was detected between larval rearing temperature and development time (Table 3.1).

	Ae. albopictus			*Ae. aegypti*		
	Summer (mean days)	**Autumn (mean days)**	**Significance (p-value)**	**Summer (mean days)**	**Autumn (mean days)**	**Significance (p-value)**
Egg - L1	2.00	14.25	6.7e-07	1.33	12.00	1.1e-03
Δt L1 - pupa	6.35	22.56	1.9e-11	5.76	17.62	2.0e-12
Δt pupa - ♀ adult	2.50	8.50	4.7e-02	2.50	8.50	4.7e-02

Table 3.1. Effect of environmental conditions (summer vs autumn) on development time (days)

Therefore, autumn profile temperatures increased the development time of larval and pupal stages, whereas the summer profile shortened development times. Temperature also significantly affected the survival of immature stages (Table 3.2).

	Ae. albopictus			*Ae. aegypti*		
	Summer (%)	**Autumn (%)**	**Significance (p-value)**	**Summer (%)**	**Autumn (%)**	**Significance (p-value)**
L1 - pupa	0.95	4.68	< 2.2e-16	0.54	25.51	< 2.2e-16
Pupa - ♀ adult	6.89	44.79	< 2.2e-16	2.94	45.45	< 2.2e-16

Table 3.2. Mortality of the immature stages of *Ae. albopictus* and *Ae. aegypti* in summer and autumn conditions.

The mortality rates for immature stages of *Ae. albopictus* and *Ae. aegypti* was significantly higher ($p < 0.05$) for autumn conditions compared to summer, especially for the pupal stage.

4.2. Quantification of infectious dose exposure

The environmental rearing condition significantly influenced the amount of virus originally ingested by the mosquitoes. The linear regression model indicated that there were no differences in the mean $\log_{10}$ viral loads ingested between *Ae. albopictus* and *Ae. aegypti*. In contrast, statistically significant differences were detected in the mean $\log_{10}$ viral loads ingested by females depending on the season (p= 0.009), with higher mean values for summer (6.2 $\log_{10}$ GEC; Table 3.3) than for autumn (5.9 log10 GEC; Table 3.3).

	Season	CHIKV strain	Viral load ($TCDI_{50}$/mL)	Nº ♀ tested	GEC ($\log_{10}$)
Ae. albopictus	Summer	S27	6.5	7	6.08± 0.33
		ITA	6.5	13	6.41± 0.25
	Autumn	S27	6.5	5	5.56± 0.19
		ITA	6.5	6	6.21± 0.31
Ae. aegypti	Summer	S27	6.5	4	6.10± 0.50
		ITA	6.5	4	6.60± 0.50
	Autumn	S27	6.5	4	5.83± 0.30
		ITA	6.5	4	6.29± 0.41

Table 3.3. CHIKV RNA quantified on whole mosquito females.

4.3. Vector competence

Vector competence for CHIKV was examined under 16 experimental conditions that resulted from the combination of 4 factors: viral load (high: 6.5 $\log_{10}$ $TCID_{50}$/mL, low: 3.5 $\log_{10}$ $TCID_{50}$/mL), viral strain (S27, ITA), mosquito species (*Ae. albopictus, Ae. aegypti*) and environmental condition (summer and autumn profiles) (Table 3.4).

Table 3.4. Infection and dissemination rates of *Ae. albopictus* and *Ae. aegypti* mosquitoes after CHIKV exposure

				Infection rate (IR)			Disseminated infection rate (DIR)			
Mosquito strain	Season	CHIKV strain	Titre ($TCID_{50}$/mL)	♀ tested	♀ Inf, IR (%)	GEC_{INF}	♀ Inf	♀ Dis, DIR (%)	$GEC_{DIS\ (body)}$	$GEC_{DIS\ (legs)}$
Ae. albopictus	Summer	S27	6,5	40	19 (48%)	7,49±2,88	19	16 (84%)	8,01±1,86	7,03±2,31
			3,5	69	0 (0%)	na	0	0 (0%)	na	na
		ITA	6,5	76	51 (67%)	8,06±1,38	51	24 (47%)	8,59±2,01	7,13±1,93
			3,5	85	4 (5%)	2,35±0,21	4	1 (25%)	8,92	7,27
	Autumn	S27	6,5	26	10 (38%)	6,33±0,70	10	3 (30%)	6,63 *	6,87±2,43
			3,5	16	0 (0%)	na	0	na	na	na
		ITA	6,5	50	41 (82%)	7,13±1,76	41	26 (63%)	7,95±0,81 **	6,45±1,78
			3,5	46	1 (2%)	6,31	1	0	na	na
Ae. aegypti	Summer	S27	6,5	40	1 (3%)	5,7	1	0 (0%)	na	na
			3,5	83	0 (0%)	na	0	0 (0%)	na	na
		ITA	6,5	39	3 (8%)	2,88±0,09	3	1 (33%)	9,1	6,6
			3,5	81	0 (0%)	na	0	na	na	na
	Autumn	S27	6,5	24	6 (25%)	4,96±1,37	6	3 (50%)	7,98±0,22	7,13±0,28
			3,5	26	0 (0%)	na	0	na	na	na
		ITA	6,5	36	15 (42%)	6,77±1,86	15	6 (40%)	8,97±2,82	7,68±2,60
			3,5	19	1 (5%)	2,13	1	0 (0%)	na	na

♀ Inf: number of infected females; ♀ Dis: number of females with a disseminated infection; *$GEC_{DIS\ (body)}$ calculated with 1 mosquito body instead of 3; **$GEC_{DIS\ (body)}$ calculated with 24 mosquito bodies instead of 26; These mosquito bodies (N=4) were employed for the experiment described in Chapter VI. na: not applicable.

The results of the logistic regression model indicated statistically significant differences in the rates of infection depending on several factors. In contrast, the differences in the rates of dissemination did not vary significantly between viral doses, CHIKV strains, mosquito strains or environmental conditions. This lack of statistical significance could be due to the smaller sample sizes available.

Viral dose

Blood meal viral load was the factor that most influenced mosquito IRs and DIRs for CHIKV. Blood meals with high viral load (6.5 $\log_{10}$ $TCID_{50}$ /mL) produced significantly higher mosquito IRs than those with low viral load (3.5 $\log_{10}$ $TCID_{50}$ /mL) (OR = 66.1; CI 95% = 30.4–174.4; p <2e-16) (Table 3.4).

Viral strain

At high dose, ITA strain produced significantly higher IRs than S27 strain (OR = 2.5; CI 95%= 1.6–4.2; p= 0.0002) (Table 3.4).

Mosquito strain

The IRs were significantly higher in *Ae. albopictus* as compared to *Ae. aegypti* (OR = 6.2; CI 95%= 3.7–10.4; p= 3.9e-12) (Table 3.4).

Environmental condition

Overall, IRs were significantly higher in autumn as compared to summer (OR = 2.1; CI 95%= 1.3–3.4; p= 0.002) (Table 3.4).

When we look at *Ae. albopictus* alone, IR and DIR were higher in summer (48%; 84%) compared to autumn (38%; 30%) for S27 strain (Table 3.4). The differences were not statistically significant for IR (p= 0.47), but were statistically significant for DIR (p= 0.011). In contrast, ITA strain displayed higher IR and DIR in autumn (82%; 63%) compared to summer (67%; 47%), although neither difference was statistically different (IR p= 0.065; DIR p= 0.176) (Table 3.4). When comparing DIRs according to the viral strain (S27 vs ITA), we found that viral dissemination of S27 strain was significantly higher (p= 0.006) than ITA strain (84% vs 47%) in summer conditions, whereas for *Ae. albopictus* infected with ITA strain, dissemination was more likely in autumn than in summer conditions (63% vs 30%), although the difference was not statistically significant (p= 0.079).

When examining viral loads in body and legs we observed that both mosquito species infected with blood meal at high viral dose exhibited two distinct profiles of infection for both CHIKV strains (Figure 3.1).

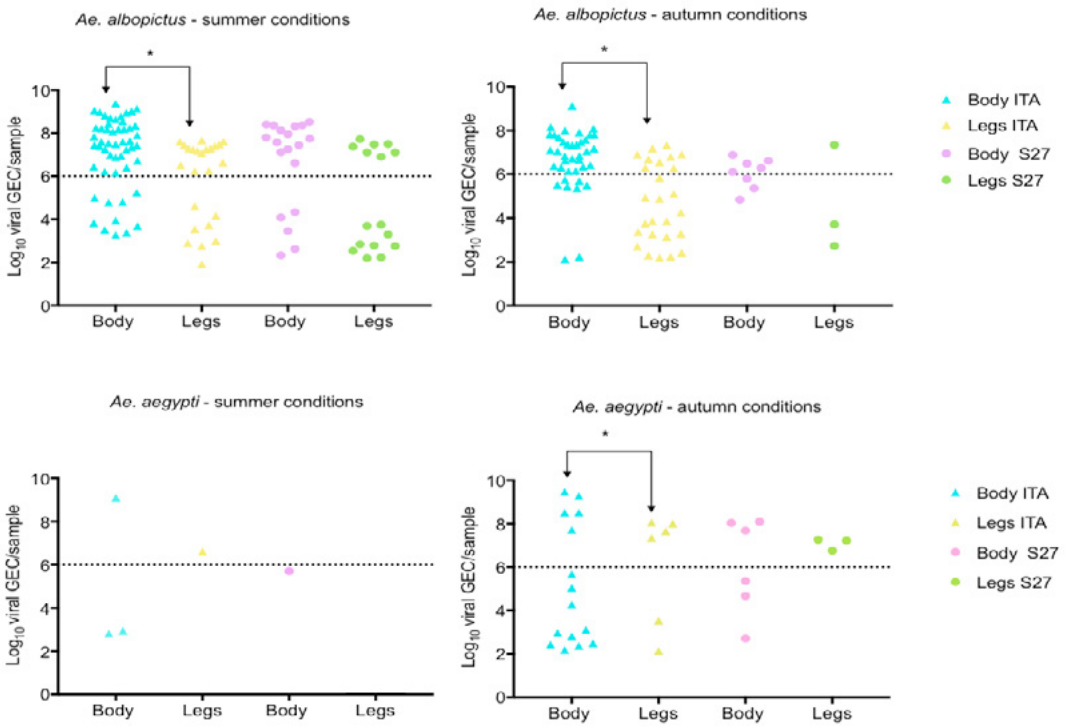

Figure 3.1. Viral load (log_{10} GEC) in bodies and legs of *Ae. albopictus* and *Ae. aegypti* infected with a high viral titre. Asterisk symbols indicate a significant positive correlation ($p < 0.05$) between the viral loads detected in the body and the legs. Triangle represents samples of the virus strain ITA and circles represent samples of the virus strain S27. Dotted lines represent the cut-off value of the viral load.

The first pattern showed either *Ae. albopictus* females (6.11 log_{10} to 9.37 log_{10} GEC/body and 6.22 log_{10} to 8.06 log_{10} GEC/legs) or *Ae. aegypti* females (7.68 log_{10} to 9.48 log_{10} GEC/body and 6.6 log_{10} to 8.06 log_{10} GEC/legs) with high viral replication levels. The second pattern showed either *Ae. albopictus* females (2.12 log_{10} to 5.80 log_{10} GEC/body and 1.92 log10 to 5.84 log10 GEC/legs) or *Ae. aegypti* females (2.18 $log_{10}$to 5.70 log_{10} GEC/body and 2.13 log_{10} to 3.53 log_{10} GEC/legs) with low viral replication levels. A cut-off value of 6-log_{10} viral load was selected. For *Ae. albopictus*, statistically significant differences ($p < 0.05$) were observed in the mean log_{10} GEC between the groups with high and low replication levels, in both the body and the legs, in both summer and autumn conditions and for the two CHIKV strains. The results also indicated significant positive correlations between the viral loads detected in the body and legs

of *Ae. albopictus* infected with ITA strain using summer conditions (p= 0.0006) and also under autumn conditions (p= 0.0002) (Fig 3.1). Moreover, there was a positive correlation, although not statistically significant (p= 0.08), in the viral loads detected in the body and the legs of *Ae. albopictus* infected with S27 strain using summer conditions. There was an insufficient number of samples to evaluate the relationship between body and legs infected with S27 strain in autumn conditions.

For *Ae. aegypti*, significant positive correlations between the viral loads detected in body and legs were only detected in the mosquitoes infected with ITA strain under autumn conditions (p= 0.016) (Fig 3.1).

5. DISCUSSION

This study aimed to assess some specific parameters that may affect the susceptibility of mosquitoes to be infected by CHIKV. We assessed the VC of a Spanish strain of *Ae. albopictus* by estimating the proportion of mosquitoes with disseminated infection. This approximation gives an estimate of transmission likelihood under the framework of the four following questions:

1. Are Chikungunya-infected humans infectious to mosquitoes during the recovery phase?

Two viral loads were tested, a "high" viral load (6.5 $\log_{10}$TCID$_{50}$ /mL) and a "low" viral load (3.5 $\log_{10}$TCID$_{50}$ /mL). Both viral loads are representative of the range of viraemia titres detected in infected travellers returning to Europe or the United States (US) from endemic countries [268, 269]. Our results showed that the level of viraemia influenced the rates of infection and disseminated infection of CHIKV. Low-level viraem ia (3.5 $\log_{10}$ TCID$_{50}$ /mL) limited the infection rate (0-5%) for both *Aedes* species. Considering that a female imbibes approximately 5 μL of blood per feeding [270], there will be mosquitoes imbibing very few virions or none, and hence the probability for a mosquito to become infected would be low but not negligible. The low viral load employed might be close to the minimum threshold necessary to trigger an infection, and it is similar to others previously defined for different *Ae. albopictus* populations from the US (≈ 4 $\log_{10}$ PFU/mL; [271, 272]). Moreover, the number of GEC measured (8.92 $\log_{10}$ GEC/body; 7.27 $\log_{10}$ GEC/legs) in the *Ae. albopictus* infected with low-level viraemia was indicative of high CHIKV replication level in the mosquito, equivalent to the infection pattern of females exposed to high-level viremia which showed high viral loads (≥ 6 $\log_{10}$ GEC) described above (Fig 3.1) which are expected to have a high probability of transmitting the virus. Therefore, as the risk of infection is low but not negligible, it would be highly recommended that patients in a recovery phase should use repellents until complete viraemia clearance is guaranteed to minimise mosquito infection risk.

2. Do the S27 and ITA CHIKV strains pose the same risk if introduced into Spain?

We found that IRs were significantly higher with the variant ITA strain compared to the S27 strain. Therefore, CHIKV mutation (E1-A226V) of the ITA strain conferred a selective advantage at the midgut infection barrier level in *Ae. albopictus* compared with the S27 strain (E1-226A) regardless of the season. We also observed that viral dissemination differed according to CHIKV strains (S27 vs ITA) and environmental conditions (summer vs autumn). The disseminated infection rate was significantly higher for S27 in summer conditions compared to autumn conditions, whereas, DIR was higher (but not significantly) for ITA in autumn conditions compared with summer. However, comparable DIRs (95% and 100%) between CHIKV strains of the ECSA lineage (with and without the E1-A226V mutation) were detected in European *Ae. albopictus* populations (from France and Italy) at constant temperatures of 28 ºC and 26 ºC, respectively [273, 274]. These differences between our results and the other European populations may be caused by the different environmental conditions assayed, but differences among the vector geographic populations and the viral strains may have played a role. These observations could suggest that the risk for autochthonous transmission may be similar regardless of the CHIKV strain introduced. Further studies are necessary to investigate what vector-virus combinations may be more efficient at maintaining transmission in temperate areas.

3. Does the tested *Ae. albopictus* strain experience efficient infection and dissemination when exposed to CHIKV?

The results of our study demonstrated that the Spanish strain of *Ae. albopictus* tested was susceptible to CHIKV infection in summer and autumn conditions. Both CHIKV strains, S27 (E1-226A) and ITA (E1-226V) were efficiently disseminated within the mosquito hemocoel, which is a standard proxy for their mosquito-to-human transmission potential. The mean viral particles in mosquitoes differed between seasons, likely due to the influence of temperature on the speed of viral replication. Overall, viral load was higher in summer than in the autumn season, except for Ae. aegypti infected with CHIKV ITA strain. We found that *Ae. albopictus* was more susceptible to CHIKV infection than Ae. aegypti, as seen in other studies [275, 276]. Both mosquito species exhibited two CHIKV infection patterns (previously described by Vazeille *et*

al., [277]): i) females strongly susceptible (≥ 6 $\log_{10}$ GEC) and ii) females weakly susceptible to infection (< 6 $\log_{10}$ GEC). A similar CHIKV infection profile was also described in Ae. aegypti [278]. Another VC study on the same *Ae. albopictus* colony yielded two distinct infection patterns for the West Nile virus [279].

4. Does a colder season reduce the risk for autochthonous transmission scenario in Spain?

It has previously been shown that rearing conditions experienced in the immature stages can modulate important characteristics of adult mosquitoes as body size, microbiota composition, nutrient reserves, blood-feeding behaviour, female fecundity and adult longevity [280, 281] and therefore may also affect the VC of adults mosquitoes [241, 282, 283]. Our results showed that larval rearing temperature significantly affected the blood meal size of adult mosquitoes, although the body sizes of mosquitoes were not determined. Mosquitoes reared in autumn conditions ingested significantly ($p= 0.009$) lower virus doses than mosquitoes reared in summer conditions. Higher dehydration during the starvation for mosquitoes reared in summer conditions might explain a larger blood intake (and thus of virus). Unexpectedly, mosquitoes reared in autumn conditions and with a lower initial virus dose resulted in higher IR ($p= 0.065$) and DIR ($p= 0.176$) for the ITA strain (E1-226V) compared to the summer profile. These results are consistent with another study where cooler temperatures (20 ºC *vs* 24 ºC and 32 ºC) during larval and pupal development led to enhance rates of infection and dissemination of a temperate *Ae. albopictus* population (from the US) for CHIKV E1-226V strain [282]. Similarly, cooler temperatures (18 ºC) in the adult stage increased the susceptibility of European *Ae. albopictus* populations (from Germany and Italy) for the mutated CHIKV strain (E1-226V) compared to warmer temperatures (24 ºC) [261]. Other studies [262, 263] showed that CHIKV strains of the ECSA lineage, with and without the E1-A226V mutation, were efficiently disseminated and transmitted by European *Ae. albopictus* populations from France and Italy at low temperatures (20 ºC). Taken together, these data suggest that, cooler temperatures during immature and adult development are associated with enhanced susceptibility of *Ae. albopictus* to CHIKV. These results suggest that the ongoing spread of *Ae. albopictus* to colder regions of Spain and central Europe might pose a threat.

