Universitat Autònoma de Barcelona

Influence of mosquito-virus interaction on Zika virus and Rift Valley fever phlebovirus transmission

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PhD Thesis

Bellaterra, 2020

Influence of mosquito-virus interaction on Zika virus and Rift Valley fever phlebovirus transmission

Tesis doctoral presentada por **Ana Isabel Núñez García** para acceder al grado de Doctora en el marco del programa de Doctorado en *Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona*, bajo la dirección de **Núria Busquets Martí** y la tutoría de **Francesc Accensi i Alemany.**

Bellaterra, 2020

La Dra. **Núria Busquets Martí**, investigadora del *Institut de Recerca i Tecnologia Agroalimentàries - Centre de Recerca en Sanitat Animal* (IRTA-CReSA) y Dr. **Francesc Accensi i Alemany**, profesor titular del *Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universidad Autónoma de Barcelona* e investigador adscrito al IRTA-CReSA.

Certifican:

Que los trabajos de investigación desarrollados en la memoria de tesis doctoral "*Influence of mosquito-virus interaction on Zika virus and Rift Valley fever phlebovirus transmission*", presentados por la licenciada **Ana Isabel Núñez García** para la obtención del Grado de Doctor en *Medicina i Sanitat Animals* se ha realizado bajo la dirección y tutoría, y autorizan su presentación a fin de ser evaluada por la comisión correspondiente.

Y porque así conste y tenga los efectos que correspondan, firman el presente certificado.

Bellaterra (Barcelona), 22 de abril de 2020

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La presente tesis doctoral ha sido realizada con el soporte de la Agencia de Gestión de Ayudas Universitarias y de Investigación (AGAUR) (FI 2017) y el Fondo social Europeo.

Este trabajo ha sido parcialmente financiado por los *Centres de Recerca de Catalunya* (CERCA), por el Ministerio de Asuntos Económicos y Transformación Digital (Gobierno de España) con el proyecto MINECO AGL2013-47257-P y por *European Union's Horizon 2020 Research and Innovation Programme* con el proyecto ZIKAlliance (Grant Agreement no.734548)

La impresión de esta tesis ha estado financiada por el programa de doctorado en *Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona*.

Diseño de portada: A. Berdugo Vega

A los que ya no están
Y a los que resuenan conmigo

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Abbreviations

AMPs: antimicrobial peptides;
API5: apoptosis inhibitory protein 5;
ATP: adenosine 5'-triphosphate;
BUSCO: Benchmarking Universal Single-Copy Orthologs;
CHIKV: chikungunya virus;
CPE: cytopathic effect;
CTL: C-type lectin-related;
DEFA: Defensin-A;
DEFC: Defensin-C;
DEG: differentially expressed genes;
DENV: Dengue virus;
DIR: disseminated infection rate;
DMEM: Dulbecco's Modified Eagle Medium;
dpe: days post exposure;
dpi: days post-inoculation;
Drosha: ribonuclease 3;
dsRNA: double stranded RNA;
E1: ubiquitin-activating enzymes;
E1: first oviposition (of the gonotrophic cycle);
E2: second oviposition (of the gonotrophic cycle);
EEEV: eastern equine encephalitis virus;
EIP: extrinsic incubation period;
FBG: pathogen-binding fibrinogen-like domain;
FBN: fibrinogen-domain immuno-lectin;
FC: Fold Change;
FEF: full-engorged inoculated females;
FIR: Filial infection rate;
FKH: Fork head;
FREP: fibrinogen-related protein;
GO: gene ontology;
hpe: hours post-exposure;
Hsp70: heat shock protein 70;
IAPs: inhibitors of apoptosis;
IMD: Immune deficiency pathway;
IR: infection rate;
JAK/STAT: Janus kinase/signal transducer and activator of transcription;
JEV: Japanese encephalitis virus;
LOQS: Loquacious or R3D1;
LRIMs: leucine-rich repeat immune proteins;
LRR: leucine-rich repeats;
MEB: midgut escape barrier;
MIB: midgut infection barrier;
miRNA: microRNA pathway;
miRNAs: microRNAs;
MLs: myeloid differentiation 2-related lipid recognition receptors;

NCBI: National Center for Biology Information;
nt: nucleotides;
Nup88: nuclear pore complex protein Nup88
PA28: proteasome activator complex subunit 3;
PFU: Plaque forming units;
PI3K: phosphatidylinositol 3-kinase;
piRNA: P-element-induced Wimpy testes gene (PIWI)-interacting RNAs;
PIWI: P-element-induced Wimpy testes gene;
PRRs: pattern-recognition receptors;
Rel: Relish;
RISC: RNA-silencing complex;
RNAi: RNA interference;
RVF: Rift Valley fever;
RVFV: Rift Valley fever phlebovirus;
SGEB: salivary gland escape barrier;
SINV: Sindbis virus;
SLEV: St. Louis encephalitis virus;
siRNA: small interfering RNA;
SRRPs: small regulatory RNA pathway members;
STKc_PAK3: catalytic domain of the protein serine/threonine kinase;
$TCID_{50}$/mL: 50 % tissue culture infective dose per mL;
TE: transmission efficiency;
TOLLPATH: Toll Pathway members;
TR: transmission rate;
UBA: ubiquitin-activating enzyme;
UBC or E2: ubiquitin-conjugating enzyme;
UBX: ubiquitin regulatory X domain;
UMP1: ubiquitin-mediated proteolysis 1;
UPP: ubiquitin proteasome pathway;
VC: vector competence;
VT: vertical transmission;
WNV: West Nile virus;
YFV: yellow fever virus;
zf-TRAF: TNF receptor-associated factor;
zf-UBR or E3: ubiquitin ligase containing the UBR box;
ZIKV: Zika virus;

Abstract

Vector-borne diseases represent a 17 % of infectious diseases in the world. Among them, those diseases caused by arboviruses (**ar**thropod-**bo**rne **vi**ruses), which circulate in the nature between arthropods (their vectors) and vertebrate hosts (their reservoirs), are currently provoking serious diseases in humans and animals. For decades, the arboviral diseases were neglected, since most of them were located in developing areas. Nowadays, environmental, ecological and socioeconomic factors (*e.g.*, globalization and climate change) have contributed to the emergence and re-emergence of arboviral diseases. The constant movement of people and merchandise has allowed the colonization and establishment of exotic mosquito species in our country such as the tiger mosquito (*Aedes albopictus)*, which is a potential vector of many arboviruses (*e.g.*, dengue virus, Zika virus or chikungunya virus). This thesis focused on conducting vector competence and transmission studies in local mosquito species for Zika virus (ZIKV) and on the study of the *Culex pipiens* transcriptome alteration after being exposed to the Rift Valley fever phlebovirus (RVFV) in order to better understand how virus-vector interaction influences on ZIKV and RVFV transmission.

Chapters I and II focused on estimating the vector competence for ZIKV of different field-collected mosquito species present in our country: *Aedes albopictus, Aedes caspius* and *Culex pipiens*. In addition, vertical transmission studies were performed to determine if the progeny of females infected with ZIKV were able to disseminate the virus. The results of these studies showed that local populations of *Ae. albopictus* were competent vectors for ZIKV and *Cx. pipiens* and *Ae. caspius* species were refractory for this arbovirus. Moreover, it was demonstrated that ZIKV was able to be transmitted to the progeny but the later could not disseminate the virus.

Chapter III focused on the study of interactions between the *Cx. pipiens* mosquito species and RVFV at molecular level, with the aim to characterize the alterations in the expression of the mosquito genes related to the immune system during RVFV infection by analyzing *de novo* transcriptome. As a result, 48 immune differentially expressed genes in mosquitoes exposed to RVFV were altered, which could serve as potential targets to control the infection, either by unbalancing the mosquito tolerance to RVFV or by inhibiting the infection in mosquitoes. The results obtained on the *Cx. pipiens* transcriptome alterations due to exposure to RVFV pave the way for future functional studies about genes involved in the control/tolerance of RVFV infection.

Overall, this thesis increased the knowledge to better design efficient strategies for ZIKV and RVFV surveillance and control.

Resumen

Las enfermedades transmitidas por vectores representan un alto porcentaje de las enfermedades infecciosas en el mundo. Concretamente, las enfermedades causadas por arbovirus (**ar**thropod-**bo**rne **vi**ruses), que circulan en la naturaleza entre artrópodos (sus vectores), y los hospedadores vertebrados (sus reservorios), pueden causar enfermedades graves en los hospedadores vertebrados, pero no causan una patología significativa en los vectores. Durante décadas las enfermedades causadas por arbovirus fueron olvidadas, ya que en su gran mayoría estaban localizadas en zonas en vías de desarrollo. En la actualidad, factores ambientales, ecológicos y socioeconómicos, como el cambio climático y la globalización, han contribuido a la emergencia y reemergencia de las enfermedades arbovirales. El constante movimiento de personas y mercancías ha dado lugar a la colonización y establecimiento de especies de exóticas en nuestro país, como el mosquito tigre (*Aedes albopictus*), el cual es transmisor de muchos arbovirus (*e.g.* el virus del dengue, el virus Zika (ZIKV) o el virus chikungunya). El desarrollo de esta tesis se centró en realizar estudios de competencia vectorial para el ZIKV y en un estudio del transcriptoma de *Culex pipiens* después de ser expuesto al phlebovirus de la fiebre del Valley del Rift (RVFV) para comprender las interacciones el virus y los mosquitos locales.

Los capítulos I y II se focalizaron en estimar la competencia vectorial para ZIKV de diferentes especies de mosquitos de campo presentes en nuestro país: *Aedes albopictus*, *Aedes caspius* y *Culex pipiens*. Además, se desarrollaron experimentos de transmisión vertical para determinar si la generación de mosquitos provenientes de hembras infectadas con el ZIKV es capaz de diseminarlo. Durante el desarrollo de estos estudios, se ha demostrado que los mosquitos locales de la especie *Ae. albopictus* son vectores competentes para el ZIKV. Sin embargo, las especies *Cx. pipiens* y *Ae. caspius* son refractarias para este arbovirus. Con respecto al experimento de transmisión vertical, se

demostró que la progenie de las hembras inoculadas con el virus de forma intratorácica fue susceptible a la infección del virus, pero no fueron capaces de diseminarlo.