The enhancement of VC by cooler temperatures is not exclusive to CHIKV and *Aedes* mosquitoes, as it has been described for other virus-vector systems (18-19 ºC) [241, 284-286]. Several hypotheses have been proposed to explain why mosquitoes exposed to colder temperatures were more susceptible to arbovirus infection. Mosquitoes reared at lower temperatures may have: i) 'either a greater number of receptors or an enhancement in virion binding, which, in either case, would make their midgut cells more susceptible to viral infection' [241], ii) a milder innate immune response of RNA interference (RNAi) due to the slow viral replication and the inhibition of the RNAi at cold temperatures [223, 287], and iii) a change in the composition or density in the gut microbiota that may alter mosquito VC [228, 288].

Our study highlights that, in temperate regions, seasonal differences in temperature affected mosquito life-history traits and susceptibility to CHIKV infection in *Ae. albopictus*. Our results show that a Spanish *Ae. albopictus* strain may become infected during summer and autumn from viraemic travellers even from a residual viraemia. In addition, mosquitoes developed a disseminated infection that might be used as a proxy to estimate transmission.
The results of this study provide new data regarding how the susceptibility of *Ae. albopictus* to CHIKV infection can be modulated by the tripartite interactions between the virus, environmental variables and the mosquito. This knowledge will contribute to enhancing risk assessment studies and mathematical models to forecast the transmission dynamics of CHIKV in temperate regions once the virus is introduced. Consequently, we expect that tailored protocols and surveillance programs could be improved considering the seasonality of climate, mosquitoes and dynamics of viraemic travellers.

ACKNOWLEDGEMENTS

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Chapter 4

Comparative virulence of two strains of the East/Central/South African (ECSA) lineage in an IFN-α/β receptor-deficient mouse model infected via the subcutaneous route

"Le virus est constitué par un parasite microscopique qu'on multiplie aisément par la culture, en dehors que le mal peu frapper"

Louis Pasteur

1. ABSTRACT

Background

Before the recent epidemics of Chikungunya virus (CHIKV) in the Indian Ocean islands, India, Europe and America, there were few animal models of CHIKV infection, so the pathogenesis of disease in humans was poorly understood. In this study, we deepen in the knowledge of CHIKV infection and pathogenesis using a mouse model.

Methods

Alpha/beta interferon receptor-deficient (IFN-α/β R-/-) mice (A129 background) were subcutaneously inoculated with three doses (10^2, 10^4 or 10^6 $TCID_{50}$) of two CHIKV strains of the East/ Central/ South Africa (ECSA) lineage: the prototype strain S27 (E1-226A) and the emergent strain ITA (E1-226V). We compared the virulence levels of two strains of CHIKV ECSA lineage in terms of morbidity, mortality in A129 mice. Furthermore, the tissue tropism of the two CHIKV strains (S27 and ITA) was evaluated from several target organs: encephalon, liver, spleen, inguinal lymph nodes, and gastrocnemius muscle.

Results

The A129 mouse model was highly susceptible to CHIKV infection by the subcutaneous route, with most mice succumbing to the disease at 2-3 days post-inoculation. We found that the prototype strain S27 exhibited a higher virulence in A129 mice as compared to the emergent strain ITA, causing death approximately one day earlier. However, both strains (S27 and ITA) yielded similar tissue tropism and histopathological lesions in the target organs.

Conclusions

This mouse model (IFNα/β R-/- A129) provides valuable information on CHIKV virulence, and it may be a suitable animal for the testing of antiviral drugs and vaccines.

Keywords: Chikungunya virus, mouse model, type-I interferon, A129, virulence, tissue tropism, pathogenesis, histopathology, transmission model.

2. INTRODUCTION

Chikungunya virus (CHIKV) is an alphavirus within the *Togaviridae* family, transmitted primarily by the mosquitoes *Aedes aegypti* and *Aedes albopictus* [135]. Phylogenetic analyses based on E1 gene sequences grouped CHIK viruses into three lineages: West African, East/Central/South African (ECSA) and Asian [105]. CHIKV is very conservative, isolates from different lineages can diverge 4.4-15.5% of nucleotide sequence identity among isolates from diferent lineage, while isolates in the same lineage can share over 99.8% [289]. In 2005, the ECSA genotype (re) emerged and caused different outbreaks throughout Indian Ocean islands, the India subcontinent, Asia and Europe, driven principally by viraemic travellers [290]. In 2013, the Asian genotype of the CHIKV was the responsible for the outbreak in the Caribbean region and the American continent [124]. Typically, chikungunya infection has been associated with mild symptoms and signs such as fever, myalgia, arthralgia and sometimes rash [115]. The acute phase of the disease is usually self-limiting, resolving within 3-4 days, whereas the chronic phase is characterised by persistent polyarthralgia and joint symptoms for periods of months or even years [291, 292]. Studies conducted after CHIKV outbreaks on La Réunion Island in 2006 and Italy in 2007 showed that 37% of CHIKV cases developed a chronic inflammatory rheumatism and 14% chronic arthritis following CHIKV disease [98]. Furthermore, severe forms of CHIKV infection, involving the Central Nervous System (CNS) were described in neonates and older patients with underlying conditions during the last outbreaks [293]. Other atypical clinical manifestations (such as cardiovascular, renal, hepatic and respiratory syndromes) were also reported [294-297]. There are currently no known vaccines or antivirals against CHIKV infection. Up to the emergence in 2005, little was known about the pathogenesis of the disease. Recent experimental studies in mouse models have demonstrated that replication of the virus depends on defective type I interferon (IFN)-signaling, [290]. Adult mice with a partial (IFN-α/β R +/-) or totally (IFN-α/β R-/-) abrogated type-I IFN patway develop a mild or severe infection respectively [298].

There are limited studies evaluating the virulence of CHIKV strains within the ECSA lineage. Previous studies have found notable differences in pathogenesis and virulence between ECSA and Asian lineages. These differences have been demonstrated using *in vitro* methods [300, 301], mouse [289, 302] and non-human primate models [289]. In Europe, the autochthonous transmission of CHIKV has been driven by two different ECSA strains of the virus: with the *Ae. albopictus*–adaptative E1-A226V mutation and without this mutation [210, 215]. Therefore, we aimed to compare the virulence levels as well as the pathogenesis

between two CHIKV strains of ECSA lineage in a mouse model. We utilize deficient in type I interferon (IFN) mice which have been shown to be highly susceptible to CHIKV infection [298]. To simulate the natural route of infection, A129 mice were inoculated with CHIKV by a subcutaneous route [303, 304]. Considering that the mosquitoes inoculated a wide range of viral doses (10^2-10^6 PFU) as they feed on a vertebrate host [305], we chose to use three doses based on the titres found in saliva of CHIKV-infected *Ae. albopictus* [306]. In addition to comparing the virulence and pathogenenicity of two ECSA strains, this study serves to assess if this mouse model may be useful for establishing a mosquito-dependent CHIKV transmission cycle.

3. MATERIAL AND METHODS

3.1. Ethics estatement

This study was performed following the European Directive, 2010/63/EU on the protection of animals used for scientific purpose. Protocols were reviewed and approved by the *Centre de Recerca en Sanitat Animal (*CReSA).

3.2. Mice

Alpha/beta interferon receptor-deficient (IFN-α/β R-/-) mice (A129 background) were obtained from B&K Universal Limited (UK) and were bred in the facilities of Centre de Biotecnología Animal y Terapia Génica-CBATEG (Universitat Autònoma de Barcelona). All mice were acclimatised for one week at *CReSA* Biosafety Level 3 (BSL-3) animal facility before each challenge, and were kept in a room under controlled environmental conditions: temperature (22 ±1 ºC), humidity (50-55% RH) and photoperiod (12-hour light/dark cycle). The animals were housed at a density of 5 or 6 per cage with a pelleted diet and water provided *ad libitum*. Approximately equal numbers of male and female mice were used in each experiment.

3.3. Virus strains

Two CHIKV strains of the ECSA lineage were used: S27 Petersfield and ITA1_TAM_E1 hereafter named S27 and ITA, respectively. The main difference in the amino-acid sequences of these strains is the change at the position 226 of the E1 glycoprotein: S27 harbours an alanine (E1-226A), whereas ITA harbours a valine (E1-226V). Other two changes have been described at E1-269 (methionine for S27 and valine for ITA) and E1-284 (aspartic acid for S27 and glutamic acid for ITA). The detailed information of these CHIKV strains is provided in Chapter III.

3.4. Mouse model of CHIKV infection

In order to better mimic viral transmission by mosquito bites, virus challenge was delivered subcutaneously (s.c.) through needle injection in the ventral side area of the mouse. Sixty-four IFN-α/βR-/- A129 mice (8-10 weeks old) were anaesthetised with isoflurane (2-3%) for 2 minutes, and were inoculated with 100 µL containing 10^2, 10^4, 10^6 $TCID_{50}$ of either CHIKV strains ITA or S27, diluted in sterile phosphate-buffered saline (PBS). The control group (n= 7) was mock-injected with 100 µL of sterile PBS. Mice were divided into six groups (n= 10-12 mice/group) according to the dose (10^2, 10^4, 10^6 $TCID_{50}$) and CHIKV strain (S27 versus ITA) received. Following inoculation, mice were assessed for weight loss, signs of disease and mortality daily. All the mice were assigned a clinical score (0-3 scale) based on previous studies of CHIKV infection in IFN-α/β R-/- A129 mice (Table 4.1). [290, 298]

Clinical parameters	Range	Score
Loss of body weight	Weight loss (1-10 %)	0-3
Mobility	Normal to ataxia	0-3
Depressive behaviour	Normal to lethargy	0-3
Euphoric behaviour	Normal to hyperactivity	0-3
Evaluation of the fur	Normal to ruffled fur	0-3
Skin lesions	Normal to erythematous patch	0-3
Total score		0-18

Table 4.1. Clinical score of CHIKV disease in IFN-α/β R-/- mice

To measure viraemia, blood samples were collected daily from the facial vein. Half of the mice in each group were bled at 0, 2, and 4 days post-inoculation (dpi) and the remaining mice at 1, 3 and 5 dpi. One mouse of the control group (n= 7) was bled daily for 5 days.

Morbidity (inferred as body weight loss), viraemia, survival rate and the clinical score of CHIKV-infected mice were measured per viral strain and dose daily. The duration of the study was 5 days, but a set of ethical clinical endpoints was defined for mice that lost ≥ 20 % of their initial body weight or lost < 20 % and exhibited ruffled fur, hunched posture and lethargy during the study. The mice that showed severe clinical signs before the endpoint were euthanized with an overdose of sodium pentobarbital (60 mg/mL) administered intraperitoneally (i.p.) followed by cervical dislocation.

3.5. Virological and histopathological analysis

To investigate the differences in viral RNA levels and tissue tropism between S27 and ITA strains, three mice per group were euthanized at the scheduled time-points (2-5 dpi). A129 mice were anaesthetised and then perfused through the intracardiac route with fetal bovine serum for 10 min at 7 mL/min to rinse out the blood-borne virus. At necropsy, encephalon, liver, spleen, inguinal lymph nodes and gastrocnemius muscle were collected in tubes with 0.5 mL of medium Dulbecco's modified Eagle's medium (DMEM) and frozen at -80°C.

To compare CHIKV pathogenesis between strains (S27 *vs* ITA), three mice per group were anaesthetised and then transcardially perfused with formaldehyde in PBS (pH 7.4) at the scheduled time-points (2-5 dpi). Table 4.2 shows all the animals euthanized and sampled for the histopathological study. At necropsy, encephalon, liver, spleen, inguinal lymph nodes, gastrocnemius muscle, joint (knee and foot) and skin were collected, fixed in formaldehyde for 2 days and then introduced into the tissue processor (Leica tp 1020) for paraffin embedding. After that, the samples collected were sliced at 4 μm thick. The sections were stained with hematoxylin-eosin (H&E) to evaluate inflammation and tissue damage. Lesions were scored using the following criteria: within normal limits (WNL), minimal, mild, moderate and severe lesions. All analyses were performed by a pathologist in a blind manner.

STRAIN	**S27**			**ITA**			**CONTROL**
Days / Doses	**10^2 $TCDI_{50}$**	**10^4 $TCDI_{50}$**	**10^6 $TCDI_{50}$**	**10^2 $TCDI_{50}$**	**10^4 $TCDI_{50}$**	**10^6 $TCDI_{50}$**	**PBS**
2 dpi	1	1	3	-	1	1	4
3 dpi	-	-	-	4	3	4	-
4 dpi	-	-	-	1	-	-	-

Table 4.2. Total number of mice euthanized after infection with three doses (10^2, 10^4, 10^6 $TCID_{50}$) of two CHIKV strains (S27 or ITA) and sampled for histopathology at different days post-virus inoculation (2 dpi, 3 dpi, 4 dpi). Mice injected with PBS were used as mock controls.

3.6. Viral extraction and detection

Blood samples from each mouse (≈ 150 µL) were collected and centrifuged at 10000 x g at 4 °C for 10 min. Serum was removed, diluted into DMEM with a working concentration of 1/10, and stored at -80 °C for further analysis. Viral RNA was extracted with NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. Tissues samples were weighed and RNA was extracted from 100 mg of tissue using TRIzol reagent (Invitrogen®) according to the manufacturer's instructions. Viral RNA levels were quantified in the sera and tissues of CHIKV-infected mice by reverse transcriptase PCR (RT-PCR) as described previously in Chapter III. All results were expressed as threshold cycle (Ct) values. The cut-off for CHIKV detection was set at a Ct value ≤ 35.

3.7. Statistical analysis

Graphical reresentations of mortality and morbodity were performed with GraphPad Prism version 7.04 (GraphPad Software, La Jolla CA, USA www.graphpad.com) and expressed as means ± SEM .

Differences in mortality rates between the two CHIKV strains was analyzed estimating the average survival rates using Wilcoxon rank sum test. Differences in viral RNA levels in the mouse sera between the two CHIKV strains was analysed using the Wilcoxon rank sum test. In all cases, p-values less than 0.05 were considered statistically significant.

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4. RESULTS

4.1. CHIKV-S27 strain is more virulent than CHIKV-ITA strain in IFN -α/βR-/- A129 mice following s.c. inoculation at three different doses (low, medium, and high)

Most of the mice (75%; 48/64) displayed clinical signs of disease (score > 3) within 2 dpi, including rapid weight loss (Figures 4.1A and 4.1C), hunched posture, ruffled fur and lethargy. Furthermore, 92% (59/64) of mice succumbed to disease or were euthanized due to severe disease between 2-3 dpi (Figures 4.1B and 4.1D). Mice that were euthanized due to disease progression were counted as death on the same day. In total, 37.5% (20/64) of CHIKV-infected mice succumbed to the infection and, 62.5% (40/64) of mice were culled on welfare grounds.

A129 mice inoculated with the prototype S27 strain displayed more servere clinical signs of illness (higher clinical scores) than those inoculated with the emergent ITA strain (Table 4.3).

CLINICAL SCORE	**S27**			**ITA**		
Days p.i	**high**	**medium**	**low**	**high**	**medium**	**low**
1	0.4 (100%)	0.5 (100%)	0.6 (100%)	0.9 (100%)	0.75 (100%)	0.4 (100%)
2	8.14 (70%)	7.70 (83%)	5.22 (90%)	4.60 (100%)	4.58 (100%)	0.50 (100%)
3	na	na	9 (10%)	11.50 (60%)	9 (66,67%)	7.9 (100%)
4	na	na	na	na	na	9

Table 4.3. Mean clinical score of A129 mice after inoculation with three doses (10^6, 10^4 and 10^2 $TCDI_{50}$) of both CHIKV strains (S27 or ITA).**na:** not applicable. The percentage of mice used for the calculation is placed in parentheses.

The higher virulence of S27 strain also became evident when comparing survival curves between CHIKV strains. The prototype S27 strain killed 90% of mice at the medium dose (10^4 $TCDI_{50}$) at 2 dpi, whereas the ITA strain killed only 20% the mice at the same dose at 2 dpi (Figures 4.1B and 4.1D). Likewise, we found that the average survival in the group of mice inoculated s.c. with the mutated ITA strain (3.06 ± 0.50 days) was significantly higher (p= 9.39e-08) compared to mice inoculated with the prototype S27 strain (2.25 ± 0.44 days), indicating that S27 strain is more virulent in these animals. Only mice inoculated with the lowest dose (10^2 $TCDI_{50}$) of either strain displayed a typical mortality dose-response curve, with mortality rates lower than the high and medium doses at 2 dpi. By contrast, ITA-infected mice with the medium dose (10^4 $TCDI_{50}$) exhibited higher mortality rate than those infected with the high dose (10^6 $TCDI_{50}$) (17% *vs* 10%) at 2 dpi (Figure 4.1B). Likewise, the mortality rates of mice inoculated with S27 strain were similar in high (100%), and medium (92%) doses at 2 dpi (Figure 4.1D).

As shown in Figures 4.1A and 4.1C, both CHIKV strains resulted in similar morbidity curves in mice inoculated with high and medium doses (6.69 ± 1.71% of mean weight loss for the high dose and 6.79 ± 0.73% of mean weight loss for the medium dose). By contrast, the s.c. injection of the lowest dose (10^2 $TCDI_{50}$) resulted in different morbidity curve between CHIKV strains, with a higher weight loss in mice challenged with ITA strain (10.85 ± 2.09%) than the group of S27 strain (3.19 ± 3.48%). In comparison, the mock-infected mice maintained a stable weight profile throught the observation period with a little weight loss of (1.16 ± 0.74%) (data not shown).