Por otro lado, el capítulo III se centró en el estudio de las interacciones a nivel molecular entre la especie de mosquito *Cx. pipiens* y RVFV, con el objetivo caracterizar las alteraciones a nivel molecular de la expresión de los genes correspondientes al sistema inmune del mosquito durante la infección por RVFV mediante un análisis del transcriptoma *de novo*. Como resultado, se obtuvieron 48 genes diferencialmente expresados en los mosquitos ante la presencia del virus que servir de diana para controlar la infección, ya sea para desequilibrar la tolerancia de los mosquitos al virus como para inhibir la infección en los mosquitos. Los resultados obtenidos del estudio de las alteraciones del transcriptoma de mosquitos de la especie *Cx. pipiens* expuestos a RVFV sientan las bases para la realización de futuros estudios funcionales de los genes involucrados en controlar/permitir la infección por RVFV.

En general, el desarrollo de esta tesis incrementa el conocimiento para mejorar el diseño de estrategias eficientes para la vigilancia de vectores transmisores del ZIKV y del RVFV.

PART I

General section: introduction and objectives

GENERAL INTRODUCTION

1. Arthropod-borne diseases

The recent globalization, particularly the increasing commercialization and the number of travelers to areas with high medical risks (*e.g.*, tropical areas), is one of the most important factors that alters the interactions between humans, animals and the environment, ultimately affecting the emergence or re-emergence of several diseases (Cleton et al. 2012). Most of these emerging diseases are arthropod-borne diseases, which are caused by a pathogen agent transmitted by an arthropod-vector and provoke relevant morbidity and mortality in the world. Importantly, most of these diseases occurs primarily in developing countries (Hill et al. 2005), which entails several problems in their monitoring and control. Additionally, the capacity of arboviruses to evolve and adapt to new arthropod vectors might be a key factor on its geographical expansion. Other factors related to the emergence of the arthropod-borne disease comprise: the genetic evolution of the pathogens, climate change (which can influence in the expansion of the vector distribution), uncontrolled use of insecticides, adaptation of the pathogens to new reservoirs (amplification hosts), rapid extension of urbanization, deforestation, lack of vector control measures and, as pinpointed above, increase of travelers (Musso and Gubler 2016).

Most arthropod-borne diseases are caused by the **ar**thropod-**bo**rne **vi**ruses (arboviruses), which use an arthropod vector in their biological transmission cycle. The arboviral transmission is mainly by blood-feeding vectors such as mosquitoes, ticks, midges and sandflies.

Currently, more than 150 arboviruses are reported to be zoonotic causing diseases in humans, domestic animals and wildlife. Most of arboviruses belong to *Togaviridae*, *Flaviviridae* and *Phenuiviridae* families, but there are also a reduce number of

arboviruses that belong to *Orthomyxoviridae* and *Reoviridae* families (Table 1) (Cleton et al. 2012).

Table 1. Arbovirus belongs to six virus families. Table adapted from Go *et al*. (2014).

RNA	**Family**	**Genus**	**Virus species of human/ veterinarian medical interest**
Single-stranded positive-sense RNA	*Togaviridae*	*Alphavirus*	*-Chikungunya virus*
	Flaviviridae	*Flavivirus*	*-Zika virus* *-Dengue virus* *-Yellow fever virus*
Single-stranded negative sense RNA	*Peribunyaviridae*	*Orthobunyavirus*	*-La Crosse orthobunyavirus*
	Nairoviridae	*Orthonairovirus*	*-Crimean-Congo hemorrhagic fever orthonairovirus*
	Phenuiviridae	*Phlebovirus*	*-Rift Valley fever phlebovirus*
	Orthomyxoviridae	*Thogotovirus*	*- Thogoto thogotovirus*
Double-stranded RNA	*Reoviridae*	*Orvivirus* *Coltivirus*	*-Bluetongue virus* *-Colorado tick fever virus*

Some of the most spread arboviruses transmitted by mosquitoes include West Nile virus (WNV), Rift Valley fever phlebovirus (RVFV), dengue virus (DENV), chikungunya virus (CHIKV), Zika virus (ZIKV) and other encephalitis with human and veterinarian medical relevance. Currently, there are 3,570 mosquito species (order Diptera; family Culicidae) (Ralph Harbach 2013), of which around 300 mosquito species can transmit human and animal arboviruses (Liang et al. 2015).

1.1. *Arboviral transmission cycles between vertebrate hosts and mosquito vectors*.

Most arboviruses are zoonotic, and their horizontal transmission presents an **enzootic or sylvatic cycle** using vertebrate hosts, such as non-human primates, rodents, birds or other mammals (Fig 1. A) (Weaver & Barrett, 2004). The enzootic cycle is maintained among wild animals and enzootic or main vectors, in which virus amplification takes place. The vertebrate host acts as an enzootic reservoir, since it does not develop symptoms of disease (*e.g.*, sylvatic DENV, yellow fever virus [YFV] or WNV). When the transmission occurs between domestic animals and the main or secondary vectors is called **epizootic or rural cycle** (Fig 1. B). In this cycle, the virus is amplified in domestic animals, also known as amplifying host, with the risk of being transmitted to humans by insect vectors, *e.g.*, JEV or VEEV. This takes place when the viral load in the amplifying host is high enough to be transmitted to a vector during a blood-feeding. Some arboviruses have changed their host-range, from enzootic reservoirs to humans giving rise to a new transmission cycle called **urban or epidemic cycle** (Fig 1. C), in which the virus circulates among humans and urban vectors (*e.g.*, *Ae. aegypti* and DENV, YFV or CHIKV). In these cases, humans are good viral sources due to its high viremia levels, and the transmission cycle occurs repeatedly. The viral transmission in the urban cycle occurs when the density of human population is enough to maintain the virus (Go et al. 2014).

According to the above described cycles, some arboviruses affect primarily humans since they have re-emerged with the increasing of the urbanization turning humans into their main reservoirs and no longer need enzootic amplification to produce a new epidemic (*e.g.*, ZIKV, DENV or CHIKV) (Weaver and Reisen 2010). Other arboviruses are compromising the animal and human health due to the virus circulation in both and the close relationship between domestic/wild animals and humans (*e.g.*, RVFV or WNV). The present

thesis focused in two arboviruses: i) ZIVK, which does not need animals for its transmission, and ii) RVFV, which requires animals to keep its transmission cycle.

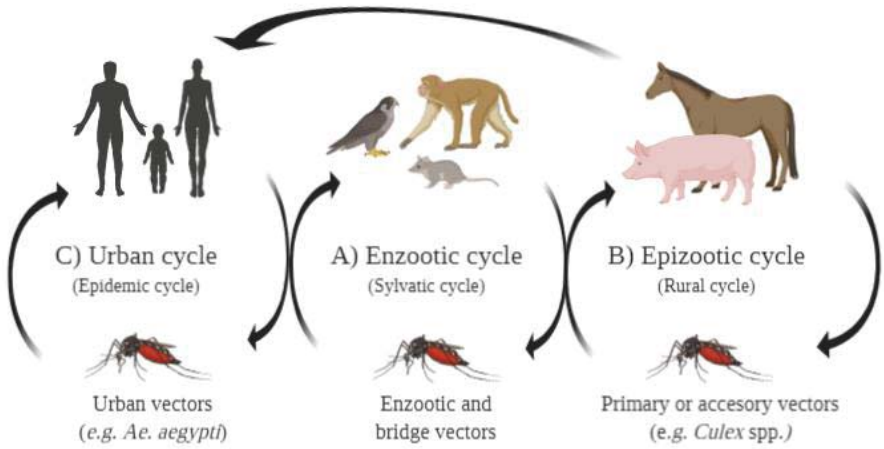

Figure 1. Representation of the transmission cycle between vectors and vertebrate hosts. Figure adapted from Go *et al*. (2014). Created with the Biorender.com

2. Zika virus

Zika virus is one of the most recent emerging arboviruses in the world. It has caused several outbreaks and epidemics in the Pacific island and American continent and has been associated with severe clinical manifestations and congenital malformations, such as microcephalies and Guillain-Barre syndrome (Basu and Tumban 2016).

2.1. *Classification, morphology, genome structure and phylogeny,*

Zika virus belongs to the *Flavivirus* genus within the *Flaviviridae* family (a genus that includes as well other 53 viral species, such as DENV, YFV and WNV [ICTV 2019]). This virus is comprised into the Spondweni serocomplex (Kuno et al. 1998).

Zika virus is an enveloped virus with an internal nucleocapsid and an external lipid bilayer. The linear single-stranded positive-sense RNA genome of ZIKV (10,794-nucleotides) and multiple copies of the viral capsid (C) protein are included in the internal nucleocapsid. The external host cell-derived lipid bilayer is protected by the viral membrane M protein and the envelope (E) protein. The ORF encodes a polyprotein cleaved into 10 proteins: 3 structural (C, prM, E) that form the virion and 7 non-structural (NS1, NS2a, NS2B, NS3, NS4a, NS4b, and NS5), which are crucial for the viral RNA replication (Fig 2) (Song et al. 2017, Boyer et al. 2018).

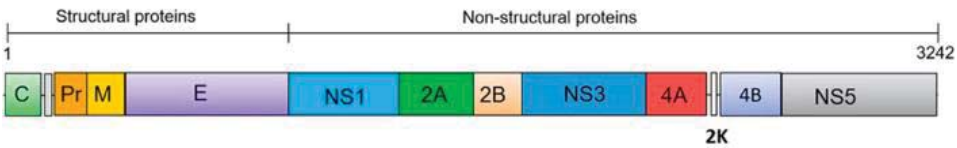

Figure 2. ZIKV structure of the genome. Extracted from Hu and Sun (2019).

A recent phylogenetic analysis based on the sequence of the polyprotein of 87 ZIKV strains characterized three ZIKV lineages: Asian, African I and African II (Saiz et al. 2017). Results from Li *et al*. (2017) suggest that ZIKV was originated in Africa followed by a spreading to Asia, Pacific islands and America given that the ZIKV strains identified in the American continent are phylogenetically close to Asian and Pacific strains.

2.2. *Epidemiology*

Zika virus was first isolated from a sentinel rhesus macaque monkey in the Zika forest of Uganda in 1947 during a yellow fever study (Dick et al. 1952). Since then the virus has been found in Asia in the 1960s causing sporadic human disease cases (Hills et al. 2017). The first large outbreak caused by ZIKV was reported in the Yap Island (Federate States of Micronesia) in 2007 (Duffy et al. 2009), which spread to other Pacific islands in 2013 (Musso et al. 2014). Finally, ZIKV reached Latin America in May 2015 (Zanluca et al. 2015) affecting almost 34,000 humans in more than 45 American countries until now (PAHO n.d., Boyer et al. 2018, WHO 2019). In February 2016, the WHO declared that Zika infection constitutes a Public Health Emergency of International Concern (PHEIC) (WHO 2016).

2.3. *Transmission cycle of the virus, hosts and vectors*

Concerning ZIKV transmission, there are two principal cycles: an enzootic or sylvatic cycle and an epidemic or urban cycle (Basu and Tumban 2016, Song et al. 2017). Enzootic cycle occurs in sylvatic habitats through the bite of arboreal infected mosquito females (*e.g*., *Aedes spp*.) to non-human primates in forests (Fig 3) (Weaver et al. 2016). In sylvatic habitats, ZIKV can also be transmitted to humans by the bite of infected mosquitoes following viral replication in humans and viremia (Basu and Tumban 2016, Song et al. 2017). Then, an urban cycle begins in towns when the virus is transmitted from an infected individual to urban mosquitoes during a blood meal and then

mosquitoes can transmit the virus to other humans (Song et al. 2017). Importantly, infected pregnant women can also transmit the virus to the fetus during pregnancy and it is also possible the sexual transmission between humans (Basu and Tumban 2016). The mosquito females can transmit vertically the virus to their progeny and venereal transmission also is possible from males to females and vice versa (Gutiérrez-Bugallo et al. 2019). Currently, it is unknown if the virus could be transmitted by sexual transmission between rhesus monkeys (Basu and Tumban 2016).

Vertebrate animals play a key role in the maintenance of the virus in the nature. At present, more than 79 vertebrate species have been identified as potential host since were natural or experimentally susceptible to ZIKV infection. Most of them are mammals, particularly primates (*e.g.*, *Macaca mulatta* and *Cercopithecus aethiops*) (Gutiérrez-Bugallo et al. 2019).

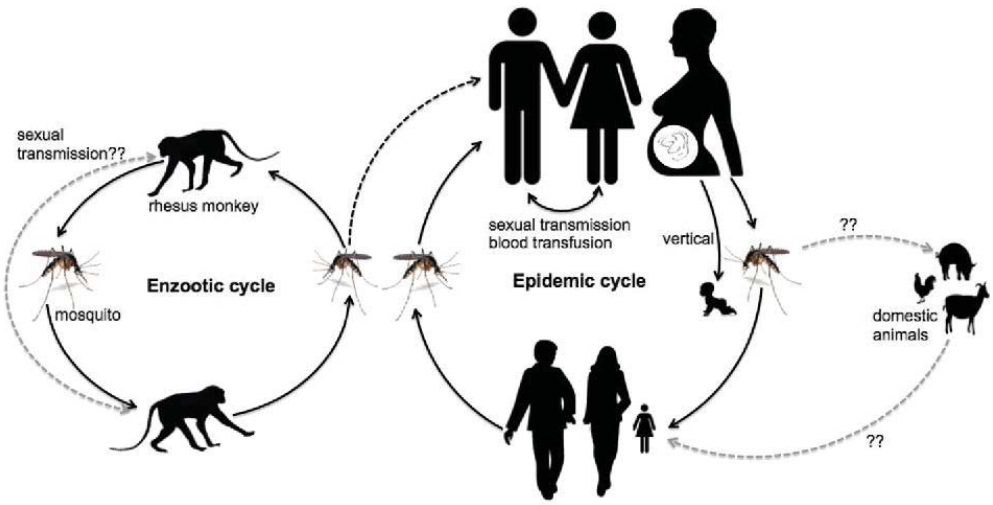

Figure 3. The cycle of Zika virus transmission. Extracted from Basu and Tumban (2016).

Zika virus has been isolated from numerous field mosquito species in African, American and Asian continents (*e.g.*, *Ae*. *aegypti*, *Aedes africanus*, *Anopheles coustani* or *Anopheles gambiae* among other species). The principal ZIKV vector is *Ae*. *aegypti*, which has been confirmed experimentally in field-collected mosquito populations from several countries of Africa, America and Asia. In addition, *Ae*. *albopictus* has been involved in ZIKV transmission, as the virus was detected in field-collected mosquitoes from Gabon and Brazil and has been experimentally demonstrated in vector competence (VC) studies (Boyer et al. 2018, Vazeille et al. 2019).

3. Rift Valley Fever phlebovirus

Rift Valley fever is an emerging arthropod-borne zoonosis disease caused by the pathogen agent RVFV. The infection causes fatal illness in wild animals and ruminants (*e.g.*, sheep or cattle), causing high economic losses in livestock production due to the high mortality and abortion rates. The virus sporadically spreads to humans and, among these infections, 1–2 % advances to major disease, causing retinitis, blindness, encephalitis, hepatitis and a hemorrhagic fever (Weaver and Reisen 2010, WHO 2020) with less than 1 % of mortality. Despite the low human mortality due to RVFV infection, the illness survivors could suffer visual and neurological disorders (Baba et al. 2016).

3.1 *Classification, morphology, genome structure and phylogeny.*

Rift Valley fever phlebovirus belongs to the *Phlebovirus* genus (family *Phenuiviridae;* order *Bunyavirales*).

The RVFV virions are spherical particles with an envelope (Ikegami 2012) containing a single-stranded negative-sense RNA genome. The envelope is formed by a lipid bilayer covered by 122 capsomers composed by Gn and Gc glycoprotein heterodimers. Three segments of single-stranded RNA compose the RVFV genome: S, M and L segments, which encodes for four structural proteins and two non-structural proteins (Fig 4). The S segment expresses with ambisense strategy the nucleoprotein (N) and a non-structural protein NSs, which is a main factor of virulence. The M segment encodes two glycoproteins (Gn and Gc) and the non-structural protein Nsm. The L segment encodes the viral RNA-dependent RNA polymerase (L protein) (Pepin et al. 2010, Ikegami 2012, Mansfield et al. 2015).

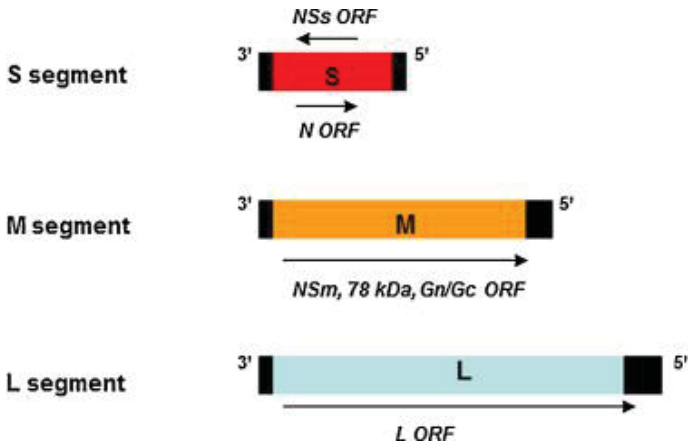

Figure 4. Genome structure of the RVFV. Extracted from: Mansfield et al. 2015.

In the last 25 years, several analyses of RVFV genetic diversity have been published. A study performed by Grobbelaar *et al*. (2011) established the 15 genetic lineages (Grobbelaar et al. 2011). This study demonstrated that the RVFV genetic diversity in all lineages is limited, with maximum differences in pairs for the S segment and partial M segments (Grobbelaar et al. 2011, Ikegami 2012). The analysis of RVFV isolates through epizootic and epidemic outbreaks shows two RVFV diversity patterns: i) outbreaks occurred in East Africa were caused by different lineages of RVFV, suggesting enzootic circulation of these lineages between mammal reservoirs and local mosquitoes (Ikegami 2012), and ii) the isolated strains during the epidemic outbreaks occurred in countries such as Egypt, Madagascar and Mauritania were closely associated to a RVFV strain of Central Africa and showed a limited genetic diversity (Ikegami 2012). This suggests that these outbreaks come from the introduction of a RVFV strain of Central African, followed by transmission between human and animals through mosquitoes (Ikegami 2012).

3.2 *Epidemiology*

The virus was identified for the first time in 1931 after an epizootic outbreak that caused sheep abortions and mortality in 1930 in the Lake Naivasha (Rift Valley province, Kenya). The first human death occurred in 1934 (Baba et al. 2016). The next large epidemic and epizootic outbreak was in South Africa in 1951 causing around 500,000 abortions and 100,000 deaths in sheep (Linthicum et al. 2016). Since then, repeated outbreaks have been reported in several African countries, such as Kenya, South Africa or Nigeria, during the rainiest years. There is a high association between RVFV occurrence and unusual water availability since all outbreaks in sub-Saharan areas (in the Africa Horn) took place after the excessive rainfall in savanna grasslands and that El Niño Southern Oscillation (ENSO) phenomenon. Due to an epizootic outbreak in Sudan in 1976, the virus arrived to Egypt causing an epidemic from 1977 to 1979 affecting 200,000 people, and 600 lethal cases were officially reported (Chevalier et al. 2010). Other minor outbreaks were also reported in 1993, 1994, 1997 and 2003 in Egypt (Ahmed Kamal 2011). The virus emerged outside Africa for the first time in 2000, causing an epizootic and epidemic outbreak in Yemen and Saudi Arabia (Linthicum et al. 2016). In the last years, in 2007-2008, 738 human cases were reported in Sudan, counting 230 victims (Chevalier et al. 2010). The last RVF outbreaks have been recently reported during January and April months in 2020 in Libya and Rwanda.(OIE, 2020).

3.3 *Transmission cycle of the virus, host and vectors*

The RVFV transmission cycle is complex due to: i) the direct transmission from infected animals to non-infected animals or humans, ii) the potential transovarian transmission in some mosquito species, and iii) the amount and different ecology of possible vectors (Chevalier et al. 2010). The RVFV cycle is summarized in Fig 5, where two overlapping cycles can be appreciated: i) an enzootic transmission cycle between primary vectors and wild animals as potential amplifying

hosts, and ii) an epidemic-epizootic transmission cycle between secondary vectors and domestic animals as amplifying hosts (Bird et al. 2009). The epidemic-epizootic cycle is associated with unusual weather events such as ENSO phenomenon, since the severe rainfalls provide a good aquatic habitat to hatch the floodwater eggs of *Aedes* mosquito species (Bird et al. 2009). In this scenario, the infected adult mosquitoes infect the domestic animals (*e.g.*, goats and sheep), which can develop high viraemias. At this point, the infected animals can infect the secondary vectors during a blood-feeding (Bird et al. 2009).