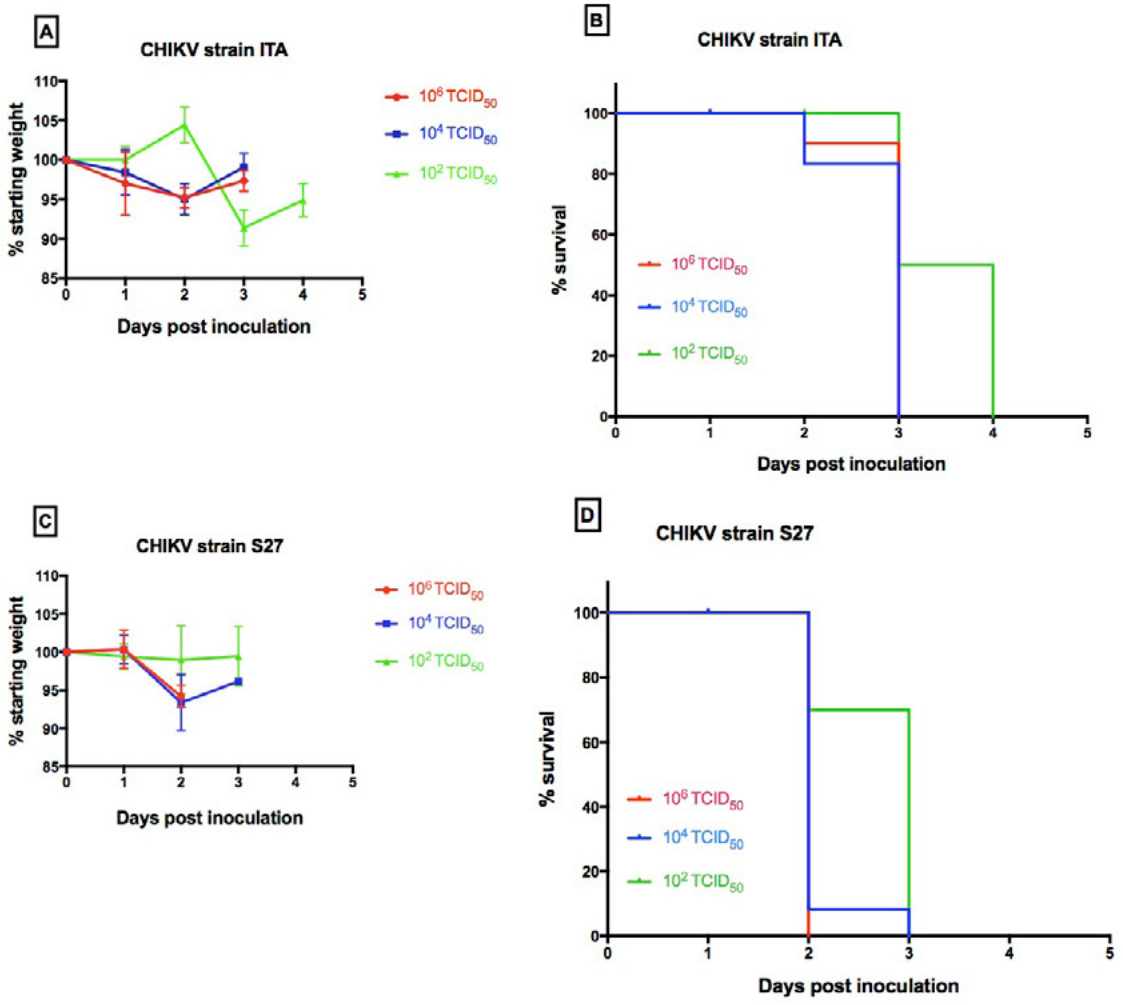

Figure 4.1. Morbidity and Survival curves of adutl IFN α/βR-/- A129 mice after s.c. infection with three doses (10^6, 10^4 and 10^2 TCDI$_{50}$) of both CHIKV strains (S27 or ITA). Morbidity curves, differences in mean weight compared to the day of challenge (Graphs A and C). Survival curves (Graphs B and D).
Mean values with error bars denoting standard deviation (SD). N=10-12 mice per strain and dose.

4.2. Viral replication and tissue tropism do not differ substantially between S27 and ITA CHIKV strains in IFN -α/βR-/- A129 mice inoculated s.c. at three different doses (low, medium and high)

To investigate the differences in CHIKV replication in CHIKV-infected mice between S27 and ITA strains, we collected blood, liver, spleen, encephalon, inguinal lymph nodes and gastrocnemius muscle at 2 and 3 dpi. The viral titers in the serum were compared on day 2 post-inoculation when mice began to show severe clinical signs of the disease. The results showed high levels of virus replication in blood samples, regardless of the challenge dose tested (10^2, 10^4 or 10^6 $TCID_{50}$) (Figure 4.2).

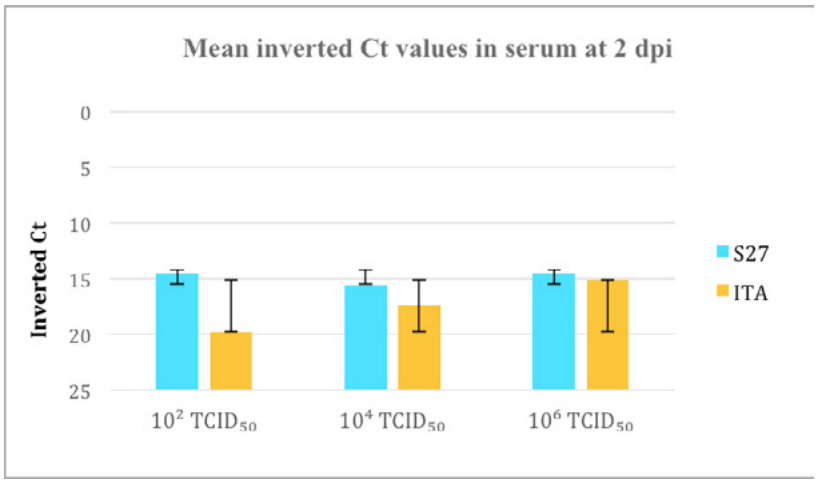

Figure 4.2. Mean inverted Ct values of CHIKV RNA in serum (N= 2-4 samples). Bars indicate the standard error.

When comparing the S27 and ITA strains, we found higher serum viral loads (lower Ct values) in S27-infected mice (Ct range 14-16) compared to ITA-infected mice (Ct range 15-20) at 2 dpi, being this difference between both strains only statistically significant (p= 0.019) for mice challenged with the lower virus dose (10^2 $TCDI_{50}$) (Figure 4.2). For high and medium dose, viral RNA levels were slightly higher (but not significantly) on average for the prototype S27 strain than for the emergent ITA strain (p= 0.8; p= 0.2 respectively).

To study tropism in CHIKV-infected mice, we collected the target tissues in those animals euthanised on 2 and 3 dpi. We found high levels of CHIKV RNA in all collected tissues (Ct range from 13 to 25 Ct), regardless of the challenge dose, as shown in Figures 4.3A- 4.3C.

We found higher amounts of viral RNA (lower Ct values) in tissues of S27-infected mice compared to ITA-infected mice. However, it should be noted that the tissues were collected from mice on different days post-infection (2 dpi for S27 strain and 3 dpi for ITA strain) and, it was not possible to conduct a statistical analysis between these two groups because viral RNA was quantified in only one A129 mouse per viral dose. As expected, uninfected tissue controls were negative by RT-qPCR.

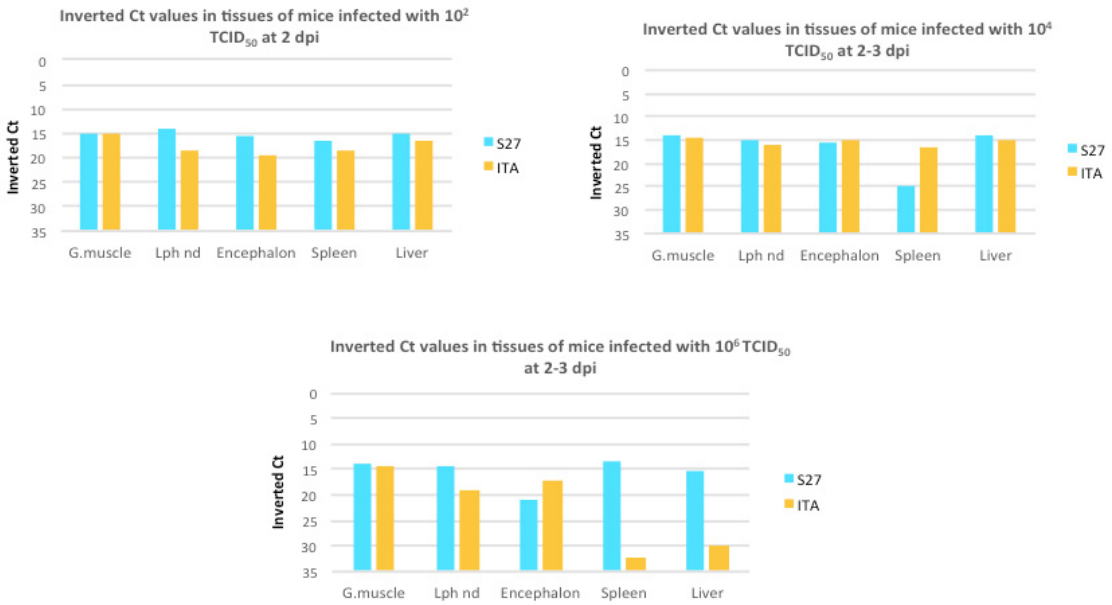

Figure 4.3 (A-C) Ct values of CHIKV RNA in mouse tissues collected at 2-3 dpi (N= 1 mouse per dose and strain). G.muscle: gastrocnemius muscle; Lph nd: Inguinal lymph nodes.

Although all organs analyzed contained viruses, the highest CHIKV RNA levels were found in the spleen, (Ct=13) and gastrocnemius muscle (Ct=14) in S27-infected mice; while the highest CHIKV RNA levels were found in the gastrocnemius muscle (Ct=14) and encephalon (Ct=15) in ITA-infected mice (Figures 4.3A and 4.3C).

4.3. Similar histopathological changes in encephalon, liver, spleen, and inguinal lymph nodes in IFN -α/βR-/- A129 mice challenged s.c. with S27 or ITA strains at three doses (low, medium and high)

To evaluate whether both CHIKV strains (S27 and ITA) cause similar tissue damage in CHIKV-infected mice, a total of 19 CHIKV-infected mice along with 4 mock-infected controls were euthanized at 2, 3 and 4 dpi (Table 4.2). Since S27-infected mice succumbed earlier to the infection than ITA-infected mice, all the tissues samples from S27 strain were at disease onset (2 dpi), whereas the tissues samples of ITA-infected mice were collected at three different days (2, 3, and 4 dpi) (Tables 4.4 and 4.5).

The most notable microscopic lesion attributable to CHIKV infection was a lymphoid depletion (lympholysis) in both, the spleen and inguinal lymph nodes, observed in 94% (18/19) of sections examined at 2, 3 and 4 dpi (Tables 4.4 and 4.5). The severity of lympholysis caused by both strains of CHIKV (S27 and ITA) was comparable (Tables 4.4 and 4.5). The lympholysis was characterized by morphologic changes in the nuclei of the lymphocytes (karyorrhexis) (Fig. 4.4C2). Additionally, mild-moderate lipidosis was observed in some sections of livers examined, in 2 of 5 S27-infected mice, and in 1 of 2 of ITA-infected mice at 2 dpi. Likewise, lipidosis was also observed in 8 of 11 mice and 1 ITA-infected mice at 3, and 4 dpi, respectively (Tables 4.4 and 4.5). However, some sections of the liver showed evidence of tissue destruction, with focal areas of hepatocyte necrosis. The presence of necrosis suggested ongoing cellular injury and was only observed in several mice infected with a low or medium dose of ITA strain at 3 dpi (3/4 mice from 10^2 $TCDI_{50}$ and 2/3 from 104 TCDI50) (Tables 4.4 and 4.5). The spleen, inguinal lymph nodes and liver from the four mock-infected mice, euthanized at day 2 post-inoculation showed a standard organ architecture (Figures 4.4A, 4.4D and 4.4 G).

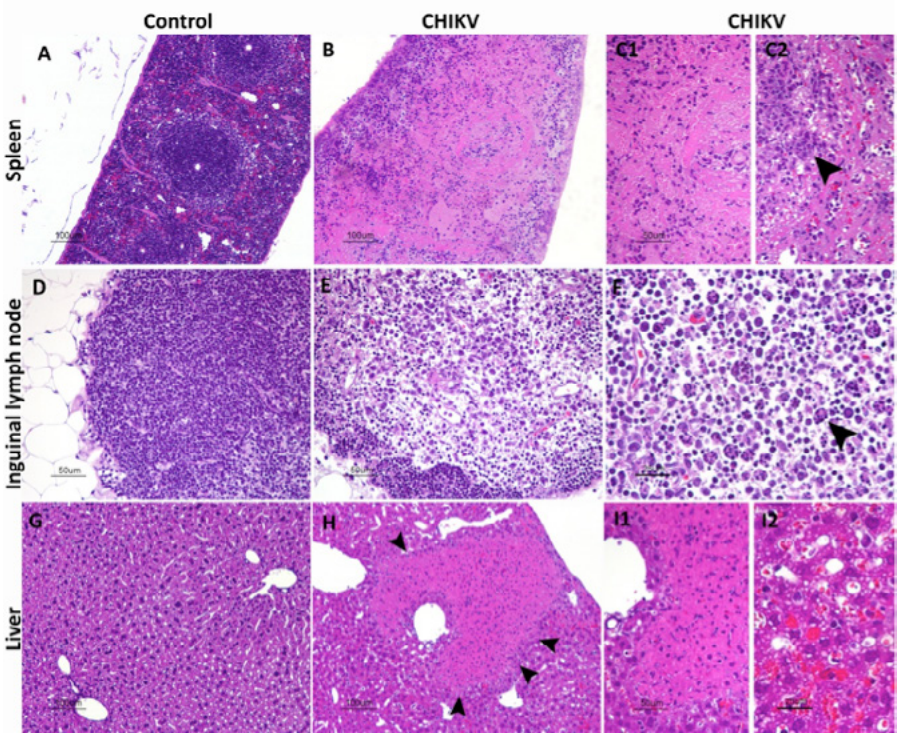

Figure 4.4. Histopathology of the spleen, inguinal lymph nodes and liver from A129 mice at day 2 after s.c. inoculation with 10^4 $TCID_{50}$ or 10^6 $TCID_{50}$ of CHIKV-ITA strain. H&E-stained sections of control mice (A, D, G), CHIK-ITA infected mice at 2 dpi with 10^4 $TCID_{50}$ (B, C1, C2) and CHIKV-ITA infected mice at 2 dpi with 10^6 $TCID_{50}$ (E, F, H, I1, I2). Spleen, inguinal lymph node and liver from control mice, were WNL (A, D, G).

C1: Detail increases of the splenic lymphoid follicle without lymphocytes and marked hyalinosis. C2: Detail of a follicle in which the lymphocytes have been replaced by dendritic cells with an epithelioid change (arrow). F: Details of lympholysis, lymphocyte nuclei can be observed in karyorrhexis and active phagocytosis of cellular debris (arrow). H: Focus of hepatocyte necrosis (arrows). I1: Detail of further increases in the area of hepatocyte necrosis. I2: Detail of hepatocytes with microvacuolation of the cytoplasm: lipoidosis. N=1 mouse per strain and dose.

Tissue	ITA (3 dpi)			ITA (4 dpi)
	10^2TCID$_{50}$ (n=4)	10^4 TCID$_{50}$ (n=3)	10^6 TCID$_{50}$ (n=4)	10^2TCID$_{50}$ (n=1)
Encephalon	Focal area of disease (1/4)	WNL	Mild spongiosis in the hippocampus	WNL
Liver	Focal hepatocyte necrosis (3/4) Mild-moderate lipidosis (2/4)	Focal hepatocyte necrosis (2/3) Mild-moderate lipidosis (2/3)	Mild-moderate lipidosis (4/4)	WNL
Spleen	Lympholisis (4/4)	Lympholisis (3/3)	Lympholisis (4/4)	Lympholisis
Inguinal lymph nodes	Lympholisis (4/4)	Lympholisis (3/3)	Lympholisis (4/4)	Lympholisis
Skeletal muscle	WNL	WNL	WNL	WNL
Skin	WNL	WNL	WNL	WNL
Joint	WNL	WNL	WNL	WNL
Others	Bone marrow with mild focal karyorrhexis (1/4)	nd	nd	nd

Table 4.5. Results of histopathological analysis of different tissues from A129 mice at 3 and 4 after s.c. inoculation with CHIK ITA strain. WNL: Within Normal Limits; nd: no data. The number of mice used in the analysis is placed in parenthesis.

There were no significant abnormalities in the encephalon of IFN -α/βR-/- A129. The only histopathological finding was a mild spongiosis in the hippocampus, which was observed in the encephalon of only 3 of 19 mice (one S27-infected mouse at 2dpi and two ITA-infected mice infected at 3 dpi; Tables 4.4 and 4.5). Additional findings of interest include the observation of a mild karyorrhexis in the bone marrow of 4/5 of S27-infected mice infected at 2 dpi, 1/2 of ITA-infected mice at 2 dpi, and 1/11 of ITA-mice infected at 3 dpi (Tables 4.4 and 4.5). No evidence of encephalitis, arthritis or myositis was found in any of the animals studied, despite the high levels of viral RNA detected by RT-qPCR at 2, 3 dpi in the encephalon and gastrocnemius muscle of these animals (Figures 4.3 A- 4.3 C).

5. DISCUSSION

Mice deficient in the receptor for IFN-α/β R-/- (A129) were highly susceptible to infection with either CHIKV-S27 or CHIKV-ITA strain by s.c. inoculation. These findings were consistent with the results (severity and lethality) obtained by Couderc *et al.*, [298] for the CHIKV-21 (E1-226V) strain following intradermal infection with 10^6 PFU in A129 mice. Both routes of exposure (s.c. and intradermal) culminated in 100% mortality with a similar mean time of survival of 3.06 ± 0.50 days for ITA strain (E1-226V) and 3 ± 0.20 days for CHIK-21 strain (E1-226V). Similar results were found when infecting the mouse A129 with two other isolates from La Runion (CHIKV-27 and CHIKV-115) and one isolate from Congo (CHIKV-117) [298]. Similar to our findings, Gardner *et al.*, [302] observed that infection of adult mice (8 weeks old) A129 mice inoculated s.c. (hind footpad) with 10^3 PFU of either CHIKV-37797 (E1-226A) or CHIKV-LR (E1-226V) resulted in 100% mortality by 4 and 5 days respectively [302]. Other study found a similar mean time to death (4 days) in adult mice (6–8 weeks old) IFN-α/β R-/- mice (C57BL/6 background) infected s.c. in the footpad with 10 FFU of the emergent CHIKV strain LR 2006-OPY1 (E1-226V) [307].

In our study, all mice, either succumbed to the disease or were culled for welfare reasons, showed signs of disease, including progressive weight loss, ruffled fur, a hunched posture, and inactivity by day 2 or 3 after infection. The disease course was rapid and aggressive, particularly in animals challenged with the prototype S27 strain. Despite this difference, both strains exhibited similar tissue tropism and histopathological changes. The higher virulence of S27 strain became mainly evident in mice inoculated with the lowest dose since high and medium challenges doses yielded a similar lethality in mice, which potentially impeded the observation of differences between both strains. The higher virulence of the prototype strain S27 in mice may be due to its extensive mouse passage history [308], whereas the emergent ITA strain was recently isolated. Although CHIKV strains have shown lineage-specific variations in virulence in the A129 mouse model and non-human primate models [289], this variation has yet to be corroborated in humans [309]. The apparently enhanced neurovirulence of the emergent CHIKV strain (E1-226V) in human infections is still under study [308].

As with previous studies [298, 302], high levels of RNA was found in the blood, and all tested tissues, due to the absence of IFN-α/βreceptor-mediated antiviral responses. In our study, CHIKV exhibited a similar tropism to that observed in other mouse models [298], non-human primate models [310], and CHIKV-infected patients [311]. The major histopathological changes

were found in the liver and spleen. Previous studies in human hepatic cell lines [301], mouse [289] and non-human primates [310] found that the liver represent a strong target for viral replication. In some cases, a liver dysfunction has been reported in CHIKV-infected humans in the acute phase [312, 313].

However, compared to other CHIKV mouse models, we not could demonstrate signs of chronic infection. At the time of necropsy, there was no evidence of tissue damage such as myositis, tenosynovitis and arthritis in the collected musculoskeletal tissue, despite detecting high levels of viral RNA. This finding may be related to the premature death of CHIKV-infected mice and is consistent with the outcomes of previous studies in IFN-α/β R-/- mice following intradermal and s.c. route [298, 307]. Experiments using models of non-lethal CHIKV infection have also been able to simulate the chronic phase of the disease in both young and adult wild-type mice. For example, Ziegler *et al.*, [314] observed arthritogenic disease manifestations using newborn and young mice (2-3 and 14-days old, respectively) of strains ICR and CD-1 following s.c. inoculation of the emergent CHIKV strain LR 2006-OPY1 (E1-226V). Another study [315] showed that young (14-day-old) C57BL/6J animals infected with 100 PFU by s.c. route with the CHIKV strain SL15649 (from Sri Lanka) developed severe arthritis, tenosynovitis, and myositis. Furthermore, viral persistence of CHIKV RNA in joints was detected 3 weeks after inoculation. Likewise, Gardner *et al.*, [316] showed that adult (six-week-old) wild-type C57BL/6 mice developed a mild musculoskeletal disease following s.c. inoculation of the emergent CHIKV strain LR 2006-OPY1 (E1-A226V). This mouse model recapitulates self-limiting arthritis, tenosynovitis, and myositis seen in humans. The data from these studies implicate the muscle together with joint tissues, as sites of virus persistence, which could contribute to chronic symptoms seen in human CHIKV infections (such as myalgia and arthralgia).

Our study extends earlier studies on CHIKV-infection in A129 mouse model, providing new data on the virulence of CHIKV when comparing mortality, morbility, clinical signs and viraemia between two strains of the ECSA lineage. We found that the prototype strain (S27) was more virulent, and caused significantly higher clinical score, mortality and viraemia than currently emergent strain (ITA), although histopathological differences were not identified.
We found that this mouse model was highly sensitive to CHIKV infection; a dose of 10^2 $TCDI_{50}$ injected s.c. was effective in inducing the death of the mice between 2 and 4 dpi. Since mosquitoes inoculate a wide range of viral doses as they probe and feed on a host, (ranging from 10 to 10^6 PFU [305]), this mouse model is suitable for establishing a mosquito-dependent transmission model.

In conclusion, CHIKV infection of IFN-deficient mice (A129 background) via the s.c. route provides a good model for studying the acute phase of the disease as it mimics some aspects of human disease during the viraemic period. As mice (IFN-α/β R-/- A129) succumbed to the disease very quickly, we could not make long-term assessments and therefore studying the chronic phase of CHIKV infection as Couderc *et al.*, [298] pointed out. This mouse model, IFN-α/β R-/- A129 ,would be useful in the early stages of research of candidates for vaccines and antiviral drugs and for establishing a mosquito-dependent transmission model.

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Chapter 5

Experimental studies on the transmission of Chikungunya virus by *Aedes albopictus*

" Si no tienes éxito a la primera inténtalo al menos dos veces más de manera que al menos el error sea estadísticmente significativo"

Anónimo

1. ABSTRACT

Background

Evaluating vector competence requires demonstration of virus transmission by mosquitoes. Knowing the potential of transmission of local mosquitoes is crucial for implementing vector control programmes. In this study, we assessed the ability of a Spanish *Ae. albopictus* strain to experimentally transmit Chikungunya virus (CHIKV) under summer and autumn conditions. The transmission of CHIKV was detected using a mouse model and an *in vitro* method.

Methods

Aedes albopictus mosquitoes either individually or in groups of 5-10 mosquitoes were tested for CHIKV transmission at 9 days after feeding on a high dose of infected blood meal (6.5 $\log_{10}$ $TCID_{50}$ /ml). These mosquitoes were allowed to feed on a mouse lacking alpha/beta interferon (IFN-α/β -/-) or a honey-soaked card. We also evaluated the effect of temperature (summer vs autumn) on transmission of CHIKV by *Ae. albopictus*. Vector competence was evaluated by estimating the infection rate by screening mosquito bodies, dissemination rate by testing legs, and transmission rate by screening mouse sera and saliva. All samples were analysed through quantitative real-time RT-PCR (RT-qPCR).

Results

A total of 126 *Ae. albopictus* mosquitoes were tested for transmission using a mouse model. Of these, 16 mosquitoes fed on mice, 4 to full engorgement and 12 partially. Among the 16 female mosquitoes, 7 had a disseminated infection (43.75 %; 7/16). None of the mice became infected after the follow-up, and the transmission efficiency was 0 % (0/126). For the *in vitro* assay, a total of 45 mosquitoes were allowed to feed on honey-soaked cards to test transmission. Among the 38 mosquitoes kept under summer conditions, 2 mosquitoes were capable of transmitting the CHIKV E1-226V with a transmission efficiency of 5 % (2/38).

Conclusions

We could not establish a transmission model of CHIKV between mice lacking interferon α/β receptor and the *Ae. albopictus* mosquito. By contrast, we demonstrate the vector competence of a Spanish strain of *Ae. albopictus* for CHIKV E1-226V using honey-soaked cards under summer conditions. While 5 % of transmission efficiency seems to be low, local transmission of CHIKV may drive in Catalonia if other parameters determining the vector capacity of *Ae. albopictus* are suitable.

Keywords: Chikungunya virus, *Aedes albopictus*, transmission, *in vivo*, mouse model, type-I interferon, A129, FTA™ card.

2. INTRODUCTION

Virus transmission by mosquitoes is a critical component of vector competence laboratory studies. The capability of detecting infectious mosquitoes is essential to understanding the epidemiology of arboviruses [317]. Early vector competence studies evaluated virus transmission by *in vivo* laboratory methods, allowing mosquitoes to feed on animal models. The transmission was then displayed by the emergence of clinical signs and symptoms of the disease. Subsequently, it was verified by the recovery of the arbovirus from the blood or by seroconversion of the host. In addition to studying virus transmission, animal models are useful to evaluate arboviral pathogenesis and to screen candidate vaccines and antiviral drugs. However, *in vivo* transmission studies have some disadvantages: laboratory models such as non-human primates and genetically modified mice are costly and require special facilities for animal housing, appropriate permissions and compliance with the Institutional animal care and personal training. Other limitations to consider are that there are no laboratory animal models for studying every vector–virus system [317], and they are not suitable for experiments involving large sample sizes due to the application of the 3 Rs (replacement, reduction and refinement). Current studies rely on the use of *in vitro* methods to circumvent these problems. Some studies employ the collection of salivary glands as evidence of virus transmission potential. However, salivary gland dissection is a time-consuming, delicate and labour-intense technique that requires well-trained personnel. Alternatively, detection of RNA or infectious particles in mosquito legs or head tissues has been used as a standard proxy for transmission potential [318]. It has been observed a positive correlation between the titre of disseminated virus and the likelihood of detecting it in saliva [319]. However, this method may overestimate the transmission rate since it does not take into account the existence of salivary gland barriers [320].

Currently, the gold standard for *in vitro* transmission assay is the collection of saliva in capillary tubes, followed by inoculation in cell culture, as it allows the detection of the presence of infectious viruses present in those tissues. However, this method may not detect small but transmissible amounts of virus and thus may underestimate the transmission rate. This technique is less sensitive than the *in vivo* transmission assay and may miss 30-50 % of saliva samples that were subsequently positive by inoculation into mice [321]. Besides, mosquitoes are allowed to salivate in the capillary tube for much longer (30-45 minutes), than is required for blood feeding in nature (approximately 3 minutes; [322]), which may overestimate the amount of virus expectorated [260]. The main limitation of these *in vitro* methods is that it requires the

sacrificing of mosquitoes to assess virus transmission. Recently, a non-lethal method to collect saliva has already used successfully in arbovirus surveillance. This system exploits the fact that female mosquitoes expectorate virus in their saliva during their feeding on sugar sources. Saliva can be collected using Flinders Technical Associates (FTA™) cards, which are filter paper cards designed to preserve both RNA and DNA without the need for a cold storage chain [323]. It is a useful tool for molecular epidemiological studies of arboviruses allowing strain identification and genotyping [320]. This method has several advantages: i) it allows the collection of saliva from a single mosquito at different time points. Therefore, enabling the estimated assessment of the extrinsic incubation period (EIP) of arboviruses (i.e., the interval between the ingestion of the virus and the earliest time at which virus is released in the saliva), ii) this method allows using the same mosquito for further transmission studies. Therefore, it could be possible to combine different transmission assays (e.g. FTA™ card and capillary tube), iii) this technique does not require the same level of trained personnel as opposed to the capillary assay, and iv) it allows the processing of large numbers of samples. However, this method presents some limitations. First, the saliva sample cannot be used for direct virus isolation in cell culture, hindering the confirmation of the viral viability. Second, the occurrence of false negatives is a possibility due to the minimal amount of saliva expelled [320]. Furthermore, it remains unknown if the amount of saliva delivered during sugar feeding differs from the amount inoculated into the vertebrate host during blood feeding.

Thus, this study aims to evaluate the transmission capability of *Ae. albopictus* experimentally. In addition, we examine the effect of temperature on CHIKV transmission. To such aims, we developed a mosquito-mediated transmission cycle between *Ae. albopictus* mosquitoes and mice lacking interferon α/β receptor (A129). Furthermore, we employed FTA™ cards as an alternative to replacing *in vivo* models. We assessed the transmission potential of CHIKV by *Ae. albopictus* in temperatures similar to those found in summer and autumn seasons in Catalonia.

3. MATERIAL AND METHODS

3.1. Ethics statement

This study was performed following the European Directive, 2010/63/EU on the protection of animals used for scientific purpose. Protocols were reviewed and approved by the *Centre de Recerca en Sanitat Animal* (CReSA).

3.2. Mice

Alpha/beta interferon (IFN-α/β) receptor-deficient mice (A129 background) were obtained from B&K Universal Limited (UK) and were bred in the facilities of *Centre de Biotecnología Animal y Terapia Génica*-CBATEG (Universitat Autònoma de Barcelona). IFN α/βR-/- A129 mice were acclimatised for one week in the CReSA Biosafety Level 3 (BSL-3) animal facility before each challenge. All mice were housed at a density of 5 or 6 per cage with a pelleted diet and water provided *ad libitum*. The animal facility was maintained under a controlled environment: 22 ± 1 ºC and 50-55% RH with a 12 h light/dark cycle. Approximately equal numbers of male and female mice were used in each experiment.

3.3. Mosquito strain and rearing

Aedes albopictus strain was collected in Sant Cugat del Vallès (Catalonia) in 2009 and reared since then as a laboratory colony. Mosquitoes were reared, throughout all development stages, under laboratory conditions that simulate natural climatic conditions in Catalonia (NE Spain). Two environmental profiles (summer and autumn) were defined for three variables: temperature, relative humidity (RH) and photoperiod. Climatic data were obtained from the Catalan Meteorological Service (http://www.meteo.cat/). Summer profile was inferred from July average temperatures, 26 ºC for 14 hours (day) and 22 ºC for 10 hours (night) and 86% RH. Autumn profile dataset was obtained from October average temperatures, 18 ºC for 12 hours (day) and 15 ºC for 12 hours (night) and 76% RH.

3.4. Virus strains

Two CHIKV strains of the ECSA lineage were used, S27 Petersfield and ITA1_TAM_E1, named S27 and ITA hereafter respectively. The main difference between sequences of both strains is an amino acid change at position 226 of the E1 envelope glycoprotein: S27 harbours an alanine (E1-226A), whereas ITA harbours a valine (E1-226V). The detailed information of these CHIKV strains is provided in Chapter III.

3.5. Infection of mosquitoes

Groups of 65 ± 16 female 7-15- day-old mosquitoes were starved for 24 h before they were blood-fed using a Hemotek feeding system (Discovery Workshops, UK) with 1-day old specific-pathogen-free chicken skin as a membrane. The infectious blood meal used in the experiments contained 6.5 $\log_{10}$ $TCID_{50}$ /ml of either S27 or ITA CHIKV strain. Partially engorged females (PEF) were discarded, and only fully engorged females (FEF) were selected using CO2 anaesthesia and transferred either individually in cardboard cages (Watkins & Doncaster, U.K.) or groups of 5-10 mosquitoes inside plastic cages. Adult mosquitoes were maintained *ad libitum* with sucrose solution (10%) administered on soaked cotton pledgets placed on the mesh screen. Cardboard and plastic cages were stored inside a climatic chamber according to the environmental profile (summer versus autumn) for an EIP of 9 days. To determine whether mosquitoes were capable of transmitting CHIKV by bite, once overcome the EIP, they were allowed to refeed on a mouse (5-10 mosquitoes per mouse) or honey-coated FTA™ cards (1 mosquito per card).

3.6. Exposure of mice to uninfected mosquitoes

According to previous studies [324], *Aedes albopictus* is the most common species associated with systemic allergic reactions to mosquito bites, raising the need to evaluate the allergenicity of the mosquito bite before studying CHIKV transmission. For that aim, we assessed the allergic reaction potential to the mosquito bite by allowing uninfected mosquitoes to take blood meals from IFN -α/βR-/- A129 mice. Ninety-six non-blood-fed 5-8 day-old female *Ae. albopictus* mosquitoes were starved for 24 h before feeding on mice. Eighteen IFN -α/βR-/- A129 mice (8-10 weeks old) were anaesthetised with a mixture of ketamine (50 mg/kg) and xylazine (5

mg/kg) administrated intraperitoneally (i.p.). Each anaesthetised mouse was placed on top of a mesh that covered each mosquito container that allowed mosquitoes to take blood meals from the abdominal area of a mouse. We evaluated the swelling and redness of mosquito bites on the mice skin with low (1 mosquito), medium (5 mosquitoes) and high (10 mosquitoes) mosquito density. Mosquitoes were allowed to feed on anaesthetised mice for 20 minutes and scored as FEF, PEF or unfed. Mice were under follow-up observation for two days in order to detect delayed hypersensitive reactions. At the end of the experiment, all mice were sacrificed with sodium pentobarbital (60 mg/mL) administrated i.p. followed by cervical dislocation.

3.7. Transmission experiments

At 9 days post-exposure (dpe) transmission trials were conducted by allowing *Ae. albopictus* either blood-feeding on a mouse or sugar-feeding on a honey-coated card.

3.7.1 Transmission of CHIKV by *Ae. albopictus* bite on a mouse

Two experiments were independently performed, mimicking the environmental conditions of Catalonia (summer and autumn). The first assay was carried out with groups of mosquitoes reared under summer conditions, whereas, the second assay was performed with groups of mosquitoes reared under autumn conditions.

Eight days after the CHIKV-infective blood meal, *Ae. albopictus* mosquitoes were starved by a 24 hours before feeding on IFN -α/βR-/- A129 mice. A total of 23 adult mice were anaesthetised i.p. with a mixture of ketamine-xylazine (approximately n= 6 mice per CHIKV strain and environmental condition). Each mouse was placed on the mesh on the top of each mosquito container that allowed mosquitoes to take blood meals from the abdominal area of a mouse (Figure 5.1.). It is important to point out that during anaesthesia, the mice were covered with a cotton piece in order to avoid a decrease in their body temperature and consequent reduction in mosquito attraction.

As a control group, four mice were exposed to mosquitoes previously fed on uninfected blood. Mosquito feeding was allowed for 60-90 minutes. In the first assay, twelve animals were bled from the facial vein for six consecutive days of post mosquito exposure (dpme). In the second

assay, eleven animals were bled in the same manner but for eleven days at 0, 1, 3, 5, 7, 9, and 11 dpme.

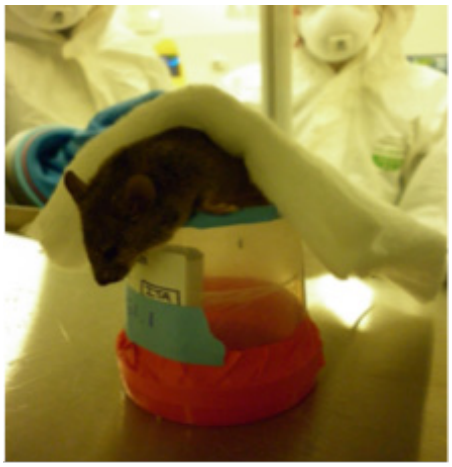

Fig. 5.1. A mouse bit by *Ae. albopictus* mosquitoes previously exposed to CHIKV.

3.7.2. Transmission of CHIKV by Ae. albopictus bite on FTA™ card

Two experiments were independently performed depending on the environmental conditions experienced by the mosquitoes (summer and autumn). The first assay was carried out with groups of mosquitoes reared under summer conditions, whereas, the second assay was performed with groups of mosquitoes reared under autumn conditions.

Mosquitoes were tested for their potential to transmit CHIKV at 9 days after the CHIKV-infective blood meal with a viral titre of 6.5 $\log_{10}$ $TCID_{50}$ /mL of the ITA strain using FTA™ cards. A honey-soaked card was placed in each cardboard cage that allowed mosquitoes fed on the honey and presumably expelled saliva into the card. The FTA™ cards (GE Healthcare, Little Chalfont, UK) were soaked with Manuka honey (Manuka Health New Zealand, New Zealand) mixed with a blue food dye since it enables visual identification of the mosquitoes that had fed on the honey (Figure 5.2).

Fig. 5.2. Mosquito with blue-dyed gut after feeding on honey-coated FTA™ card.

After 48 h of exposure (from 7 to 9 dpe), each card was collected and eluted in 0.3 mL of PBS and stored at −80 ºC.

3.8. Samples

All mosquitoes used in the transmission experiments were sacrificed by freezing at -80ºC. Each mosquito was individually dissected by separating the legs from the rest of the body. Both samples (bodies and legs) were transferred into tubes with 0.5 mL Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland). Mosquito samples were homogenised at 30 Hz for 1 min using TissueLyser II (Qiagen GmbH, Germany) and stored at −80 ºC until tested for CHIKV.

Mouse blood samples (≈ 150 μL) were collected and centrifuged at 10000 x g at 4 °C for 10 min. Serum was removed, diluted 1/10 with medium DMEM and stored at -80 °C for further analysis.

3.9. Viral detection and quantification

Viral RNA was extracted using NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. Quantitative real-time RT-qPCR (RT-qPCR) was performed as described previously in Chapter III. The amount of CHIKV RNA in the mouse sera, mosquito bodies, legs, and saliva was measured by RT-qPCR and expressed as genome-equivalent copies (GEC).