The RVFV transmission from animal to animal can also occur through several routes: i) via milk, from infected animal to lactating animals, ii) via direct contact from infected animals, fluids or tissues to healthy animals and iii) during vaccination, due to the re-use of needles. The transmission to humans can take place: i) by the bite of infected mosquitoes, ii) through exposure to infectious aerosols and iii) via direct contact during the slaughtering or necropsy of viremic animals (Bird et al. 2009). Because humans do not develop very high viraemia, they are considered dead-end hosts, however this hypothesis is yet to be confirmed (Chevalier et al. 2010, Lumley et al. 2017).

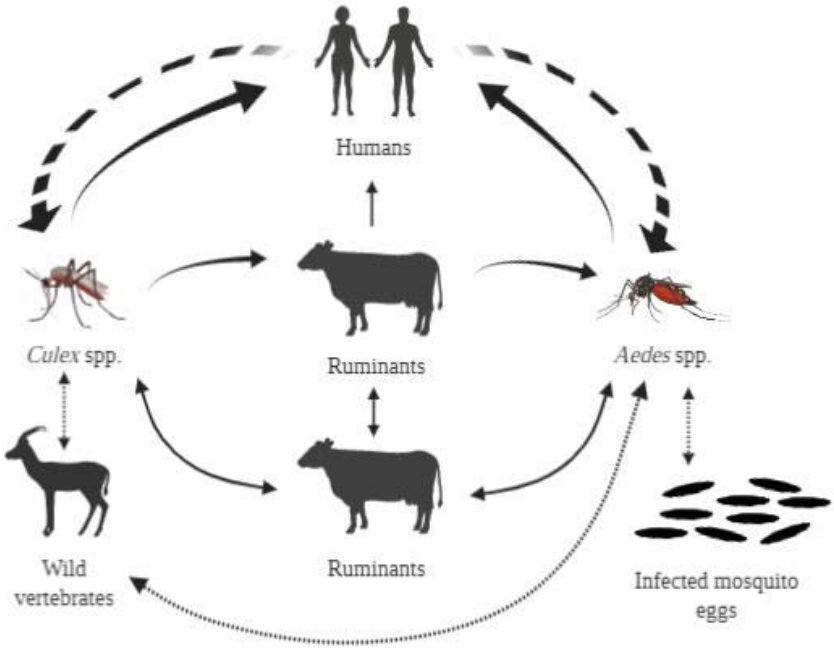

Figure 5. The Rift Valley fever virus transmission cycle. The arrows represent the transmission direction between hosts and vectors. The continue lines represent the routes demonstrated by laboratory experiments and dotted lines show the routes still to verify by experimental assays. Adapted from Lumley *et al*. (2017) and created with the Biorender.com.

The main vertebrate hosts are domesticated camels and domestic ruminants, particularly goats and several species of ungulate wild animals, including African buffalo, common warthog and giraffe (Linthicum et al. 2016).

Since the identification of RVFV in 1931, mosquito species have been involved in the viral transmission, among other arthropods, *e.g*., ticks, sandflies and biting midges. Currently, the virus has been isolated from at least 53 species in eight genera belonging to Culicidae family (Linthicum et al. 2016, Lumley et al. 2017). The *Aedes* and *Culex* genus are the most relevant vectors for RVFV *cf.* other genus, such as *Anopheles*, *Coquillettidia*, *Eretmapodite*, *Mansonia* and *Ochlerotatus*. In addition, *Aedes macintoshi* mosquitoes and other

species as *Aedes vexans* could transmit the virus vertically to their progeny (Chevalier et al. 2010). The infected eggs of some *Aedes* genus are thought to survive during the drought periods between epizootic cycles, giving rise to infected adults after rainy season. The virus is supposed to be preserved in an enzootic cycle among rainfall years and rainy seasons by vertical transovarially transmission in dryness resistant eggs of *Aedes* species, particularly *Aedes macintoshi* (Weaver and Reisen 2010). Nonetheless, this vertical transovarially transmission hypothesis remains to be demonstrated, as pinpointed previously by Lumley *et al*. (2017).

4. Mosquito biological cycle and virus transmission by mosquitoes

The life cycle of mosquitoes is composed by four development stages: egg, larvae, pupae and adult stages (Fig 6). The mosquito gonotrophic cycle, encompasses blood-feeding, egg maturation and oviposition (which can occur several times during adult female life). The whole gonotrophic cycle takes 2-7 days, depending on temperature (Paaijmans and Thomas 2011). Eggs maturation requires that females ingest blood that is protein-rich source (Raikhel 2005). Some species lay the eggs on the water surfaces and usually hatch in one or two days; however, the egg-habits vary widely between mosquito species as some mosquito species lay the eggs on moist soil surfaces where later flooding is required to hatch them. In the case of eggs laid on soil surface, the hatch may occur some months or years later. The larvae development is integrated by three molts until they became the fourth-stage larva. After several days, depending on factors such as water temperature, larvae molts to become pupa. Few days later (1-2 days), the adult mosquito (imago) emerges from the pupa; in general, the males emerge before than females. The entire life cycle is completed approximately in ten days during summer environmental conditions. There are various factors that influence the length-time of the cycle, such as the water temperature or larval and food density in the water (Higgs and Beaty 2005).

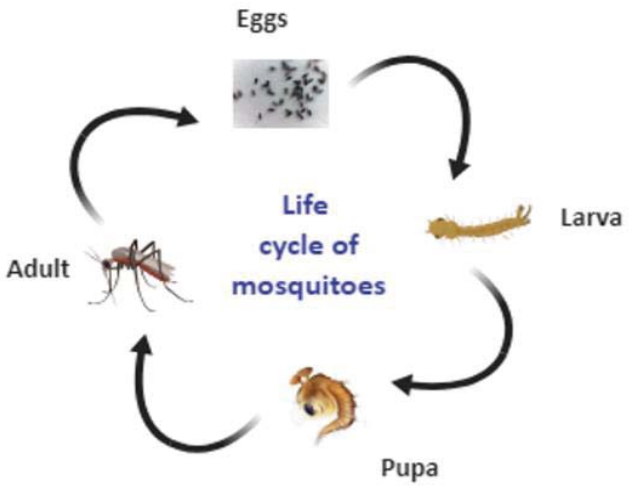

Figure 6. The mosquito life cycle. Created with Biorender.com.

The life cycle of arboviruses within the mosquito starts when an adult female bites a viremic animal or human, the arbovirus passes into the midgut and infects and replicates in the midgut epithelial cells crossing through the midgut infection barrier (MIB). Then, the virus disseminates to the hemolymph (within the hemocoel) to infect secondary organs, such as muscles, ovaries or fat body. Once the salivary glands become infected and shed virions into the salivary ducts, the virus could be transmitted to vertebrates during blood feeding. This is the most common route of arbovirus transmission and is known as horizontal transmission (Musso and Gubler 2016). However, the horizontal transmission may also be venereal between an infected male to non-infected female and vice versa (Campos et al. 2017). Moreover, vertical transmission (VT) has also been described from infected females to their progeny (Weaver and Reisen 2010). The VT in mosquitoes occurs through two mechanisms: i) by transovarial transmission, in which the virus infects germinal tissues and ii) by trans-ovum transmission, in which takes place at the time of the fertilization or through viral contamination in the egg surface during oviposition (Rosen 1987a, Higgs and Beaty 2005, Sánchez-Vargas et al. 2018).

5. Mosquito species as arbovirus vectors in Spain

Several autochthonous mosquito species in Spain can potentially act as arbovirus vectors. For instance, *Culex pipiens* (Linnaeus, 1758) from Spain has been proved experimentally to be able to transmit WNV (Brustolin et al. 2016) and RVFV (Brustolin et al. 2017). Also, *Aedes caspius* (Pallas, 1771) from France was reported as potential vector of RVFV (Moutailler et al. 2008). In addition, during the last 40 years, some exotic mosquito species have been reported in Europe as invasive species because they have established and adapted to the environmental conditions of European continent. Among these invasive mosquito species in Europe are included species such as *Aedes aegypti, Aedes japonicus, Aedes triseriatus and Aedes albopictus* (Skuse, 1895), most commonly known as the Asian tiger mosquito. Spanish *Ae. albopictus* populations were experimentally reported to transmit several arboviruses, such as DENV or RVFV (Brustolin et al. 2017, 2018). The present thesis has focused on three mosquito species (*Culex pipiens, Aedes caspius* and *Ae. albopictus)* from Spain in order to shed light in their capacity to transmit arboviruses.

Culex pipiens is a common mosquito that is widely distributed in urban and sub-urban temperate regions of the European countries (Amraoui et al. 2016). *Culex pipiens* species can be found in three forms morphologically no differentiable: i) *Cx. pipiens* form *molestus* (Forskal 1775), which feeds preferentially on mammals, ii) *Cx. pipiens* form *pipiens* with preferential blood-feeding behavior on birds and iii) *Cx. pipiens* hybrid form, which present both behavioral patterns. This behavior of the hybrid form was hypothesized to be a bridge vector for WNV (Farajollahi et al. 2011). *Culex pipiens* form *pipiens* is heterodynamic (undergoes diapause), anautogeneous (a blood-meal is necessary to lay the first eggs), and eurygamous (requires open spaces to mate). Conversely, *Cx. pipiens* form molestus is homodynamic (active during the year), autogeneous (does not requires a blood-meal to lay the first eggs) and stenogamous (mates in limited areas) (Amraoui

et al. 2012, Brugman et al. 2018). *Culex pipiens* form *molestus* is crepuscular feeder and also bites during the night (Schaffner et al. 2001). This species has been shown to play a key role in the re-emergence of WNV, Usutu virus and Sindbis virus in Europe (Brugman et al. 2018). The successful overwintering of WNV in Italian mosquito populations from 2008 to 2011 was an important epidemiological change (Calzolari et al. 2013, Brugman et al. 2018). Additionally, Spanish *Cx. pipiens* mosquitoes have recently been reported as potential vectors of zoonotic arboviruses, such as WNV (Brustolin et al. 2016) and RVFV (Brustolin et al. 2017).

Aedes caspius is an anthropophilic and floodwater mosquito species widely distributed in the Western Palaearctic region (Robert et al. 2019). This species is permissive to fluctuating levels of salinity in larval breeding areas so can be found in several habitats, including coastlands, irrigated lands, channels, wetlands and rice fields (Bellini et al. 1997). These mosquitoes are known to aggressively feed on humans and animals during the day and night both outdoors and indoors (Soliman et al. 2016). In this way, its role as a vector may be key for the knowledge of transmission cycle of particular diseases in Europe, especially in view of its anthropophilic behavior. In addition, previous studies with French *Ae. caspius* populations (from the Camargue) have experimentally demonstrated their potential vector competence for arboviruses, such as CHIKV and RVFV (Moutailler et al. 2008). However, French and Spanish *Ae. caspius* were not competent vectors to WNV and ZIKV, respectively (Balenghien et al. 2008, Gutiérrez-López et al. 2019).

Aedes albopictus represents a great threat to the public health and is catalogued as one of the most invasive insect species in the world (Jolyon M. Medlock et al. 2012). This specie was originated in Southeast Asia forests and has colonized several European (*e.g.* Italy, Greece, France or Spain) and African countries, (*e.g.*, Nigeria, Gabon, Equatorial Guinea or Cameroon), American continent (from Argentina

to USA), several Pacific Islands (*e.g.*, Fiji, Salomon Islands or Hawaii) and Australia in the last four decades (Paupy et al. 2009). *Aedes albopictus* is an anthropophilic and daytime mosquito that possesses a huge capacity of adaptation to new environments due to its ecological plasticity. *Aedes albopictus* mosquito populations are able to survive at low temperatures and their eggs can undergo latency period during the winter in temperate countries through its great ability to synthesize lipids, which makes them resistant to cold temperatures (Paupy et al. 2009). Additionally, *Ae. albopictus* is an opportunistic and zoophilic species preferring to feed on mammals, although mosquito females can feed on most of vertebrate groups (*e.g.*, reptiles, birds or amphibians) (Paupy et al. 2009). This zoophilic behavior increases the propagation risk of several arboviruses between animals and humans.

Due to the globalization along with the climate change and the great ability of mosquito vectors to colonize and adapt to new environments, it is crucial to implement efficient vector surveillance and control strategies in each country in order to prevent the introduction/transmission of arboviruses. In the present thesis, we focused on the interaction between: i) the three mosquito species above-mentioned (*Ae. caspius*, *Ae. albopictus* and *Cx. pipiens*) and ZIKV and ii) *Cx. pipiens* and RVFV.

6. Vectorial capacity and vector competence

The ability of mosquitoes to transmit pathogens is called VC or vector capacity. However, the vectorial capacity concept is more complex than VC and is commonly defined as the number of infectious bites a host receives daily, and mathematically represented by the following formula (Macdonald 1956):

$$Vectorial\ capacity = ma^2bp^n / -log_e p$$

Where, ***m*** = is the number of mosquitoes in proportion to the host; ***a*** = refers to the probability to blood-feeding rate/day; ***b*** = represents the transmission rate among the exposed females; ***p*** = refers to the mosquito survival rate/day and ***n*** = is the time that the virus need to complete the cycle in mosquitoes, in other words, the extrinsic incubation period (EIP) (Azar and Weaver 2019). The vector capacity concept serves to encompass a balance between the extrinsic factors, such as the environmental conditions, density of mosquito population, the mosquito longevity, host density, and the genetic variation of the pathogen and intrinsic factors, such as the physiology of the vector (*e.g.*, immune system or microbiome) (Azar and Weaver 2019).

Vector competence is defined as the arthropod intrinsic ability to acquire, maintain and transmit a pathogen agent (Azar and Weaver 2019). To estimate the VC in arthropods the infection (IR), disseminated infection (DIR) and transmission rates, (TR) and transmission efficiency (TE) are usually assessed. The IR refers to the virus ability to infect and replicate in the midgut epithelial cells. The DIR corresponds to the viral capacity to cross the midgut basal lamina and pass into the hemocoel, disseminating through it and infecting secondary organs. The TR represents the presence of infectious particles in saliva, so the virus can be transmitted to vertebrates during a blood meal. Finally, the TE estimates the transmission in the nature

since shows the proportion of mosquitos with infectious saliva among the total number of fully-engorged females.

Several factors affect vector competence and are grouped in extrinsic factors, which include the environmental conditions and pathogen genotype, and intrinsic factors, such as the physiology of the vectors, (*e.g.*, immune system and natural barriers in vectors to arboviruses) and the microbiome (Azar and Weaver 2019).

6.1. *Extrinsic factors of vector competence*

The extrinsic factors do not depend directly on the vectors. The environmental conditions and the genotype of the pathogen (viral genetic evolution) are the main factors that influence the arbovirus transmission (Azar and Weaver 2019). Several studies have documented significant effects of adult rearing temperature on VC for zoonotic arboviruses. The temperature directly affects viral replication and transmissibility by the mosquito; moreover, some studies showed that the variability of aquatic temperature during mosquito development cycle indirectly affects the VC in adults (Ciota and Keyel 2019). It has been demonstrated an inverse relationship between the temperature of larval rearing and infectivity in *Aedes* spp. mosquitoes exposed to RVFV, Venezuelan equine encephalitis virus and CHIKV (Turell 1993, Westbrook et al. 2010); however this relationship was not evidenced for RVFV in *Cx. pipiens* mosquitoes (Brubaker and Turell 1998). This relationship between the temperature of larval rearing and viral infectivity within the mosquito could be due to the fact that the temperature modifies mosquito life cycle and size, which have also been associated to VC alteration (Paulson and Hawley 1991). Moreover, recent studies showed that temperature during larval stage significantly alter the immune and stress gene expression in adult mosquitoes (Ciota and Keyel 2019). Therefore, the larval development temperature may also affect the VC.

The genotype of the pathogen is also an extrinsic factor to take in account for VC assays. DENV studies in *Ae*. *aegypti* mosquitoes have demonstrated that the VC is influenced by the mosquito-virus genetics interactions, since the same mosquito species exposed to different DENV-1 viruses presented different results for VC (Lambrechts et al. 2009).

6.2. *Intrinsic factors of vector competence*

The intrinsic factors depend directly on the physiology of the vector. The main intrinsic factors are the genotype of mosquito population, the mosquito microbiome and the vector-virus genotype interaction, which is mainly affected by the immune system and the natural tissue barriers in mosquitoes (Franz et al. 2015).

6.2.1. *Natural barriers of the mosquitoes to arbovirus infection and transmission*

Mosquitoes present physical barriers to protect themselves against pathogens, which affect directly the VC because these barriers can limit infections. The barriers within mosquitoes are the peritrophic membrane, the midgut barrier and the salivary gland barrier. At the same time, midgut and salivary gland barriers are both divided into an infection (MIB and SGIB) and an escape barrier (MEB and SGEB) (Fig 7).

After an infectious blood meal, most of virions travel to the midgut to infect the midgut epithelial cells (Fig 7. B). As previously described by Vogels *et al*. (2017), the first barrier that arboviruses meet is the peritrophic membrane, a chitinous sac that contains the blood meal during the digestion. This membrane is not a permanent barrier as its purpose is only for digestion and endures a few hours. Ingress of some arboviruses, particularly the flaviviruses, into the midgut epithelial cells probably occurs by the binding to membrane-associated cell receptors (Fig 7. B 1). Then, arboviruses replicate into the midgut epithelial cells (MIB) (Vogels et al. 2017). In this stage, the viral

replication can be influenced by the proteolytic enzymes of the digestion, the innate antiviral responses (*e.g.*, RNA interference [RNAi]) and the midgut microbiota (Azar and Weaver 2019).

After infection and replication in midgut epithelial cells, the virions need to cross through the basal lamina and disseminate to the haemocoel to infect secondary organs or tissues via haemolymph (*e.g.* salivary glands) (Vogels et al. 2017). In fact, virions can reach the haemocoel by: i) passing through the basal lamina with/without a replication cycle of the virus in the midgut epithelial cells, ii) via infection of the midgut tracheae or iii) by direct passage into the haemolymph (Fig 7. B 3-6). The basal lamina is a dense proteinaceous matrix that acts as physical barrier in the midgut blocking microorganisms, such as bacteria, fungi and viruses (Vogels et al. 2017). Although, the basal lamina is permeable to small molecules, its density varies among mosquito species, thus affecting the passage of virions into the haemocoel (Franz et al. 2015). Another route to reach the haemolymph is through tracheae infection. The tracheae network crosses the lamina basal and directly connect to the midgut epithelial cells providing to the virions a way to evade the basal lamina, as it was described in *Cx. pipiens* exposed to RVFV (Romoser et al. 2005). Finally, it has been described that a few mosquitoes may have a permeable midgut, which allows the direct virion passage into haemocoel after an infectious blood meal (Houk et al. 1979, Vogels et al. 2017).

In the case that the virus infects the midgut epithelial cells but is not able to escape and disseminate to the haemocoel, the barrier is called MEB.

After viral dissemination via haemolymph, virions reach the secondary organs or tissues, such as the fat body, haemocytes and ovaries. The ovary infection is key for VT in some mosquito-virus pairings and the infection of the salivary gland cells is crucial for viral horizontal transmission, known as SGIB (Fig 7. A). Once the virions

reach and infect the salivary glands, the viral titer rises in saliva (Fig 7. A 1-3) (Vogels et al. 2017). This is important because SGIB has been proposed to be a dose-dependent or dose-independent pathogen transmission in varying mosquito-virus pairings, *e.g.*. dose-dependent in Western equine encephalitis virus-*Culex tarsalis* pairing and dose-independent for Japanese encephalitis virus (JEV) and WNV in *Cx. pipiens* (Houk et al. 1981, Turell et al. 2006, Franz et al. 2015). Several studies demonstrated that the liberation of virions in the lumen of the salivary glands may need the apoptosis induction (Fig 7. A 4) (Clem 2016, Vogels et al. 2017). In addition, it is important to recall that the infection of the salivary glands may occur without the infection of secondary organs or tissues via tracheal (Brustolin et al. 2017, Azar and Weaver 2019). On the other hand, several studies have reported salivary glands infected without shedding virions into the saliva, pinpointing the presence of a SGEB (Paulson et al. 1989, Franz et al. 2015, Kumar et al. 2018, Azar and Weaver 2019).