Infection, dissemination and transmission rates were calculated for the Spanish *Ae. albopictus* strain. The infection rate (IR) was defined as the proportion of mosquitoes with CHIKV-positive bodies (abdomen, thorax and head) among the number of tested mosquitoes. The dissemination rate (DIR) was defined as the proportion of mosquitoes with CHIKV-positive legs among those with CHIKV-infected bodies. The Transmission rate (TR) was defined as the proportion of mosquitoes with CHIKV RNA in saliva or the proportion of mouse sera with CHIKV RNA among the number of mosquitoes with disseminated infection. Ultimately, Transmission Efficiency (TE) was defined according to the proportion of mosquitoes with CHIKV RNA in saliva or the proportion of mouse sera with CHIKV RNA among the total number of mosquitoes tested.

3.10. Statistical Analyses

The mean value of the viral load ($\log_{10}$ GEC) in the mosquito bodies and legs of mosquitoes disseminated that were able to transmit CHIKV was compared with the values of those mosquitoes that had a disseminated infection but they were no able to transmit the virus. The normality of viral loads ($\log_{10}$ GEC) in each group was assessed using the Shapiro-Wilk test. In the case of normally distributed data, a mean comparison was carried out using a Student's t-test, while for non-normally distributed data, the non-parametric Wilcoxon rank-sum test was employed. Differences in mosquito biting rates were analysed using a chi-square test. All calculations were carried out using R software (http://cran.r-project.org/).

4. RESULTS

4.1. Low level of inflammatory response was observed in adult IFN α/βR-/- A129 mice bitten by uninfected *Ae. albopictus* mosquitoes

Seventy-four *Ae. albopictus* mosquitoes out of 96 (77%) took their first blood meal from mice (n= 18). On average, the biting rate (i.e. number of bites per mouse exposure) was 4.11 bites, with 2/3 of the mice receiving more than one bite. Two out of the 18 mice (11%), bitten by 4 and 6 mosquitoes, respectively, developed a hypersensitivity response characterised by inflammation and redness at the bite site. By contrast, three mice bitten by > 7 mosquitoes did not show signs of an allergic reaction. Therefore, the mouse model used (IFN α/βR-/- A129) responded slightly to *Ae. albopictus* mosquito bites.

4.2. Low feeding rate of *Ae. albopictus* on IFN α/βR-/- A129 mice could have contributed to our inability to detect transmission of CHIKV by mosquito bite

Mosquitoes that were exposed to a blood meal (6.5 $\log_{10}$ $TCID_{50}$/mL) were tested for their ability to transmit the virus by allowing those to feed on mice. A total of 126 females were exposed to 23 mice (5-10 mosquitoes/ mouse). Among the 126 mosquitoes, 75 females (59%) were infected with CHIKV, and of them, 45 (61%) displayed a disseminated infection (Table 5.1). Of the 126 mosquitoes that fed on the first-blood meal, 45 had a disseminated infection (36%).

Assay	Season	CHIKV strain	♀ alive 9 dpe	First blood meal IR (%)	First blood meal DIR (%)
1	summer	S27	31	17/31 (55)	15/17 (88)
		ITA	32	21/32 (66)	9/21 (43)
2	autumn	S27	25	7/25 (28)	2/7 (29)
		ITA	38*	30/37 (79)	20/30 (67)
Total			126	75/126 (59)	45/75 (61)

Table 5.1. Infection and disseminated infection rates to CHIKV strains (S27 and ITA) of *Ae. albopictus* at 9 dpe
* During the analysis process, one out of the 38 mosquitoes was lost.

The majority of the 126 mosquitoes refuse to feed on mice as a second blood meal, resulting in a feeding rate of only 13% (16/126) during the second blood feeding. Visual evaluation on stereomicroscope identified 16 mice-fed mosquitoes, four fully engorged and 12 partially engorged (Table 5.2). Among the 16 mice-fed mosquitoes, ten were infected (62%) and out of these, seven (70%) had a disseminated infection and could be competent to transmit the virus on the second blood meal (Table 5.2). Thus, the 7 infected mosquitoes fed on 4 mice. On average, each mouse received 1.75 potentially infectious bites. Despite of that, RT-qPCR assays of bitten mice sera showed that none of these bites transmitted detectable CHIK virions to the mice.

Season	2nd blood meal from mouse	Status of FEF				Status of PFE			
		FEF	NI	NDI	DI	PEF	NI	NDI	DI
Summer	14	2	0	1	1	12	4	2	6
Autumn	2	2	2	0	0	0	-	-	-
Total	16	4	2	1	1	12	4	2	6

Table 5.2. Feeding behaviour of Ae. albopictus mosquitoes with regard to the infection status by CHIKV. NI: Not infected; NDI: Non-disseminated infection; DI: Disseminated infection.

We found that environmental conditions used in our study (summer and autumn) influenced the mosquito's biting rate, and hence the probability of transmission of CHIKV (Table 5.2). The biting rate was significantly higher ($p= 0.00029$) for mosquitoes reared at summer conditions ($n= 14$), in comparison to those submitted to autumn conditions ($n= 2$). By contrast, we found that the biting rate was not affected by the infection status of a mosquito. The proportion of infected mosquitoes (13.3%; 10/75) that reefed on mice was similar to that observed in the uninfected ones (11.8%; 6/51) (Tables 5.1 and 5.2).

4.3. High feeding rate of *Ae. albopictus* on FTATM cards allowed us to detect transmission of CHIKV by mosquito bite in summer conditions

A total of 45 *Ae. albopictus* females, which had taken a CHIKV-infected blood meal (6.5 $\log_{10}$ $TCID_{50}$ /mL) 7 days previously, were allowed to feed individually on a honey-coated FTA™ card. All Ae. albopictus females ($n= 45$) fed on the honey-coated FTA™ cards in both seasons, summer and autumn. In the summer season, there were 38 Ae. albopictus females. Of these, 26 females (68%) were infected, and 14 of them (54%) showed disseminated infection. Two out of 14 mosquitoes that had disseminated infection expectorated detectable CHIKV particles in the saliva, giving a TR of 14% (Table 5.3). Therefore, two out of 38 mosquitoes transmitted CHIKV resulting in 5% of TE at 9 dpe (Table 5.3). When comparing CHIKV viral loads be-

tween mosquitoes that did and did not transmit the virus we found that the mean CHIKV viral load in the bodies of the mosquitoes that transmitted CHIKV (9.18 ± 0.39 $\log_{10}$ GEC) was higher than viral load (8.2 ± 1.18 $\log_{10}$ GEC) of those mosquitoes with a disseminated infection that did not transmit the virus. A similar pattern to the body was observed in the legs viral load, with higher values in transmitter mosquitoes (7.52 ± 0.26 $\log_{10}$ GEC), than the leg viral load of non-transmitters (6.6 ± 1.26 $\log_{10}$ GEC). However, due to the small sample size tested, as only two mosquitoes transmitting detectable viral particles, those differences were not statistically significant (p= 0.20 and 0.26 respectively for body and legs). The amounts of CHIKV secreted in the two saliva samples were 2.27 $\log_{10}$ GEC and 3.75 $\log_{10}$ GEC, respectively.

In the autumn season, there were 7 *Ae. albopictus* females. Among them, 4 (57%) were infected, and 1 mosquito (25%) showed disseminated infection.

However, the TR of CHIKV in *Ae. albopictus* at 9 dpe was 0% (0/1) under autumn conditions.

Assay	Season	♀ alive d7	First blood meal d0		Sugar feeding d7-d9	
			IR (%)	DR (%)	TR (%)	TE (%)
1	Summer	38	26/38 (68)	14/26 (54)	2/14 (14)	2/38 (5)
2	Autumn	7	4/7 (57)	1/4 (25)	0/1 (0)	0/7 (0)
	Total	45				

Table 5.3. Infection, dissemination, and transmission rates of CHIKV ITA strain of *Ae. albopictus* mosquitoes at 9 dpe under summer and autumn conditions.

5. DISCUSSION

Type I Interferon receptor-deficient A129 mice have been previously employed to study CHIKV pathogenesis [298, 302]. Few mouse models have established a mosquito-mediated CHIKV transmission cycle [116, 275, 325]. One of these studies employed adult (6-8 weeks) immunodeficient mice [325], whereas the other two studies employed suckling mice (2-3 days) wild type [116, 275]. In this study, we could not demonstrate that CHIKV could be delivered to mice by mosquito bites, as only 16 mosquitoes had fed on them. Among these, 7 (13%) had a disseminated infection. Even though most of these 7 seven mosquitos took only a partial blood meal (6/7; 86%), it is known that PEF may transmit as many viral particles as mosquitoes feeding to engorgement [326] since most saliva is expectorated during probing. Interestingly, our results showed that none of the mice was infected by CHIKV. It is important to point out that the strain of mosquitoes used in this study has been colonised for several generations, which is known to affect their blood-feeding and oviposition behaviour [327]. Furthermore, those females were not allowed to complete their gonotrophic cycle, i.e. the time interval between two consecutive blood meals. Therefore, we hypothesize that altogether such factors led to the low feeding rate observed for those mosquitoes on mice. In the field, it is observed that *Ae. albopictus* mosquitoes tend to take multiple blood meals during a single gonotrophic cycle, a phenomenon named gonotrophic discordance [328, 329]. However, most laboratory-bred species are gonotrophic concordant and will not take a second blood meal before egg-laying [327].

The Spanish strain of *Ae. albopictus* was experimentally able to transmit CHIKV ITA strain (E1-226V) using FTATM cards. The infection and dissemination rates observed in this study (68% and 54%, respectively) were lower than previously reported for CHIKV E1-226V in European *Ae. albopictus* populations (75-100% for IRs and DIRs) [273, 274, 330-332], but similar to the findings of Haddad *et al.*, [333] with a disseminated rate of 60 %. The TE reported in the earlier works [273, 274, 331, 333] ranged from 23-85% at constant temperatures (26-28 ºC) and were higher than the TE (5%) of the Spanish strain of *Ae. albopictus* used in the present study under fluctuating summer conditions (daytime temperature of 26 ºC and night-time temperature of 22 ºC).

Our results point to a low TE (5%) for the CHIKV ITA (E1-226V) strain, which might suggest the existence of a midgut escape, salivary gland barrier(s), or both. The virus must overcome

these barriers in the mosquito before being secreted in the mosquito saliva when biting a host. The failure to transmit the virus may be related to a lack of dissemination from the midgut, a low-level salivary gland infection, a lack of virus released into the saliva, or yet to our inability to detect small quantities of virus expelled into FTA™ card. Other factors, including the mosquito midgut microbiota (e.g., *Wolbachia* spp.) may have also played a role as well. When comparing CHIKV viral load (GEC) between mosquitoes that did and did not transmit the virus we have not observed a significant difference, indicating that factors other than virus concentration in mosquito bodies and legs may determinate viral transmission.

The differences between our results and those found in other European mosquito populations may be explained by differences in the genetic background of the respective mosquito populations, in the viral strain used [328], viral dose employed during oral infection [176], incubation period, the method of exposure to virus [334] and also by the chosen environmental conditions [263]. Besides the methodological approach, with molecular method instead of cell culture, the main difference among the studies was the environmental conditions. While previous studies were conducted at constant temperature (26-28 ºC), our study detected transmission using a fluctuating temperature profile (daytime 26 ºC; night-time 22 ºC). Previous studies have found that fluctuating temperature regimes affect mosquitoes' VC for arboviruses compared to constant temperatures [240, 287]. For example, it has been reported that fluctuating temperatures (daytime 31 ºC for 13.5h and night-time 23 ºC for 10.5h) inhibited disseminated infection of CHIKV in *Ae. albopictus* relative to constant temperature (27 ºC) [335].

In this study, we could not demonstrate CHIKV transmission under autumn conditions (daytime 18 ºC; night-time 15 ºC). However, previous VC studies showed that European *Ae. albopictus* populations can transmit CHIKV E1-226V efficiently at low temperatures (18, 20 and 21 ºC). The TE values ranged between 70 and 80 % [263] and TRs ranging from 37.5 to 63.3% [261]. Future studies should be done to address this question with larger sample sizes.

Based on our results, we confirmed that the A129 mouse model is suitable for transmission studies due to the low rate of allergic reactions to *Ae. albopictus* mosquito bites. VC studies require demonstrating transmission of pathogens by mosquitoes, being the use of animal models and adequate approach to confirm that. However, we found that not all mosquitoes' strains bite animals under experimental conditions, hindering our efforts to use an animal model as a way to demonstrate CHIKV transmission in this study. Furthermore, the *in vitro* assay (FTA™ card),

shortened experimental time yet it may have failed to detect small but transmissible amounts of virus. Overall, our results indicate that, the *Ae. albopictus* strain from Catalonia (NE Spain) is a competent laboratory vector of CHIKV in summer conditions. Although we have a low TE (5%, n= 2/38) for CHIKV, other ecological and anthropogenic factors need to be assessed. Considering the number of imported cases of CHIKV disease recorded each year [259], the high densities of *Ae. albopictus* and its preference for human blood in urban zones [246, 336], the risk of CHIKV transmission in Catalonia may be higher. In fact, the precedent CHIKV outbreaks in Italy and France [337] highlighted the vulnerability of the Mediterranean region to the transmission of CHIKV.

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Chapter 6

Assessment of Chikungunya infection of immature stages of a European *Aedes albopictus* strain

" Research is to see what everybody else has seen,
and to think what nobody else has thought"

Albert Szent-Györgyi

1. ASTRACT

Background

Vertical transmission of Chikungunya virus (CHIKV) in *Aedes* mosquitoes is a known mechanism of persistence of the virus in nature. In this study, we investigate the ability of CHIKV to survive and persist for long periods in temperate areas by alternative infection routes that could occur during the aquatic life stages.

Methods

Larvae of *Ae. albopictus* were exposed to CHIKV through two infection pathways: by adding a viral suspension (5 $\log_{10}$ $TCID_{50}$ /mL) to the rearing water or by adding dead CHIKV-infected *Ae. albopictus* imagoes' provided as a complementary food source (containing from 2.73 to 7.19 $\log_{10}$ genome-equivalent copies of CHIKV). We assessed whether the emerged mosquitoes were capable of transmitting the CHIKV by bite on a honey-soaked card. Furthermore, the survival of CHIKV in the rearing water was evaluated by real-time RT-quantitative PCR (RT-qPCR). All samples, adult mosquitoes and cards were assessed by RT-qPCR.

Results

Under laboratory conditions tested, CHIKV RNA was detected in water for up to 8 days. However, no evidence of transstadial transmission was detected in adults since none of the emerging mosquitoes (0/117) tested was positive by RT-qPCR.

Conclusions

Our studies suggest that CHIKV could persist at breeding sites, but we failed to demonstrate its ability to infect the larvae. Despite this negative result, as a low number of larvae were tested, it could be a source of larvae infection. Future studies should be done in order to evaluate the transstadial transmission of CHIKV in *Ae. albopictus* populations using larger samples.

Keywords: *Aedes albopictus*, mosquito larvae, Chikungunya virus, transstadial transmission, persistence, vertical transmission.

2. INTRODUCTION

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus that is maintained in the field mainly through transmission cycles between the vectors *Aede*s mosquitoes and the human hosts (i.e., horizontal transmission) [135]. On the African and Asian continents, CHIKV epidemics are characterised by sudden outbreaks that occur cyclically with inter-epidemic periods of 7-20 years [257]. Understanding how CHIKV is maintained in endemic areas during these inter-epidemic periods may help to prevent new resurgences of this virus and increase the preparedness of public health authorities to respond more efficiently to outbreaks. During the first half of the 20th century, laboratory experiments were conducted to assess whether an infected female mosquito could transmit an arbovirus to its offspring, a mechanism known as vertical transmission. Many of these early experimental studies failed to detect vertical transmission of arboviruses [338]. Subsequently, isolation of arboviruses from adult mosquitoes reared from field-collected eggs, larvae and pupae confirmed the existence of natural vertical transmission. Venereal transmission, that is, the passage of an arbovirus in semen from vertically infected males to females during copulation, might be another contributor to the maintenance of arboviruses in nature [339]. Although vertical transmission is the primary means of virus survival in nature, other mechanisms of arboviral persistence have been evidenced for an increasing number of arboviruses. Previous laboratory experiments have demonstrated that *Aedes* mosquitoes could be infected as larvae when they are placed into a viral suspension of DENV [340], La Crosse virus [341], St. Louis encephalitis virus [342, 343], West Nile virus [343], Yellow fever virus [344], and Zika virus [345]. Other experiments have shown infection of mosquito larvae from ingestion of infected tissues for DENV [346], and Rift Valley Fever virus [347] or by feeding on infected dead larvae or from virus-contaminated cell cultures for *Ae. albopictus* Parvovirus [348]. Such findings demonstrate that transstadial transmission (passage of a virus from one instar to the next [349]) occurs in larval habitats. There has been no evidence that these modes of infection take place in nature. However, field-infected eggs, larvae, or adults do not provide reliable evidence to confirm that arbovirus RNA can reach larvae cells by vertical transmission.

Susceptibility of *Aedes* mosquito larvae to CHIKV has not yet been evaluated although CHIKV vertical transmission in the laboratory has been demonstrated [140, 141, 350, 351] and confirmed in nature [138, 139]. Most of these studies were performed in mosquitoes from tropical areas where vector populations are active throughout the entire year, whereas there is little information about how the CHIKV survive in nature in temperate areas during an outbreak. In Europe, *Ae.*

albopictus mosquitoes are only active during the warm months, whereas during the colder months, they are overwintering as eggs, which provides a temporary reservoir for the viruses [252]. Only two studies have assessed the possible CHIKV overwintering through vertical transmission in European *Ae. albopictus* populations [331, 350]. Therefore, it would be valuable to investigate what role the transstadial transmission may play in CHIKV perpetuation in an epidemic setting. In a recent study, our laboratory group demonstrated the transstadial transmission of CHIKV from infected water to emergent adult [352]. We evaluated the susceptibility of first-instar larvae of *Ae. albopictus* to CHIKV when were exposed in a viral suspension (5 $\log_{10}$ $TCID_{50}$ /mL) added to filtered and unfiltered rainwater collected from Barcelona. We confirmed transstadial transmission of CHIKV by detection of CHIKV RNA in 1 out of 46 emergent adult mosquitoes analysed by RT-qPCR. Infective virus (5.15 $\log_{10}$ $TCID_{50}$ /mL) was isolated in Vero cells, providing substantial evidence that active viral replication had occurred in the mosquito infected as larvae. Such findings prompted us to investigate whether this mode of infection may contribute to the persistence of CHIKV in nature.

We hypothesised that during a natural CHIKV outbreak, a large number of infected adult mosquitoes might die at the breeding sites and their carcasses would be a source for larvae infection. One scenario might be that the infected mosquito tissues would shed the virus into the water. Another possible route of infection might include the consumption of infected carcasses. Previous studies have shown that dead invertebrate carcasses could be a food source in larval habitats in nature [346, 353].