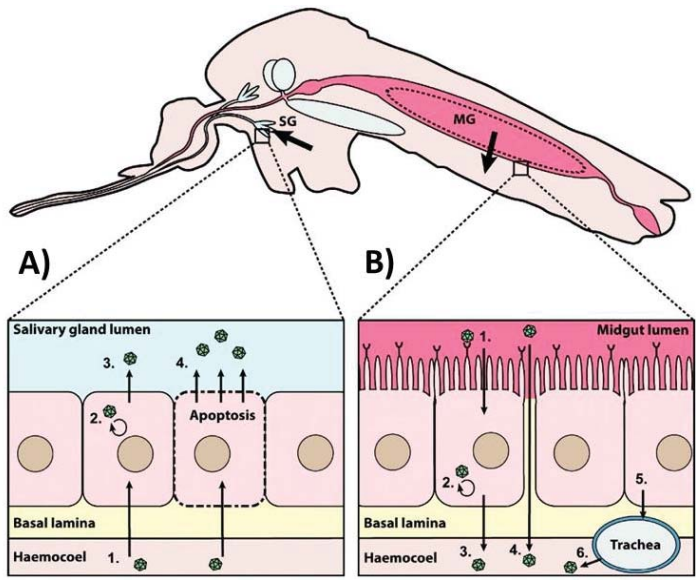

Figure 7. Arboviral infection cycle of the natural tissue barriers in mosquitoes. The arrows show the direction of the virion passages.MG: Midgut; SG: Salivary glands. Extracted from Vogels *et al*. (2017).

6.2.2. *Mosquito microbiome*

In the last years, the study of microbiome involvement in VC has acquired importance. The mosquito microbiome is influenced by multiple variables (*e.g.*, its microbiome parents, sex, larval environment, life stage, geographic origin, etc.) (Azar and Weaver 2019). The microbiome influences the mosquito physiology, such as development, metabolism, nutrition and immunity (Jupatanakul et al. 2014a). The association between the mosquito microbiome and arboviruses is mainly mediated through the mosquito innate immune system. Recent microbiome studies have focused on the most important arbovirus vectors (*e.g.*, *Ae*. *aegypti*, *Ae*. *albopictus*, and *Culex quinquefasciatus*). For example, a study performed with *Ae*. *aegypti* mosquitos showed increases of DENV-2 midgut titers in antibiotic-treated mosquitoes (aseptic mosquitoes) in comparison with non-treated mosquitoes (Xi et al. 2008). On the other hand, studies with unique bacterial species (*e.g.*, *Chromobacterium Csp_P*, *Proteus*, *Paenibacillus*, and *Wolbachia*) have been related to the reduction of the *Ae*. *aegypti* vector competence for DENV-2 evidencing the crucial role of the microbiome in VC (Bian et al. 2010, Ramirez et al. 2012, 2014, Azar and Weaver 2019). In this scenery, the manipulation of mosquito microbiota could be a relevant strategy to control arbovirus transmission, but pilot studies are needed to evaluate ecological impacts.

6.2.3. *Mosquito immune system*

The mosquito immune system is a strong defense response against extern pathogens. The three classical immune signaling pathways are the Toll, Immune deficiency (IMD) and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways, which play a key role in antiviral defense inducing immune effectors (Fig 8). The Toll and IMD pathways were first identified in *Drosophila melanogaster* (Kumar et al. 2018). The Toll signaling pathway is activated by pattern recognition receptors (PRRs) (*e.g.*, peptidoglycan recognition proteins

[PGRPs]), which detect the Gram-positive bacteria, fungi and viruses. The PRRs trigger the Toll pathway through the proteolytic Spaetzle cleavage, which adheres to Toll protein receptors and activates the signaling cascade. It results in Cactus protein phosphorylation and degradation. Cactus degradation makes possible the translocation of Rel1 to the nucleus to activate the transcription of Toll-regulated genes (Sim et al. 2014, Kumar et al. 2018). The result of the signaling cascade is the production of antimicrobial effectors also known as antimicrobial peptides (AMPs). Toll pathway has a significant role of in the control of DENV infection in *Ae*. *aegypti* (Xi et al. 2008). The IMD pathway is activated through the pathogen attachment to PGRP-LC, a transmembrane receptor, which activates the cascade via IMD, FADD, and Dredd proteins. The result is Rel2 phosphorylation, which gets into the nucleus to activate the transcription of genes regulated by IMD pathway (Kumar et al. 2018). Similarity to Toll pathway, the result is AMPs production. The IMD pathway has been considered the most efficient response against *Plasmodium* (Garver et al. 2012).

The JAK/STAT pathway was first identified in vertebrates and its role is crucial in their innate immunity. It has been evidenced its similar role in insects. The JAK/STAT pathway is induced by the attachment of Unpaired peptide ligand in the extracellular region of Dome receptors producing Hop proteins activation. The activated Hop proteins phosphorylate each other, and afterwards, phosphorylate the STAT transcription factors. Subsequently, phosphorylated STATs proteins dimerize and translocate to the nucleus to activate transcription of JAK-STAT- regulated genes. (Sim et al. 2014, Kumar et al. 2018). The role of JAK/STAT pathway was first described in *An*. *gambiae* infected with *E*. *coli* and *Micrococcus luteus*. Recent studies showed that this pathway plays a key role against arbovirus, such as DENV in *Ae*. *aegypti* mosquitoes, which suggest that it is conserved in both invertebrates and vertebrates (Souza-Neto et al. 2009, Jupatanakul et al. 2017, Kumar et al. 2018).

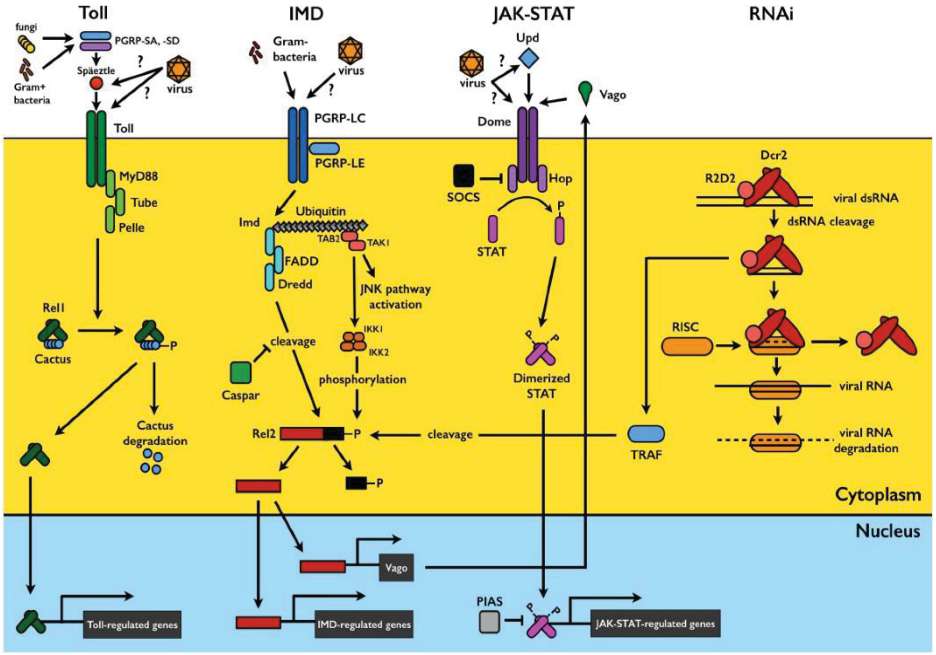

Figure 8. The representation of the mosquito immune innate pathways. The Toll, IMD and JAK/STAT pathways and the RNAi mechanism. Extracted from Sim *et al*. 2014.

Although the above-mentioned pathways develop a crucial role in the control of viral infections, the major antiviral defense response in insects is the RNAi pathway (Fig 8), which involves the generation of RNA molecules of different characteristics: small interfering RNA (siRNA), the P-element-induced Wimpy testes gene (PIWI)-interacting RNAs (piRNA) and microRNAs (miRNAs). These RNA molecules interact with the RNA-silencing complex (RISC), a multiprotein complex, to induce the antiviral response (Kumar et al. 2018). The siRNA pathway is known to be the major innate immune defense against arbovirus infections, such as DENV and CHIKV in *Ae*. *aegypti* (Sánchez-Vargas et al. 2009, McFarlane et al. 2014). This pathway is triggered mainly by three major components: Dicer2, R2D2, and Argonaute-2 (Bellés 2010, Kumar et al. 2018). The siRNA pathway is activated when a virus-derived long dsRNA is identified and cut by Dicer2 into siRNAs, which are loaded on RISC. Then, RISC unrolls the

RNA and degrades one of the siRNA strands, employing the other strand for the single-stranded viral RNA targeted degradation with the complementary sequence to the siRNA (Sim et al. 2014). In parallel, Dicer2 also activates TRAF, which triggers Rel2 leading the Vago transcription activation, a peptide that activates JAK/STAT pathway (Sim et al. 2014). Recent studies have suggested that miRNA and piRNA, which are involved in preserving the integrity of the genome, may also be implicated in antiviral defense (Vodovar et al. 2012, Blair and Olson 2015). As examples, one study conducted in *Ae. albopictus* exposed to DENV-2 showed that miR-252 acted as an antiviral (Yan et al. 2014), and other study demonstrated antiviral response involving the piRNA system in *Aedes* mosquitoes exposed to Semliki forest virus (Varjak et al. 2017).

Finally, apoptosis process and ubiquitination pathway have been identified to be involved in the immune response mechanisms. Apoptosis is a mechanism that guides to programmed cell death in many organisms removing infected or damaged cells to maintain homeostasis and can also acts as a defense mechanism participating in the regulation of viral infections (Vaidyanathan and Scott 2006, Kumar et al. 2018). The apoptosis process participates in the regulation of viral loads in mosquitoes (Kumar et al. 2018). Some viruses present genes with anti-apoptotic activity that suppress the apoptosis activity during viral infections (Kumar et al. 2018). Conversely, a recent study performed in *Ae. aegypti* exposed to SINV demonstrated that the apoptosis induction increased the viral infectivity in mosquitoes, whereas the apoptosis inhibition resulted in a decreased infection (Wang et al. 2012, Kumar et al. 2018). Therefore, how the apoptosis is involved in the immune system response is still controversial.