The present study aimed to investigate whether breeding mosquito sites might act as a reservoir of CHIKV during an outbreak in the summer season. We tested two possible infection routes: exposure of water from larval rearing sites containing CHIKV viral supernatant, and exposure to CHIKV via the consumption of infected carcasses of *Ae. albopictus* imago's. Furthermore, for the first time, the susceptibility of mosquito larvae to CHIKV was tested in an experimental setting aiming to reproduce field conditions of *Ae. albopictus* breeding sites mosquitoes in temperate areas. In such experiments, environmental conditions in the larval rearing trays mimicked summer season in Catalonia, and the aqueous larval habitat was infected with a viral dose that might shed by wild-infected mosquitoes. In addition, the mosquitoes that were used as a source of food were previously infected per os with a blood meal representative of viraemia level recorded in patients.

3. MATERIAL AND METHODS

3.1. Mosquito larvae

Aedes albopictus eggs were obtained from a colony maintained in the laboratory since 2009 and collected initially from Sant Cugat del Vallès, Catalonia (NE Spain). Mosquito eggs were synchronously hatched in autoclaved water under laboratory-controlled conditions: fluctuating temperatures 26 ºC day-22 ºC night; relative humidity (RH) 86%; a photoperiod of 14h light: 10h dark. These climatic conditions are representative of the current climatic range occupied by *Ae. albopictus* in Catalonia during July (summer season). Newly hatched larvae (≤ 24h old) were collected for larval assays.

The parental generation of larvae was screened for *flavivirus* and alphavirus by reverse transcription nested polymerase chain reaction (RT-nPCR) and confirmed to be non-infected [265, 266]. Experimental infections were performed at *Centre de Recerca en Sanitat Animal* (CReSA) Biosafety Level 3 (BSL3) facilities.

3.2. Experimental exposure of mosquito larvae to CHIKV

3.2.1. Design

Aedes albopictus mosquito larvae were exposed to CHIKV by transferring first-instar larva (L1) into water containing virus suspension or infected cadavers of adult mosquitoes. Larval development was monitored daily, and emerging adults were tested for virus transmission, allowing them to feed on honey-soaked FTA™ cards.

3.2.2. Larvae rearing water infected with CHIKV

Viral strains. Two CHIKV strains of the ECSA lineage were used in this study: S27 Petersfield, and ITA1_TAM_E1, named S27, and ITA hereafter respectively. Both CHIKV strains were previously used in the vector competence (VC) studies (Chapters III and V).

Viral suspensions. Two viral suspensions were prepared by inoculating either CHIKV strains (S27 or ITA) into the water to achieve a starting viral concentration of 5 $\log_{10}$ $TCID_{50}$ /mL.

3.2.3. Carcasses of CHIKV-infected mosquitoes

Carcasses of adult mosquitoes infected with CHIKV were obtained from a previous VC experiment (Chapter III). Adult mosquitoes (7-15 days) were orally infected with an infectious blood meal containing 6.5 $\log_{10}$ $TCID_{50}$ /mL of either CHIKV strain (S27 or ITA) using a Hemotek membrane feeding system (Discovery Workshops, UK). As a negative control, a group of mosquitoes were fed on an uninfected blood meal. Further details on mosquito infections are provided in Chapter III. At 9 days post-exposure (dpe), the mosquitoes were killed by freezing at -80 °C and were subsequently dissected to remove their legs from the rest of the body and confirm their infection status. We measured whether mosquitoes have a disseminated infection by screening the legs for CHIKV. Fourteen samples of mosquito leg were randomly selected for each CHIKV strain and screened for CHIKV by real-time RT-quantitative PCR (RT-qPCR). The bodies of these leg samples (n= 14 per CHIKV strain) were kept at 4 ºC. Based on the RT-qPCR results, two leg samples with the lowest Ct values for each strain of CHIKV were chosen. The amount of CHIKV RNA from the leg samples, expressed in genome-equivalent copies (GEC), was 2.73 and 6.43 $\log_{10}$ GEC for each of the two leg samples infected with the prototype S27 strain and 5.78 and 7.19 $\log_{10}$ GEC for each of the two leg samples infected with the emerging ITA strain. First-instar larvae of mosquitoes were exposed to CHIKV-infected bodies as a food source. In order to help the larval feeding process, the thoraces of mosquitoes were crushed with a pipette. In a previous experiment, we confirmed that *Ae. albopictus* larvae (L1) were able to feed upon uninfected dead mosquitoes placed on the water surface (data not shown).

3.2.4. Procedure

Six groups of 100 first-instar larvae were placed into each tray with 250 mL of dechlorinated tap water under simulated summer conditions (26 ºC-22 ºC; 86% RH; 14h: 10h, light: dark). Larvae were exposed to CHIKV by the 2 routes described above: via viral suspension inoculum (G1 and G2) or dead infected imagoes (G4 and G5) into water. Control larvae groups were run in parallel to each treatment and consisted of larvae exposed to virus-free medium, Dulbecco's modified Eagle's medium (DMEM) (G3) or to uninfected cadavers of mosquito (G6).

For each group, water samples of 0.5 mL were taken at 0, 4, 6, 8, 10, and 12 days and stored at -80 ºC. The quantities of food given were 0.1, 0.2, 0.3, and 0.4 mg/larva Tetramin® fish food depending on the larval stage (L1-L4). The total quantity of food in each tray was adjusted

to the number of surviving larvae. An optimal amount of food was provided to groups G1, G2 and G3, whereas, groups G4, G5 and G6 were starved of larval food for 3 days to force cannibalism of the first-instar larvae on mosquitoes carcasses. After 72 hours of starvation, groups G4, G5 and G6 were returned to their regular diet. During the experiment, the initial volume of water in the larval rearing trays was not maintained at a constant level.

Containers were monitored daily until all individuals had emerged as adults or died as an immature stage. Larvae dying during the experiment were removed. The newly emerged adults, both male and female, were held up to 1-2 days post-emergence. Adult mosquitoes from groups G1 and G2 were fed with honey-soaked FTA™ card to detect potential transmission of CHIKV, whereas emerging adults from the control group (G6) were fed with sucrose solution (10%) administered on soaked cotton pledgets. Mosquitoes were sacrificed using an excess of CO2, separated according to sex and stored individually at -80 ºC for later testing. Mosquitoes found dead were also stored at -80 ºC.

For each treatment, it was measured the number of dead larvae and pupae, number of missing larvae due to cannibalism, larval mortality rate (the percentage of dead larvae (including the larvae disappeared), pupation rate (the percentage of larvae that reached the pupal stage) and the emergence rate (the percentage of pupae that successfully emerged as an adult mosquito). The number of males and females emerging was recorded and used to determine the sex male/female (M/F) ratio.

3.3. Viral detection

Viral RNA from water samples and mosquito samples were extracted using NucleoSpin® RNA Virus (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's instructions. The presence of CHIKV RNA was detected by an in-house quantitative real-time RT-PCR (RT-qPCR) used in the routine diagnostic of CHIKV infection by the National Centre for Microbiology, Institute of Health Carlos III (CNM-ISCIII). A standard curve was generated using duplicates of 10-fold serial dilutions of DNA plasmid containing a partial region of the CHIKV genome. Quantification of viral RNA was done by comparison of the threshold cycle (Ct) values of the samples to the standards according to the ΔCt analysis and expressed as genome-equivalent copies (GEC). Limit of detection was 5.37 GEC of plasmid per reaction. Results were given as GEC for mosquitoes and water samples.

4. RESULTS

4.1 Detection of CHIKV in water

Water containing virus inoculum was analysed by RT-qPCR at the time of inoculation (0 dpi) and after 4 and 8 days (Table 6.1). Water samples from control groups (G3 and G6) and for trays with infected mosquitoes as food larvae source (G4 and G5) were negative for CHIKV.

Group	Treatment group	GEC/0 dpi	GEC/4 dpi	GEC/8 dpi
G1	Viral suspension S27 strain	6.60	3.78	3.79
G2	Viral suspension ITA strain	7.07	6.85	6.72
G3	Control medium DMEM	undet	undet	undet
G4	Infected mosquitoes S27 strain	undet	undet	undet
G5	Infected mosquitoes ITA strain	undet	undet	undet
G6	Control uninfected mosquitoes	undet	undet	undet

Table 6.1. CHIKV viral load (log_{10} GEC) in water. dpi, days post-inoculation; undet, undetermined.

A reduction in CHIKV RNA, as indicated by decreasing GEC values (from 6.60 to 3.78 log_{10} GEC), was observed on the 4th day for S27 strain (G1) (Table 6.1), whereas the viral load of the ITA strain (G2) remained stable (range 6.72-7.07 log_{10} GEC) through 8-day observation period (Table 6.1).

4.2 CHIKV was not detected from adult mosquitoes

The total number of adults mosquitoes emerged was 117 mosquitoes (71 males and 46 females). The M/F ratio ranged from 1.3 to 2 (Table 6.2). The proportion of larvae that survives to pupation (PR) or adult emergence (ER) did differ between treatments. The method used for larvae exposure to CHIKV influenced larval survivorship and rates of pupation and emergence (Table 6.2). Pupation (PR) and emergence (ER) rates were higher in trays infected through viral suspension (PR 69-72%, ER 68-87%) compared to those infected with imago carcass (PR 12% and ER 33%) (Table 6.2).

The time to reach the pupal stage was longer for the groups in which infected mosquito carcasses were added to the water (9-10 days) than the groups contaminated with viral suspension (7-8 days) (data not shown). A high larval mortality rate was observed in both control groups (94 and 100%) as well as in the groups with food-deprived conditions (80 and 100%). (Table 6.2). We observed that the water in all trays evaporated up to 50% of the original volume. In the trays where infected imagoes were added, most parts of mosquito carcasses were not consumed.
All the adult mosquitoes emerged (n= 117) were tested by RT-qPCR, however, none of them was found to be CHIKV-positive.

Group	Treatment group	n° larvae (L1)	Mortality		n°missing larvae	Larval mortality arte	PR (n° pupae)	ER (n° adults)	adults mosquitoes		M/F
			n° larvae	n° pupae					n°♂	n°♀	
G1	Viral suspension S27 strain	100 L1	6	10	12	18%	72% (72)	87.5% (63)	36	27	1.3
G2	Viral suspension ITA strain	100 L1	1	23	6	7%	69% (69)	68.12% (47)	29	18	1.6
G3	Control medium DMEM	100 L1	35	0	65	100%	0%	_	_	_	_
G4	Infected mosquitoes S27 strain	100 L1	36	0	64	100%	0%	_	_	_	_
G5	Infected mosquitoes ITA strain	100 L1	22	8	58	80%	12% (12)	33.33% (4)	4	0	_
G6	Control uninfected mosquitoes	100 L1	52	2	42	94%	4% (4)	100% (4)	2	1	2

Table 6.2. Life-history traits of the mosquito strain *Ae. albopictus* exposed to CHIKV.
PR: Pupation rate; ER: Emergence rate.

5. DISCUSSION

Water is a vital component in the mosquito life cycle and might be a vehicle for the transmission of pathogens. Potential contaminating sources are diverse and might include tissues and body fluids of infected hosts in contact with water of breeding sites. As an example, it has been suggested that animals infected with Rift Valley fever virus might die in the vicinity of mosquito larvae breeding habitats contaminating the water where larvae could acquire the virus [347]. There is evidence that mosquito larvae mosquitoes may acquire Zika virus from human urine discharge into the environment, resulting in Zika virus infection of adult mosquitoes [345]. Based on the viral load found in pools of field-collected mosquitoes infected with arboviruses, with low titer ($\leq 3 \log_{10}$ PFU/mL) and with high titer ($> 3 \log_{10}$ PFU/mL) [354], a concentration of 5 $\log_{10}$ $TCID_{50}$ /mL of CHIKV in water was chosen for the experiments performed in this study.

We detected CHIKV RNA in water, but do not necessarily represent infectious particles, up to 8 days post-inoculation under summer conditions. Although virus survival is overestimated by molecular methods, in a previous study, we found that CHIKV could survive up to 8 days (1.8 $\log_{10}$ $TCID_{50}$ /mL) in filtered (22 μm) rainwater kept at room temperature (≈ 22 ºC) [352]. However, the same study also observed that CHIKV persistence was reduced to 4 days in unfiltered rainwater [352]. The presence of indigenous microbial populations (Monera and Protista) [355] or other microbes such as *Escherichia coli* and other thermotolerant coliforms [356] in mosquito larval habitats could limit CHIKV survival in the unfiltered rainwater. There are many environmental factors affecting virus survival in natural aquatic habitats, such as the chemical and physical properties of water (temperature, pH, salinity, and organic matter) and the presence of organic matter. Besides, the sunlight can inactivate the viral population and, the rainfalls may decrease the concentration of the virus in the water.

As previously described [229], infection of mosquito larvae by arboviruses would require a high viral dose as the infection is lost in each moult of the larval instar. Although mosquito larvae expel most viruses during moult to adult, some of them may be transstadially transmitted to the adult gut. For example, most West Nile virus infections (≈ 75%) were lost during moulting, metamorphosis, or both in a previous vertical transmission study testing larvae and pupae [357]. A previous study by Whitman *et al.*, [344] showed that a high initial dose of 33x106 minimum lethal doses (M.L.D) for mice/ cc of Yellow fever virus was necessary for *Ae. aegypti* new-born

larvae (L1) to retain the virus during development to adulthood. However, other studies showed that larvae of *Ae. aegypti* were infected with DENV and Zika virus at concentrations as low as 4 $\log_{10}$ FFU/mL and 1-2 $\log_{10}$ PFU/mL, respectively [345, 346]. These different results might be due to differences existent among mosquito strains, viruses and the experimental conditions of these studies.

The moulting phase could be a key element in the success of transstadial transmission of the virus, as the larval peritrophic membrane would not be permeable to virus-sized particles (such as CHIKV (50-60 nm) [177]). During metamorphosis of fourth-instar larvae to the pupal stage, the larval peritrophic membrane is destroyed, leaving access to the virus to the pupal tissues where it establishes a productive infection [346, 355, 358]. To better understand the larval infection process, it would be required the use of an electronic microscope in order to determinate the fate of the ingested virus, which would help in unveiling which factors control transstadial passage of arboviruses in mosquito larvae.

Under the conditions of this study, we found that *Ae. albopictus* larvae exposed to CHIKV failed to become infected regardless of the way used for infection. This negative result could be due to i) our small sample size, as 110 mosquitoes emerged from the trays with viral suspension and, only 4 adult mosquitoes emerged from the trays with infected mosquito carcasses, ii) a stressful environment during the growth. A limitation of food and water resulted in increased mortality and cannibalism and, iii) the low number of infected mosquitoes carcasses provided as a food source.

Our study was not able to demonstrate the transstadial transmission of CHIKV in a Spanish *Ae. albopictus* strain. Although transstadial transmission may be an inefficient mechanism to infect mosquitoes, it may contribute, even at a low rate, to increase the number of infected mosquitoes. This infection route, combined with the horizontal transmission, could have an impact on spreading the virus. Our group will be addressed this question in future studies with larger sample sizes.

In summary, our results suggest that CHIKV can survive and remain infective for a few days in aquatic larval habitats. Mosquito breeding sites may become a reservoir for the virus during an outbreak and may contribute to the epidemic potential of this virus.

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Chapter 7

Experimental study of the susceptibility of a European *Aedes albopictus* strain to dengue virus under a simulated Mediterranean temperature regime.

" La verdadera grandeza de la ciencia acaba valorándose por su utilidad"

Gregorio Marañón

1. ABSTRACT

Dengue virus (DENV) has re-emerged in Europe driven by the geographic expansion of the mosquito species *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae) and the introduction of the virus by viraemic travellers. In the present study, the vector competence (VC) of *Ae. albopictus* collected in Catalonia (northeast Spain) was evaluated for two different DENV strains, DENV-1 and DENV-2, the serotypes responsible for all outbreaks of dengue that have occurred in Europe. Mosquitoes were reared under environmental conditions mimicking the mean temperature and humidity recorded in July on the Mediterranean coast of Catalonia. Mosquitoes were fed on an artificial infectious bloodmeal and, after 14 days post-exposure, infection, dissemination and transmission rates (IR, DIR, TR) and transmission efficiency (TE) were determined by testing the virus in the body, legs and saliva. The tested *Ae. albopictus* strain was found to be susceptible to both DENV-1 and DENV-2 strains and to be able to transmit DENV-1. This is the first time that the VC of *Ae. albopictus* for DENV has been tested in Europe in this specific context (i.e., mimicking the Mediterranean temperature and humidity recorded in Catalonia in July). This study confirms the potential of *Ae. albopictus* to start autochthonous DENV transmission cycles in the Mediterranean basin.

Keyword: *Aedes albopictus*, dengue virus, Mediterranean climate, transmission, vector competence, Europe.

2. INTRODUCTION

Dengue virus (DENV) is a vector-borne *Flavivirus* of the family *Flaviviridae* and is mainly transmitted by *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse). Dengue virus is the most widespread of the arboviruses that affect humans, with more than 390 million cases of infection estimated per year [359]. Infection with any of the DENV serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) can either be asymptomatic (in 75% of the cases) or result in one of the three clinical forms of (increasing severity) dengue fever, dengue haemorrhagic fever and dengue shock syndrome [360].

Imported cases of DENV infection have been reported in several European countries in recent years [361]. In 2010, two different autochthonous outbreaks occurred in, respectively, France [214] and Croatia [212]. In 2012-2013, a large epidemic was reported in the Portuguese island of Madeira, which has been recolonized by Ae. aegypti [154]. Further autochthonous outbreaks were reported in France in 2013, 2014 and 2015 [155-157]. *Aedes albopictus* was indicated as the vector involved in all local transmissions in continental Europe, and the DENV strains belonged to either serotype 1 or serotype 2. *Aedes albopictus* was first introduced in Europe in 1979 [75]. Since then, it has spread rapidly to several European countries, especially in the Mediterranean basin, and was first identified in Spain in 2004 [76].

To assess the risk for local transmission events, it is essential to evaluate the vector competence (VC) of local mosquito populations. This is particularly important for *Ae. albopictus* as this species' VC for DENV has been shown to vary substantially among populations of different geographic origins [362, 363]. Previous studies in European *Ae. albopictus* populations have been performed at a constant temperature (28 ºC) to simulate the mean temperature in tropical countries where DENV is endemic. Only a few studies have addressed the effects of realistic temperatures on VC for DENV, and all of them were carried out in *Ae. aegypti* [240, 287, 364, 365]. These studies support the notion that local temperatures may alter VC. Therefore, measuring VC while simulating environmental conditions at the mosquito collection site is the best approach to estimating the risk for an outbreak in the case of DENV introduction. Consequently, this study was performed using the mean temperature and humidity recorded in July on the Mediterranean coast of the Iberian Peninsula to estimate the risk for autochthonous transmission of DENV.

3. MATERIAL AND METHODS

3.1. Mosquito strain

The strain *Ae. albopictus* used in this study was collected in Sant Cugat del Vallès in 2009 and reared in the laboratory to obtain a stable colony. Mosquitoes were reared under conditions that mimicked the environmental field conditions in their natural habitats of Catalonia during the month of July of the summer season. Climatological data were provided by the Meteorological Service of Catalonia (www.meteo.cat). Mean day and night temperatures were calculated with respect to the summer photoperiod in Catalonia ((26 ºC for 14 h (light) and 22 ºC for 10 h (dark)). An average relative humidity of 86% was calculated.

3.2. Virus strains

Two DENV strains were tested: (a) strain BE 56 (hereafter named DENV-1), a human isolate that belongs to DENV-1 serotype, collected during the dengue epidemic in Madeira (2012-13), and (b) strain 20112953 (hereafter named DENV-2), a human isolate belonging to the DENV-2 serotype and collected in 2015 in Thailand.

Virus stocks of DENV-1 were produced following two passages on C6/36 cells, whereas DENV-2 was obtained after one passage on C6/36 cells. Supernatants were collected and stored at -80 ºC prior to their use for mosquito oral feeding.

3.3. Vector competence assay design

To investigate VC, four different rates were considered: infection rate (IR); disseminated infection rate (DIR); transmission rate (TR), and transmission efficiency (TE). The IR was defined as the proportion of mosquitoes with virus-positive bodies (abdomen, thorax and head) among the tested mosquitoes. The DIR was defined as the proportion of mosquitoes with infected legs among those with infected bodies. The TR was defined as the proportion of mosquitoes with DENV RNA in saliva among the number of mosquitoes with disseminated infection. Transmission efficiency was defined according to the proportion of mosquitoes with

DENV RNA in saliva among the total number of mosquitoes tested.

Female mosquitoes aged 7-10 days, and not previously blood-fed before were fed using the Hemotek feeding system (Discovery Workshops, UK) with a pathogen-free chicken skin as a membrane. The bloodmeal was prepared by mixing DENV-infected bovine blood with heparin and ATP ($5x10^{-3}$ M) as phagostimulant (Sigma-Aldrich, St. Louis, MO). The viral load was 5.5 $\log_{10}$ $TCID_{50}$ /mL. The viral titre was chosen based on the reported viraemia level reached in human patients infected with DENV [366]. After blood feeding, 10% of fully engorged females were randomly collected and killed to confirm virus exposure. Then, fully engorged females were selected under carbon dioxide (CO2) anaesthesia and individually transferred to cardboard cages (Watkins & Doncaster, Leominster, UK). Females were kept for an extrinsic incubation period (EIP) of 14 days inside the climatic cabinet. Sucrose solution (10%) was administered on cotton pledgets placed on the mesh screen. At 14 days post-exposure (dpe), all mosquitoes were anaesthetized using CO2 and dissected. The legs and wings were detached from the body, and both parts were separately homogenized in 0.5 mL of Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland). The samples were homogenized at 30 Hz for 1 min using TissueLyser II (QIAGEN, Hilden, Germany) and stored at -80 ºC until tested for DENV. The same protocol was used in all assays aimed at estimating the mortality rate, as well as in those designed to obtain IR and DIR data.

To estimate transmission, two separate assays were designed. In the first assay, transmission was tested using FTA™ cards at two different time-points: 9 and 14 dpe. On those days, cotton pledgets were replaced by FTA™ cards (GE Healthcare, Little Chalfont, UK). The FTA™ cards were soaked with Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zeeland) mixed with a blue alimentary colorant. After collection, FTA™ cards were resuspended in 0.3 mL of phosphate-buffered saline (PBS) and stored at -80 ºC until tested. In the second assay, transmission was tested at 14 dpe with two different methods: (a) using FTA™ cards, as described above, and (b) performing a direct salivary extraction using a capillary technique, as previously described [367]. Briefly, after the dissection of the legs and wings, the proboscis was inserted into a P20 pipette tip filled with 7 µL of a 1:1 solution of fetal bovine serum (FBS) and 50% sucrose solution. To stimulate salivation, 1µL of 1% pilocarpine (Sigma-Aldrich Corp.) prepared in PBS at 0.1% Tween 80, was applied to the thorax of each mosquito. After a period of 60 min, the solution containing the saliva was expelled into 1.5-mL tubes containing 193 µL of DMEM; 150 µL were used for viral RNA extraction and the remaining 50 µL were used for DENV isolation.

3.4. Virus detection

Viral RNA was extracted from bodies, legs, FTA™ cards and saliva samples with the NucleoSpin® RNA Virus Kit (Macherey-Nagel GmbH & Co. Düren, Germany) following the manufacturer's recommendations.

The viral RNA was detected by real-time reverse transcription polymerase chain reaction (RT-PCR) as previously described [368] with minor modifications. A fragment of 88 pb from the 3' UTR region was amplified using primers DF (AGGACYAGAGGTTAGAGGAGA), DR (CGYTCTGTGCCTGGAWTGAT) and probe DP (6FAM-ACAGCATATTGACGCTGGGARAGACC-TAMRA). Amplification was performed using the AgPath One-Step ID RTPCR Kit (Ambion, Applied Biosystems, Inc., Foster City, CA, USA), and a 7500 Fast Real–Time PCR System (Applied Biosystems, Inc.) programmed as follows: 45 ºC for 10 min; 95 ºC for 10 min, and 40 cycles at 97 ºC for 15 s and 55 ºC for 30 s.

3.5. Virus isolation and titration

Virus isolation was performed in a monolayer of C6/36 cells. Cells were incubated for 6-7 days (28 °C, 5% CO2). As a cytopathic effect was not observed, DENV replication was detected in the supernatant using the real time RT-PCR.

Both DENV stocks were titrated in a monolayer of C6/36 cells. Routinely, eight wells were infected for each 10-fold dilution, and 20 μL quantities of inoculum were spiked into each well. After that, 150 μL Modified Eagle's medium (MEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 2% FBS (Life Science Co., London, UK), 2 mM L-glutamine, non-essential amino acids, 1,000 U/mL of penicillin, 10 mg/mL of streptomycin and 500 U/mL of nystatin (all from Sigma Corp.) were added per well and the plates were incubated at 28 ºC and 5% of CO2 for 7 days. Calculation of the viral titre was performed by virus detection in each well using the real-time RT-PCR described above. Cycle threshold (Ct) values ranged from 18.38 to 22.45 in those wells in which the virus replicated. The TCID50 / ml was calculated using the method of Reed and Muench [369].

3.6. Statistical analyses

Assays to estimate IR and DIR were conducted using mosquitoes were from the same colony and an identical protocol (i.e. they were two replicates of the same experiment), Thus, the results from the first and second assays could be combined and the differences between DENV-1 and DENV-2 evaluated using a chi-square test.

In addition, the ability of each strain of DENV to replicate in different mosquito tissues (body and legs) was also evaluated using real-time reverse quantitative PCR (qPCR). Mean Ct values of the bodies and legs infected with DENV-1 and DENV-2, respectively, were compared. Briefly, the normality of the data was assessed using the Shapiro-Wilk test, and then data were compared using the t-test (in the case of normally distributed data), or the Wilcoxon rank sum test (in the case of non-normally distributed data). All calculations were carried out using R statistical software (http://cran.r-project.org/).

4. RESULTS

4.1. Mosquito infection.

The results of the assays for the estimation of the mortality, the IR and the DIR are shown in Table 7.1.

The finding of positive bodies indicated that the virus was able to successfully cross the midgut infection barrier successfully 14 dpe. Positive legs showed mosquitoes in which the virus was also able to cross the midgut escape barrier. Assessment of infection showed that the cumulative IRs for DENV-1 and DENV-2 were 53% (49/93) and 33% (26/78), respectively. The statistical analysis revealed that the IR was significantly higher for DENV-1 than for DENV-2 ($p= 0.041$). The cumulative DIR values were also higher for DENV-1 than for DENV-2 at 53% (26/49) and 35% (9/26), respectively (Table 7.1). However, the difference was not statistically significant ($p= 0.12$).

	Mortality		IR		DIR	
	DENV-1	DENV-2	DENV-1	DENV-2	DENV-1	DENV-2
First assay	7/50 (14%)	15/50 (30%)	27/43(63%)	9/35 (63%)	10/27 (37%)	5/9 (56%)
Second assay	0/50 (0%)	6/49 (12%)	22/50 (44%)	17/43(40%)	16/22(73%)	4/17 (24%)
Total	7/100 (7%)	21/99 (21%)	49/93 (53%)	26/78 (33%)	26/49 (53%)	9/26 (35%)

Table 7.1. Mortality, infection rates (IRs) and disseminated infection rates (DIRs)
Rates were obtained at 14 days post-exposure for dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in the first and second assays

Viral loads of DENV-1 and DENV-2 were analysed in bodies and legs. The mean ± standard deviation (SD) Ct values of the bodies infected with DENV-1 and DENV-2 were 23.0 ± 2.8 and 25.6 ± 3.5, respectively (Figure 7.1).

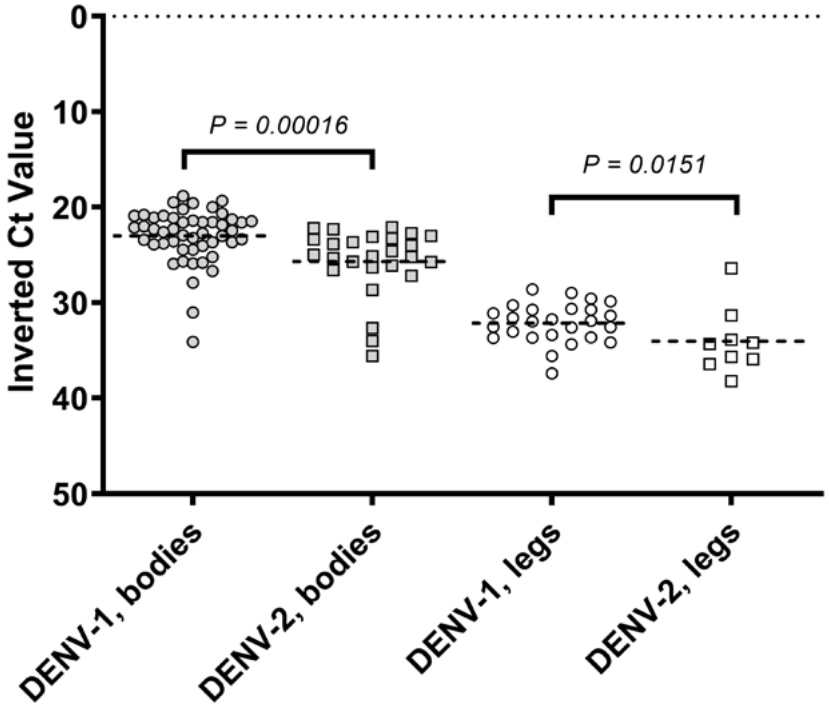

Figure 7.1. Viral loads of dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in bodies and legs of infected mosquitoes. The mean Ct value for each group is represented by a discontinuous line. p-values indicate a statistically significant higher viral replication for the DENV-1 strain than for the DENV-2 strain in bodies (p= 0.00016). No statistical significant difference (p= 0.153) between viral replications in legs was evidenced.

The result of the Wilcoxon rank sum test indicated that the difference in the mean Ct values between DENV-1 and DENV-2 was statistically significant (p= 0.00016). This result suggested that DENV-1 was able to replicate more efficiently than DENV-2 in the body of *Ae. albopictus*. By contrast, the mean ± SD Ct values of the legs infected with DENV-1 and DENV-2 were 32.1 ± 2.0 and 34.0 ± 3.4 respectively (Figure 7.1). The result of the t-test indicated that the difference in mean Ct values between DENV-1 and DENV-2 was not statistically significant (p= 0.151). Therefore, although DENV-1 was able to replicate more efficiently than DENV-2 in mosquito bodies, females with disseminated infection had similar amount of virus in the legs regardless of viral strain.

4.2. DENV transmission

In the first assay, the FTA™ cards were collected at 9 dpe and at 14 dpe. The FTA™ cards collected at 9 dpe from both groups (DENV-1 and DENV-2) showed negative findings (Table 7.2).

However, two FTA™ cards collected at 14 dpe from two mosquitoes exposed to DENV-1 were positive. These specimens also showed positive legs, indicating the presence of a disseminated infection. Therefore, the TR for DENV-1 was 20% (2/10). Conversely, at the same time-point (14 dpe), all the FTA™ cards from mosquito exposed to blood-infected with DENV-2 were negative.

In the second assay, all the FTA™ cards and saliva samples were collected at 14 dpe. The FTA™ cards and the saliva samples from mosquitoes exposed to both viral strains tested negative by RT-qPCR. Moreover, direct isolation in Vero cells from saliva samples gave negative results.

Given the results obtained using molecular techniques with FTA™ cards, the estimated TE for DENV-1 was 4.6% (2/43).

	FTA™ at 9 dpe		**FTA™ at 14 dpe**		**Saliva**	
Assay	DENV-1	DENV-2	DENV-1	DENV-2	DENV-1	DENV-2
1	0/43	0/35	2/10	0/5	na	na
2	na	na	0/16	0/4	0/50	0/43

Table 7.2. Transmission rates of dengue virus (DENV) serotypes 1 (DENV-1) and 2 (DENV-2) in the first and second assays. Positive samples of FTATM cards (9 dpe and 14 dpe) and saliva (14 dpe) by retrotranscriptase quantitative polymerase chain reaction. dpe, days post-exposure; na, not applicable

5. DISCUSSION

For the first time in Europe, the VC of *Ae. albopictus* for DENV was estimated under a protocol that mimicked the temperature conditions present in a Mediterranean area during the summer month of July. Recent data suggest that the accurate assessment of the risk for DENV transmission of a mosquito population requires the incorporation of local temperature in the VC experiments [240]. The results of the present study provide evidence that the *Ae. albopictus* strain tested is susceptible to oral infection with both DENV-1 and DENV-2 at a viral load (5.5 log10 TCID50 /mL) within the range of viremia (3-8 $\log_{10}$ $TCID_{50}$ /mL) observed in humans [370]. The IR was statistically higher for DENV-1 than for DENV-2, which indicates that susceptibility is dependent on the serotype of DENV, as observed by Gubler and Rosen [362].

The *Ae. albopictus* strain tested in the present study was more susceptible to DENV-1 infection in terms of disseminated infection (DIR: 53%) than the French population of *Ae. albopictus* (DIR: 28-45%) exposed to 105.3 FFU /mL of blood-meal and maintained at a constant temperature (28 ºC) [273]. Such variation may be explained by the different environmental conditions assayed, but geographical differences in the vector population and the virus strain cannot be ruled out.

Previous studies examining VC in Mediterranean populations of *Ae. albopictus* (from France, Italy and Lebanon) [330, 332, 333] for the same Asian DENV-2 genotype and conducted under identical environmental conditions (constant temperature of 28 ºC) showed a wide range of DIRs of 12-69%, 14-38% and 32-47%, respectively. The fluctuating conditions of the present study (daytime temperature of 26 ºC and nigh-time temperature of 22 ºC) produced a DIR of 35% for the DENV-2 strain, which is within the range of DIRs reported in the earlier works [330, 332, 333].

In addition to differences in IRs between DENV strains, the present study found a significant difference in viral infectivity. The comparison of mean Ct values in the body for both DENV strains indicated a statistically significantly higher viral replication for DENV-1 than for DENV-2, which may reflect differences in the immune response to viral infection [371]. By contrast, there were no statistically significant differences between strains tested in the mean Ct values for the legs. This suggests that the efficiency of viral replication in the midgut did not determine the amount of virus in other tissues after dissemination, as reported in previous studies [372].

The use of FTA™ cards was originally developed for field studies [323, 373]. However, FTA™ cards may be also used in VC assays and allow the collection of saliva from a single mosquito at different time-points without requiring the specimen to be killed. The technique does not require trained personal, whereas the case of the capillary technique does, and reduces the operative time required to collect the sample. The principal disadvantage of the technique is that the saliva cannot be used for direct virus isolation in cell culture. Thus, although the use of FTA™ cards cannot replace virus isolation, it represents a good strategy for evaluating transmission capacity at intermediate time-points without the need to create subgroups of specimens and sacrifice them periodically.

The transmission capacity of each mosquito was tested by using FTA™ cards at two different time-points (9 dpe and 14 dpe). At 9 dpe no mosquitoes were able to transmit either strain. However, at 14 dpe, the mosquito strain tested was able to successfully transmit the DENV-1. By contrast, DENV-2 was not detected in mosquito saliva at 14 dpe at the conditions used in the present work. These negative results do not imply to that DENV-2 transmission will not occur under different conditions. Thus, the extended period needed to complete the EIP of DENV (from 9 dpe to 14 dpe) may have an impact on the VC of this Spanish mosquito strain, potentially reducing the risk for outbreak and providing a larger time window in which to implement surveillance and vector control measures. In previous VC studies using Mediterranean mosquito populations [273, 333], EIP varied although the mosquitoes were held at a constant temperature of 28 ºC. The DENV-1 strain presented a shorter EIP and could be transmitted by a French population of *Ae. albopictus* at 9 dpe (67%) [273]. By contrast, DENV-2 seemed to require a longer EIP to achieve transmission [273]. The same strain was not detected in saliva of a Lebanese *Ae. albopictus* population at 10 dpe, but achieved at 38% TR at 21 days [333]. It is important to note that, by contrast with the previous studies, the present experimental infections were performed under a protocol that mimicked fluctuating temperature conditions, which strengthens the findings of this work. The average temperature within the current profile (22-26 ºC) is 24 ºC, which is 4 ºC lower than temperatures used in the comparable studies mentioned above, and which may potentially result in lower infection and transmission rates, and also lengthen the EIP [374]. The results of previous studies in Ae. aegypti VC for DENV under realistic temperature regimes indicate that fluctuations around low temperatures (20 ºC) enhanced the mosquito's VC [287]. This was not reported with fluctuations around high temperatures (26 ºC) [364, 365]. In Europe, DENV epidemics have occurred in the Mediterranean area and during the summer. These environmental conditions allowed *Ae. albopictus* mosquitoes to acquire and subsequently

transmit DENV from viraemic travellers to local populations [375]. The results of the present study confirm that the *Ae. albopictus* strain from Catalonia is competent for DENV under the temperature conditions that prevail on the Mediterranean coast of Catalonia during the summer. Although the present results point to a low TE (4.6%) for DENV-1, this parameter is only one of the multiple components used to determine VC. Additional ecological (mosquito population densities, feeding behaviour, daily mosquito survival) and anthropogenic (frequency of arrival of viraemic hosts, population density) factors must be considered to determine the real risk for DENV transmission in the Mediterranean area.

CONCLUSIONS

The present results indicate that the tested *Ae. albopictus* strain was susceptible to both DENV-1 and DENV-2. The mosquito strain tested was also able to transmit DENV-1, demonstrating that a local transmission event is possible in the Mediterranean region. This study contributes to knowledge of the VC of *Ae. albopictus* for DENV, which may be useful in the development of DENV risk models and surveillance programmes.