The Ubiquitination pathway contributes to the degradation of apoptotic proteins, among other biological processes. The degradation of apoptotic proteins by this pathway is essential for the maintenance of cellular health and its deregulation is related to some diseases, such as

tumors, diabetes and inflammation. The ubiquitin ligases and deubiquitinating enzymes are responsible for the regulatory functions of this mechanism (Gupta et al. 2018).

7. Knowledge gaps about ZIKV and RVFV in Spanish mosquitoes

Although both ZIKV and RVFV, which were originated in Africa, exhibit different transmission cycles (the former uses humans and the later needs animals as reservoirs), both are emergent viruses and a deep knowledge on the VC of local mosquito species (both autochthonous and invasive) are required to develop efficient surveillance programs. An additional step forward to combat these arboviruses is to further investigate molecular interactions between viruses and mosquitoes (*e.g.*, immune responses) to characterize intrinsic factors of the VC.

7.1. *Gaps of knowledge on ZIKV transmission*

There are several VC studies of European *Ae. albopictus* (Di Luca et al. 2016, González et al. 2019, Gutiérrez-López et al. 2019, Hernández-Triana et al. 2019, Vazeille et al. 2019), *Ae. caspius* (Gutiérrez-López et al. 2019) and *Cx. pipiens* (Boccolini et al. 2016, Heitmann et al. 2017) populations for ZIKV. However, the genetic differences between mosquito populations and ZIKV strains influence the VC of European mosquito populations (Kumar et al. 2018, McKenzie et al. 2019). The present thesis addresses several gaps of knowledge concerning the VC of three Spanish mosquito species: *Ae. albopictus*, *Ae. caspius* and *Cx. pipiens* for ZIKV in order to improve the proper development of the arboviruses control and surveillance strategies. Additionally, there is a great lack of understanding about the capacity of viral transmission of the progeny of infected females, which could play a key role in the maintenance of the viral cycle. Therefore, it is essential to evaluate if the Spanish mosquito species and their progeny could transmit ZIKV in order to establish an accurate vector control and surveillance programs.

7.2. *Gaps of knowledge on molecular interactions in RVFV infection*

Although the susceptibility of Spanish *Cx. pipiens* mosquitoes has already been experimentally demonstrated to RVFV, there is a great

lack of understanding about *Cx*. *pipiens*-RVFV molecular interactions, which are key to study the innate immune mechanisms in mosquitoes. By expanding our knowledge on *Cx*. *pipiens* and RVFV interactions it may be possible to identify potential target genes involved in mosquito innate viral infection-response. This knowledge can be useful to avoid the mosquito infection or modulate its tolerance to the virus, and consequently its transmission to vertebrates.

OBJECTIVES AND CHAPTERS

The main aims of the present thesis are to investigate the transmission capability of field-collected mosquito populations (*Ae*. *caspius*, *Ae*. *albopictus* and *Cx*. *pipiens*) for ZIKV in Catalonia and the characterization of the *Cx*. *pipiens*-RVFV interactions at molecular level, which is crucial to better understand the VC of this mosquito species for RVFV.

To do so, this thesis is organized in three chapters with the following specific objectives:

Chapter 1: The main objective of this chapter was to assess the VC of an *Ae*. *caspius* mosquito population from El Prat de Llobregat (Catalonia, Spain) for two ZIKV lineages, Suriname strain (Asian lineage) and MR766 strain (African I lineage) to measure its potential role in ZIKV transmission.

Chapter 2: This chapter focused on the evaluation of VC of two Spanish *Ae*. *albopictus* populations collected from the field from Rubí and El Prat de Llobregat municipalities for ZIKV Dak84 strain (African I lineage) and Martinique strain (Asian lineage) and one field *Cx*. *pipiens* population from Cerdanyola del Vallés for Dak84 strain (African I lineage). In addition, the viral dissemination by the offspring of intrathoracically inoculated *Ae*. *albopictus* females with ZIKV was assessed for both African and Asian ZIKV lineages to better estimate the risk of ZIKV VT, providing useful information to health authorities for both local mosquito species to develop strategies in the control and surveillance programs for ZIKV in Spain.

Chapter 3: In the present chapter, the main objective was to evaluate the effect of the RVFV in exposed *Cx*. *pipiens* females using transcriptome *de novo* analysis. Particularly, this study focused on the identification of differentially expressed genes associated with mosquito immune response mechanisms after RVFV exposure in order

to better understand some RVFV-*Cx*. *pipiens* interactions and potential mechanisms involved in the control of RVFV infection.

Part II.

Chapters

Chapter I

Chapter I. European *Aedes caspius* mosquitoes are experimentally unable to transmit Zika virus

Ana I. Núñez[1], Sandra Talavera[1], Carles Aranda[1,2], Lotty Birnberg[1], Raquel Rivas[1], Núria Pujol[1], Marta Verdún[1], Anna-Bella Failloux[3] and Núria Busquets[1]. (*Parasites & Vectors*, 2019; 12:363).

ABSTRACT

Background: *Aedes caspius* (Pallas, 1771) is a floodwater mosquito species widely distributed in the Western Palaearctic. As an anthropophilic species, its role as an arbovirus vector may be the key for understanding the transmission cycle of certain diseases in Europe such as Zika virus (ZIKV). Concerning vector competence for ZIKV, studies related to *Ae*. *caspius* are still scarce. ZIKV is an arbovirus that has provoked a widespread epidemic in the Pacific region (2007–2013) and in the Americas (2015–2016). ZIKV is associated with serious neurological injuries (*e.g*., microcephaly) and Guillain-Barré syndrome. Due to the ZIKV epidemics in the American continent, some viraemic travellers coming from endemic countries have been reported in Europe. More knowledge is therefore required to define the susceptibility of autochthonous mosquito species such as *Ae*. *caspius* for ZIKV in order to improve arbovirus surveillance and control programs. In the present study, the vector competence of a European population of *Ae*. *caspius* was evaluated for two ZIKV lineages, the Suriname ZIKV strain (Asian lineage) and the MR766 ZIKV strain (African I lineage). Females were tested at 7, 14 and 21 days post-exposure (dpe) to infectious blood meals. An *Ae*. *aegypti* PAEA strain was used as a positive control.

Results: *Aedes caspius* presented low susceptibility to ZIKV infection and the virus was only detected by RT-qPCR in body samples. Low viral loads were detected for the MR766 strain at 7 dpe and for the Suriname strain at 14 and 21 dpe. *Aedes caspius* was unable to produce a disseminated infection and virus transmission at any of the tested time points. Using *Ae*. *aegypti* PAEA strain, infection, dissemination and transmission rates were calculated for the Suriname ZIKV strain (Asian lineage) at each time point. For the MR766 ZIKV strain (African I lineage), while only infection rates were estimated at each time point, no dissemination or transmission were detected in either species.

Conclusions: The results of the present study reveal that the tested *Ae*. *caspius* population has a strong midgut escape barrier that limits the dissemination or transmission of the virus. As such, it seems unlikely that European *Ae*. *caspius* mosquitoes could be involved in ZIKV transmission if ZIKV was introduced into Europe. This information may help in designing a better strategy to European surveillance and control programs for ZIKV.

Keywords: Spatial patterns, power laws, patch-size distributions, facilitation.

INTRODUCTION

Aedes caspius (Pallas, 1771) is a floodwater mosquito species widely distributed in the Western Palaearctic (Robert et al. 2019). This mosquito species is tolerant to varying levels of salinity in larval breeding places (Bellini et al. 1997) and so is present in different habitats, including coastlands, irrigation channels, swamps and rice fields. *Aedes caspius* is an anthropophilic species and a crepuscular feeder and is known to bite during the day and night (Soliman et al. 2016). These mosquitoes usually feed aggressively on humans and animals, both indoors and outdoors. (Napp et al. 2018). Thus, its role as an arbovirus vector may be the key for the knowledge of transmission cycle of certain diseases in Europe, especially given its anthropophilic behavior. The vector competence of some *Ae*. *caspius* populations exposed to different arboviruses has been tested in previous studies. *Aedes caspius* populations from the Camargue (France) were experimentally found to be potential vectors of arboviruses such as chikungunya virus (CHIKV) (Vazeille et al. 2008) and Rift Valley fever phlebovirus (RVFV) (Moutailler et al. 2008). However, *Ae*. *caspius* from Camargue and from Andalusia (Spain) were unable to transmit West Nile virus (WNV) (Balenghien et al. 2008) and Zika virus (ZIKV) (Gutiérrez-López et al. 2019), respectively.

The ZIKV is an arthropod-borne virus belonging to the genus *Flavivirus* (family *Flaviviridae*). The virus is primarily transmitted in a zoonotic cycle between mosquitoes and non-human primates in Africa, although sexual (Mansuy et al. 2016) and perinatal (Besnard et al. 2014) ZIKV transmission have also been confirmed in humans. The virus has been associated with severe clinical manifestations and congenital malformations including microcephaly (Cauchemez et al. 2016) and Guillain-Barré syndrome (Cao-Lormeau et al. 2016). ZIKV was isolated for the first time from a rhesus macaque monkey in the Zika forest (Uganda) in 1947 (Dick et al. 1952). The virus was subsequently found in Asia in the 1960s. In 2007 there was an outbreak

of ZIKV in Yap Island, Micronesia (Duffy et al. 2009), which spread to Pacific islands in 2013 (Musso et al. 2014) before reaching Latin America in 2015 (Zanluca et al. 2015). Nowadays, all isolated ZIKVs are grouped into three lineages: Asian; African I; and African II (Saiz et al. 2017). ZIKV has been isolated from numerous African mosquito species in field (Zanluca and dos Santos 2016) but *Aedes aegypti* is the main vector of ZIKV in urban areas (Boyer et al. 2018). In addition, several *Aedes* species from all continents have been observed to transmit the virus experimentally: *e.g*. *Aedes vittatus*, *Aedes vexans* (Boyer et al. 2018), *Aedes polynesiensis* (Richard et al. 2016) and *Aedes albopictus* (Wong et al. 2013, Di Luca et al. 2016, Ciota, Bialosuknia, Zink, et al. 2017, Pompon et al. 2017, Kumar et al. 2018).

Due to the ZIKV epidemics in the American continent, some viraemic travellers coming from endemic countries have been reported in Europe (Millet et al. 2017), especially during the summer months (Rocklöv et al. 2016), raising important alarms for human health. In this context, more in-depth knowledge is required about the susceptibility of autochthonous mosquito species such as *Ae*. *caspius* for ZIKV to improve arbovirus surveillance and control programs. For this reason, in the present study we evaluated the vector competence of an *Ae*. *caspius* mosquito population from El Prat de Llobregat (Catalonia, Spain) for two ZIKV lineages, Suriname (Asian lineage) and MR766 (African I lineage) to measure its potential role in ZIKV transmission.