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Chapter 8

GENERAL DISCUSSION

"Science is a quest for understanding.
Quest: a long or ardous search for something"

Jocelyn Bell Burnell

In the last decade, Chikungunya and Dengue viruses, have become global pathogens. Locally acquired chikungunya and dengue infections have been reported in tropical, subtropical and temperate regions of the world [375]. There are rising concerns about how easily viraemic travellers are able to introduce arboviruses from endemic into naïve-areas, in which potential competent mosquitos are present. The risk of mosquito-borne diseases outbreak in Spain and other European countries has led to an increasing interest in factors determining the competence of local European *Ae. albopictus* populations for arboviruses. In order to understand virus transmission, it is crucial to determine VC of local mosquito populations. This thesis provides insight into three factors, host viraemia, viral strain, and environmental conditions which can influence the VC of a Spanish strain of *Ae. albopictus* for CHIKV (Chapter III). The level of viraemia in CHIKV-infected patients is generally high (10^9-10^{12} viral particles/mL) and typically lasts 4–6 days [376, 377], but can persist up to 12 days after the onset of symptoms [378], thereby extending the period of human infectiousness to mosquitoes. CHIKV infections among travellers returning to the US exhibit viraemia (from 3.9 to 6.8 $\log_{10}$ PFU/mL) [269], which are of a sufficient magnitude to infect *Ae. albopictus* mosquitoes, in accordance with the results presented in Chapter III. Previous studies have shown that *Ae. albopictus* populations (from the US) may transmit CHIKV after blood-feeding in viraemia titres as low as 3.9 $\log_{10}$ PFU/mL [271, 272]. These results indicate that such a low titre threshold is enough for infection and transmission in this mosquito, which may heighten the risk of local transmission. Thus, it is necessary to seek new antiviral drugs to minimise CHIKV replication during the acute phase. In recent years, significant efforts have been made to identify some compounds with anti-CHIKV properties [379]. Certain molecules, including flavipiravir, 6-azauridine and silymarin, show antiviral activity when tested *in vitro* [380]. Others drug such as ribavirin and suramin reduce viral burden in CHIKV-infected mice [381, 382]. However, despite such advances, no effective drug currently exists for the treatment of Chikungunya infection in clinical settings. Under these circumstances, we recommend surveillance of chikungunya-infected travellers returning to areas where *Ae. albopictus* exist. Patients should employ measures to avoid mosquito bites for two weeks in order to minimise the risk of infecting mosquitoes and thereby prevent local transmission.

Traditionally, the transmission cycle of CHIKV takes place in tropical or warmer temperatures, and therefore, there is little empirical data on mosquito VC under colder temperatures. In general, warmer temperatures (from 23-29 ºC [383]) facilitate arbovirus transmission due to shorter development times of the mosquito's life cycle, faster viral reproduction and dissemination within

the vector, as well as higher biting-rate and a shortening of the gonotrophic cycle and EIP [384-386], while cooler temperatures are considered less conducive to transmission. According to our study, warmer temperatures (22-26 ºC) increase the development rate in the larval stage, survival to adulthood and the willingness of mosquitoes to blood-feeding, as well as the amount of blood intake (Chapters III and V). Unexpectedly, cooler temperatures (15-18 ºC) enhance CHIKV infection and dissemination rates, possibly leading to a higher probability of transmission than mosquitoes kept at warmer temperatures (22-26 ºC) (Chapter III). Although the dissemination rate constitutes a relevant parameter that provides an estimate of the transmission capacity of a population, it does not necessarily determine transmissibility. The increased likelihood of infection at lower temperatures could be associated with the heightened susceptibility of midgut cells to viral infection, a decreased mosquito immune response, and a change in the diversity and density of the gut microbiota triggered by cooler temperature [223, 228, 241, 287]. Considering the ongoing spread of *Ae. albopictus* to Central Europe [387], and the prolonged active period of *Ae. albopictus* in the Mediterranean region, which is capable of extending up to December [388], we recommend evaluating VC at cooler temperatures (e.g., ≤ 22 ºC). Recently published studies from laboratory experiments find that the optimal temperature transmission of dengue and malaria is at cooler temperatures than previously predicted [287, 389, 390]. These novel findings highlighted the need to research the thermal biology of MBDs.

The two viruses, CHIKV and DENV, have several similarities in terms of vector species, ecology and clinical symptoms at onset (as seen in Chapter I), however our results suggest (Chapters III, V, and VII) that they differ in their epidemic potential. At the population level, the transmission of CHIKV and DENV (taking into account all mosquitoes exposed to an infectious blood meal) was 5% for the CHIKV ITA strain and 4.6% for DENV-1 at 9 and 14 dpe respectively (Chapters V and VII). The transmission was attempted using FTATM cards at 9 dpe for CHIKV and at two different time-points, 9 and 14 dpe, for DENV. We found that DENV-1 took longer to reach de salivary glands (14 dpe) than CHIKV-ITA strain (9 dpe), as evidenced in previous studies [305, 366]. This extended EIP of DENV-1 may have a significant impact on DENV epidemiology, given that only a proportion of infected mosquitoes would be able to survive to transmit the virus. This difference may partially explain why that whilst the number of imported dengue cases in Europe is much higher than the imported chikungunya cases (18,817 cases versus 3,672 cases from 2008 to 2017) [391-395], the number of locally-transmitted human cases due to CHIKV is 18 times higher than those resulting from DENV (736 versus 41 laboratory-confirmed cases) (updated August 2019; see Tables 1.2 and 1.3,

pages 46-47; Chapter I). Thus, this data altogether with our VC results suggest that CHIKV may have a higher epidemic potential than DENV in European populations of Ae. albopictus. This idea is also supported by previous VC studies that have confirmed that European *Ae. albopictus* mosquitoes were a more efficient vector for CHIKV in comparison with DENV under laboratory conditions [329, 332].

We observed great variability in *Ae. albopictus*' VC for CHIKV and DENV among different European *Ae. albopictus* populations (see Chapters V and VII). These studies varied in mosquito strains, viral strains and environmental conditions. This provides evidence of the three-way interaction between mosquito genotype, virus genotype and environmental factors (G x G x E). Furthermore, differences among laboratories relating to rearing protocol, the use of laboratory colony or field-mosquitoes, infection method, and type of sample analysis may produce a variation in VC outcomes. Ideally, laboratory procedures should be standardised in order to be able to compare the VC results of different laboratories. Once this issue is addressed, these studies would provide valuable information about virus-vector interactions that could be useful for searching new methods of reducing the viruses transmission.

It is important to point out that a significant limitation of this study is the fact that the mosquitoes used in the experiments derived from a well-established laboratory colony. Nowadays, it is well known that colonisation may alter the VC of mosquitoes because some of the genetic variability may have been lost at the time of colonisation (sample effect) or in later generations (genetic drift) [396-398]. However, according to Amraoui *et al.*, [399] 'using mosquito colonies for VC studies can be considered as a proxy for measuring the genetic ability of our species to transmit a given pathogen'. Future studies should test the VC of field-collected *Ae. albopictus* mosquitoes or their progeny (F1-F5 generations) for the purpose of validating the results of these VC studies.

Another concern is that there is experimental evidence that *Ae. albopictus* can replicate and deliver both CHIKV and DENV in their saliva after oral infection [400]. In 2007, Gabon faced a simultaneous CHIKV/DENV outbreak driven by *Ae. albopictus* [108]. Therefore, further studies are necessary to evaluate the dynamics of transmission and virulence when both viruses are infecting mosquitoes and human hosts as a result of a simultaneous or sequential infection. The *Ae. albopictus'* VC to CHIKV and DENV found in this study was low (5 and 4.7% respectively). However, the risk of transmission and spread of these arboviruses in Catalonia

and other parts of Spain should not solely rely on the vector competence outcome. Other factors must be considered in order to assess the risk of transmission such as vector density, human-biting rate, length of gonotrophic cycle, mosquito lifespan, vertical transmission, the proportion of viraemic travellers, immunologically naïve population, infectious asymptomatic people, and favourable environmental conditions. Local transmission of DENV from imported cases has already occurred in Spain. After almost a century without reported dengue outbreaks in Spain (the last one taking place in Andalusia in 1928 [49]), two small clusters of dengue fever were reported: in Andalusia (summer 2018) and Catalonia (autumn 2018) [158, 401]. In addition, DENV was isolated from wild *Ae. albopictus* mosquitoes caught around the residence of an imported case in Catalonia, confirming the circulation of DENV in local populations [402].

Recent studies have underscored the importance of incorporating realistic environmental conditions in the laboratory VC studies [71, 240, 280], especially when we wish translating these findings into the natural setting. Our VC studies were undertaken mimicking the environmental conditions recorded in Ae. albopictus' habitats in Catalonia, which strengthens the findings of this work.

Moreover, other *Aedes* native species such as *Aedes caspius* (Pallas, 1771), *Aedes detritus* (Haliday, 1833), *Aedes geniculatus* (Olivier, 1791), *Aedes vexans* (Meigen, 1830); as well as the invasive species *Ae. koreicus* and Ae. japonicous should also be considered and investigated as potential vectors of CHIKV and DENV in Spain (current distribution in Europe: https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps). A previous study found that field-collected *Ae. caspius* and *Ae. detritus* from France were susceptible to infection by CHIKV (E1-226V) (IRs of 25 to 67% respectively), whereas *Ae. vexans* was refractory to CHIKV infection [403]. The abundance of these species in Spanish rural environments and their anthropophilic feeding behaviour [404-406] suggest these species should be assessed as potential vectors for CHIKV. Another study has recently revealed that the native European mosquito *Ae. geniculatus* (from Albania) is highly susceptible to CHIKV (E1-226V) and could transmit the virus experimentally [407]. The new invasive species *Ae. koreicus* has recently been found in Belgium, and Italy [408, 409]. This species was competent for CHIKV (E1-226V) under laboratory conditions [410]. Another candidate species of interest is the invasive mosquito *Ae. japonicous*, a species recently detected in Spain in 2018 [411], and which has shown potential to be a vector for both CHIKV and DENV [412]. Taking into account these results, we recommend

examining the capability of other members of *Aedes* genera to act as CHIKV or DENV vectors in Spain. The virus adaptation to new vectors cannot be excluded, as previously observed in the first outbreak of CHIKV on La Réunion Island.

Experimental animal models for CHIKV infection, such as mice and non-human primates, have been widely used to address questions regarding CHIKV infection in human hosts. Many research groups have used mouse strains for studying CHIKV pathogenesis, in the most part, defective in type 1 IFN signalling [298, 413]. Our study provides new insights into the virulence of two strains of CHIKV belonging to the ECSA lineage in the mouse strain IFN-α/β R-/- A129. We found that the African S27 strain (E1-226A) was more virulent and caused greater mortality in A129 mice than the emergent ITA strain (E1-226V). The S27 strain led to a higher weight loss, earlier death, and higher disease score compared to the ITA strain, despite displaying similar histopathological changes (Chapter IV). Further studies are required in order to identify the molecular and immunological mechanisms behind these differences in virulence. Since the mouse model showed a low allergic reaction to *Aedes* mosquito bites, it could be useful for establishing a mosquito-mediated transmission model and to test antiviral drugs (Chapter V). In fact, novel candidate vaccines and therapeutic options against CHIKV have been evaluated using the A129 mouse model (reviewed in [414]).

Understanding how CHIKV may survive within their vector breeding sites in temperate climates may be useful in establishing additional control measures. In Europe, where there is no evidence of vertebrate reservoirs of CHIKV, one of the primary mechanisms by which CHIKV could be maintained in nature is through vertical transmission [350]. Other possible pathways obtained from laboratory experiments include transstadial passage of certain arboviruses from larvae to adult mosquitoes [345, 346, 415]. In Chapter VI, we assess the possibility of whether exposure of larval *Ae. albopictus* to CHIKV can lead to the emergence of infected adult mosquitoes. In the laboratory, we found that CHIKV RNA can survive up to 8 days in the water, constituting a potential source of infection for immature stages of mosquitoes (Chapter VI). Our laboratory group had previously found that *Ae. albopictus* could become infected with CHIKV by breeding in infectious water with a viral suspension of 5 $\log_{10}$ $TCID_{50}$ /mL [416]. However, in the course of preparing this thesis, we failed to confirm this route of infection in a second replicated experiment (Chapter VI). Although transstadial transmission of CHIKV was not detected under the laboratory conditions tested, it constitutes a route of infection that is worth exploring.

We can speculate that the transstadial transmission may be a mechanism for the survival of viruses within mosquito populations. This mechanism may contribute to the amplification of the virus through horizontal transmission in two ways: i) female mosquitoes infected as larvae may be capable of transmitting the virus to a human host during their first blood meal, and/or ii) male mosquitoes infected as larvae may be able to transmit the virus to uninfected females via venereal transmission. These infected females are indeed able to initiate horizontal transmission. We are unaware if a transstadial infection is equivalent to one acquired through horizontal transmission. It has been reported that pathogens transmitted horizontally tend to be more virulent than those transmitted vertically [338]. However, previous studies showed that the imagoes of infected larvae were capable of subsequently transmitting viruses (such as West Nile virus and Zika virus) horizontally in laboratory conditions [342, 344].

Recent findings indicate that arboviruses such as DENV, West Nile virus, Japanese encephalitis, Yellow fever virus and CHIKV may persist in biological fluids such as urine during the acute phase of the disease [417] providing additional means for the maintenance of arboviruses in the environment. The presence of infectious virus from human urine could raise the question as to whether larvae may become infected in larval breeding sites contaminated with urine from viraemic patients, as it has been described for the Zika virus [344]. Further studies are necessary to assess the ability of viruses to survive in water.

Despite growing public health concerns about chikungunya and dengue human infections, there are significant gaps in the epidemiology of CHIKV and DENV outbreaks in temperate regions. The incidence of CHIKV and DENV in the Mediterranean area of Europe shows seasonal patterns [387]. Transmission occurred mostly during the warmer months of the year, between July and September, which coincides with the breeding season of mosquito vector (Tables 1.2 and 1.3, pages 46-47; Chapter I). Based on environmental conditions observed during the recent outbreaks, extreme weather events may be identified as one of the risk factors for arbovirus transmission in the Mediterranean area. In some MBDs, a link between the emergence of the disease and climate change has recently been proved. For example, extreme temperatures during the summer of 2010 were considered environmental precursors of West Nile fever outbreaks in humans in Europe [418]. Heavy rainfall may have contributed to the autochthonous CHIKV transmission in Montpellier (France) during the autumn of 2014 [206]. In other cases, we can only speculate. For example, temperatures above the seasonal average in the summer of 2014 combined with a dry period may contribute to local mosquito abundance and therefore, to

transmissibility of CHIKV in 2017 in Italy and France [211]. Extreme rainfall events registered during the summer of 2014 may have also enabled the transmission of DENV in the south of France [155]. Although climate change itself is not the cause of these outbreaks, it could further the spread of mosquito-borne infection by increasing vector density and accelerating virus reproduction within mosquito vectors. Climate change predictions of more prolonged and more intense heatwaves, flooding events and droughts during the summer, could facilitate the spread of some mosquito-borne diseases to new geographic areas [419]. Unveiling the relationship between climatic factors and these outbreaks could assist in improving surveillance and may be a useful tool in identifying the periods of the year with a higher risk of arbovirus transmission. A platform currently exists which is aimed in assisting in the analysis of the risk of different arboviral diseases, which could possibly invade and spread into Catalonia (http://arbocat.cat/en/home/).

As invasive species, *Ae. albopictus* continues its expansion across Spain [246], implying that new locations would be at risk of MBDs. This mosquito species tends to proliferate in artificial containers in urban environments, in close association with humans. Furthermore, *Ae. albopictus* tends to take multiple blood meals during a single gonotrophic cycle [420], increasing the risk of arbovirus transmission to several people once infected and the likelihood of co-infections in both mosquito and human hosts. By far, vector control is the most effective method available for preventing and controlling an outbreak [421]. However, reducing the presence of the vector in the environment has not been proved sufficiently useful. Recently, it has appeared the use of genetically modified mosquitoes to control MBDs and the use of vector microbiome (symbiotic bacteria), or insect-specific viruses (ISVs) to modulate vector-virus interactions, thereby alter the susceptibility of arboviruses [422, 423]. In the case of symbiotic bacteria, the most studied example is the bacterium *Wolbachia* spp. The *Ae. albopictus* mosquito strain used in the VC studies were found naturally infected with *Wolbachia* spp, which open a new research path. Future studies should be undertaken in order to explore the interactions Wolbachia-arbovirus as a means of modulating VC. The majority of known ISVs have been isolated from mosquitoes associated with arbovirus pathogens of the families Flaviviridae (e.g., DENV) and Togaviridae (e.g., CHIKV) [423]. In a similar manner of *Wolbachia, i*t could be used as strategy for reducing VC of mosquitoes for arboviruses [423].

In summary, the results presented in this thesis add knowledge on vectors and arboviruses' circulating in Catalonia. These studies represent a starting point for future studies in the field of public health entomology.

Chapter 9
CONCLUSIONS

"En el lenguaje científico la claridad es la única estética permitida"

Gregorio Marañón

CONCLUSIONS

I. Vector competence studies showed that the *Ae. albopictus* strain from Catalonia is competent for CHIKV and DENV under the laboratory conditions tested. Our results indicate that low-level transmission of CHIKV and DENV by this species would be possible in Catalonia and other regions with similar climatic conditions.

II. The seasonal climatic patterns influenced the rate of larval development, survival to adulthood, and size of the blood meal in *Ae. albopictus*.

III. Rearing the whole life cycle under colder conditions enhanced infection and dissemination rates of a Spanish strain of *Ae. albopictus* for CHIKV when compared to warmer conditions. This viral adaptation to low temperatures might further facilitate the transmission of CHIKV in colder months and higher-latitude regions of Europe biting rate.

IV. Viraemia levels were an essential factor in *Ae. albopictus* susceptibility to CHIKV infection. Our results indicate that Ae. albopictus could become infected with CHIKV and develop a disseminate infection, a prerequisite for transmission, at low level of viraemia. This information is essential in assessing risk of an outbreak in case of CHIKV introduction in Spain.

V. The use of A129 mice with genetic deficiencies in the type I interferon signalling pathway proved helpful in the comparison of the virulence of two CHIKV strains of the East/Central/African lineage. Subcutaneous inoculation of CHIKV in A129 mice showed that the African prototype strain was more virulent and caused higher mortality and viraemia levels than the emergent viral strain. A129 mice could be useful in establishing a mosquito transmission model and for studying new antiviral drugs.

VI. Attempts to demonstrate transstadial transmission of CHIKV in immature stages of *Ae. albopictus* failed under the laboratory conditions tested. The small sample size used, and the likely low transstadial transmission rate may have influenced the outcome. This issue remains to be investigated with a larger sample size in temperate populations of *Ae. albopictus*.

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