MATERIAL AND METHODS

Mosquito rearing

Aedes caspius larvae were collected from El Prat de Llobregat (Catalonia, Spain) in October 2017. Larvae were reared in trays containing dechlorinated water supplemented with fish food (Goldfish, Tetra GmbH, Melle, Germany) until the adult stage. Emerging adults were maintained at 26/22 °C (day/night) to simulate summer

environmental conditions at the latitude where the mosquitoes were captured, a relative humidity of 80 % and a light/dark photocycle of 14:10 h. Mosquitoes were fed *ad libitum* with 10 % sucrose solution. The F0 generation was used for experimental infection. An *Ae*. *aegypti* PAEA strain, from Paea (Tahiti, French Polynesia), colonized since 1994, was reared in the same conditions and used as a positive control of ZIKV vector.

Virus production and titration

Suriname and African MR766 ZIKV strains provided by the European EVAg project were used in the present work. The Suriname ZIKV strain (EVAg no. 011V-01621; Asian lineage) was isolated from a placental material of a patient in Netherlands in 2016 who came from Suriname during the ZIKV epidemic (van der Eijk et al. 2016) and the African MR766 ZIKV strain (African I lineage) was isolated from a rhesus monkey (*Macaca mulatta)* in 1947 in Uganda (Dick et al. 1952). These strains were propagated and titrated to obtain the 50 % tissue culture infective dose per ml ($TCID_{50}$/ml) in African green monkey kidney (VERO) cells.

Experimental infection of mosquitoes

Forty-eight hours before exposure of mosquitoes to the infectious blood meal, the 10 % sucrose solution was removed to increase mosquito appetite. Totals of 480 *Ae*. *caspius* and 275 *Ae*. *aegypti* females (7–10 days old) were exposed to infectious blood at 7 $\log_{10}$ $TCDI_{50}$/ml of both Suriname and MR766 strains and 20 females of each species were exposed to non-infectious blood. For blood-meal preparation, rabbit blood was washed and mixed with adenosine 5'-triphosphate (ATP) (5×10^{-3} M) (Sigma-Aldrich, St. Louis, MO, USA) and virus (infectious) or cell culture medium (DMEM) (non-infectious). Females were exposed to a Hemotek® artificial feeding system (Discovery Workshop, Accrington, UK) at 37.5 ± 0.5 °C for 30 min. After exposure, females were anesthetized with CO_2. Fully engorged females

were selected and maintained in groups of ten in cardboard cages (Watkins & Doncaster, Leominster, UK) under the same rearing environmental conditions. Throughout the experiment, the mosquitoes were maintained with permanent access to 10% sucrose solution in cotton pledges. After a period of 7, 14 and 21 days post-exposure (dpe) to infectious blood, females were anesthetized with CO_2, legs and wings were dissected and saliva was extracted using the capillary technique as previously described (Dubrulle et al. 2009). The number of mosquitoes tested at each time point is summarized in Table 1. Body, leg and wing samples were stored in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) (Lonza Group AG, Basel, Switzerland) and the saliva samples in 0.190 ml of DMEM at -80 °C until ZIKV detection. Infection (IR), disseminated infection (DIR) and transmission rates (TR) were estimated to evaluate the vector competence (Talavera et al. 2018). The IR represents virus replication in the midgut epithelial cells. The DIR shows that the virus was able to cross the midgut barrier and reach the hemocoel. The TR shows that the virus was able to cross the salivary glands barrier. We also measured the transmission efficiency (TE), which refers to the rate of mosquitoes with infectious saliva among the total mosquitoes assayed. The experimental infection was performed at IRTA-CReSA BLS3 facilities.

ZIKV detection

Virus detection from leg, wing and body samples was carried out using 1/10 and 1/100 dilutions in 96-well plates containing a Vero cell monolayer. Saliva samples were titrated directly in 96-well plates in a Vero cell monolayer. Vero cells were maintained with DMEM supplemented with 2 % FCS and 2 % of penicillin /streptomycin /nystatin (1,000 U/ml, 10 mg/ml and 500 U/ml, respectively; Sigma-Aldrich) and incubated for seven days at 37 °C and 5 % CO_2 until cytopathic effect observation.

Prior to viral RNA extraction, the samples were homogenized using a TissueLyser II (Qiagen GmbH, Hilden, Germany) at 30 Hz for

1 min. Viral RNA was extracted from the samples using NucleoSpin® RNA Virus (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Zika RNA was detected by reverse-transcription quantitative PCR (RT-qPCR) using the primers ZIKA 1086 and ZIKA 1162c defined previously (Lanciotti et al. 2008) and AgPath-ID™ One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA). The nucleic acids were detected with a Real-Time PCR 7500 Fast System (Applied Biosystems) with the following amplification protocol: 45 °C for 10 min; 95 °C for 10 min; then 45 cycles at 95 °C for 15 s and at 60 °C for 45 s. The RT-qPCR sensibility was 0.451 $TCID_{50}$/reaction for detection of MR766 and 0.035 $TCID_{50}$/reaction for Suriname ZIKV strains.

Table 1 Summary of assays

Mosquito species tested	**ZIKV strain tested**	**Titer of ZIKV ($TCID_{50}$/ml)**	**No. of mosquitoes tested per time point**			
			7 dpe	14 dpe	21 dpe	Total
Ae. caspius	Suriname	7 log_{10} $TCID_{50}$/ml	30	30	30	90
	MR766	7 log_{10} $TCID_{50}$/ml	30	30	30	90
	Negative control	No virus	–	–	8	8
Ae. aegypti	Suriname	7 log_{10} $TCID_{50}$/ml	20	20	20	60
	MR766	7 log_{10} $TCID_{50}$/ml	20	20	19	59
	Negative control	No virus	–	–	11	11

dpe, days post-exposure

RESULTS

Feeding rates were 56 % (280/500) and 77.45 % (213/275) for *Ae. caspius* and *Ae. aegypti*, respectively.

For the *Ae. caspius* population, ZIKV was detected by RT-qPCR only in body samples for both virus strains used in the present study (Fig. 1). As detailed in Table 2, the infection rate (IR) in *Ae. caspius* for the Suriname ZIKV strain was 3.33 % and 10 % at 14 and 21 days post-exposure (dpe), respectively. For the MR766 ZIKV strain, the IR was only 3.33 % at 7 dpe. In addition, both ZIKV strains were unable to induce a disseminated infection and transmission in *Ae. caspius*.

In our positive control (*Ae. aegypti* PAEA strain), infection was detected at 7 dpe for both ZIKV strains tested by RT-qPCR and cytopathic effect while dissemination and transmission were only found for the Suriname ZIKV strain at 14 and 21 dpe (Table 1 and Fig. 1). The transmission efficiency (TE) of *Ae. aegypti* for the Suriname ZIKV strain based on the cytopathic effect in Vero cells was 15 % (3/20) and 5 % (1/20) at 14 and 21 dpe, respectively.

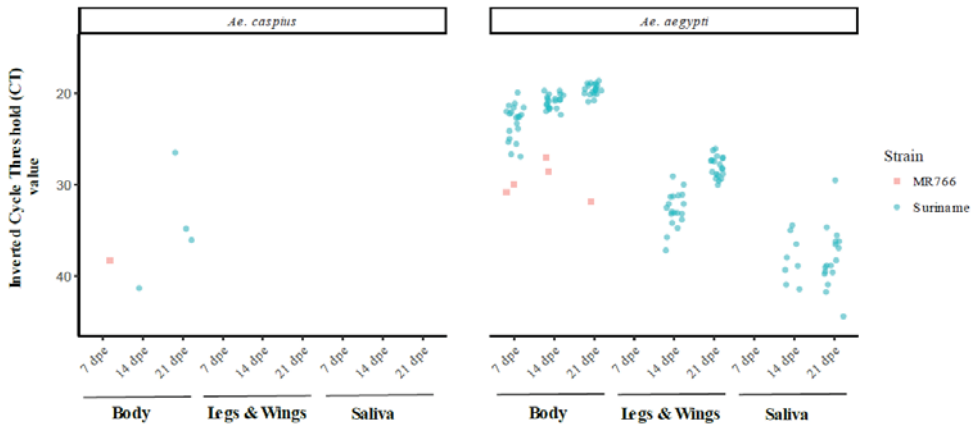

Fig. 1 Results of the RT-qPCR for both mosquito species tested. The CT values are blue for the Suriname strain and red for MR766.

Table 2. Infection, disseminated infection and transmission rates of *Ae*. *caspius* from Catalonia and *Ae*. *aegypti* PAEA populations artificially fed with Suriname and MR766 ZIKV strains at a titer of 7 $\log_{10}$ $TCID_{50}$/ml. Viral detection was performed by the visualization of cytopathic effect on cells and RT-qPCR assays.

Dpe	Species	Suriname						MR766					
		IR (%)		DIR (%)		TR (%)		IR (%)		DIR (%)		TR (%)	
		CE	RT-qPCR	CE	RT-qPCR	CE	RT-qPCR	CE	RT-qPCR	CE	RT-qPCR	CE	RT-qPCR
7	*Ae*. *caspius*	0/30 (0)	0/30 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/30 (0)	1/30 (3.33)	0/0 (0)	0/1 (0)	0/0 (0)	0/0 (0)
	Ae. *aegypti*	17/20 (85)	20/20 (100)	0/17 (0)	0/20 (0)	0/0 (0)	0/0 (0)	2/20 (10)	2/20 (10)	0/2 (0)	0/2 (0)	0/0 (0)	0/0 (0)
14	*Ae*. *caspius*	0/30 (0)	1/30 (3.33)	0/0 (0)	0/1 (0)	0/0 (0)	0/0 (0)	0/30 (0)	0/30 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)
	Ae. *aegypti*	20/20 (100)	20/20 (100)	9/20 (45)	19/20 (95)	3/9 (33.3)	8/19 (52.6)	2/20 (10)	2/20 (10)	0/2 (0)	0/2 (0)	0/0 (0)	0/0 (0)
21	*Ae*. *caspius*	0/30 (0)	3/30 (10)	0/0 (0)	0/3 (0)	0/0 (0)	0/0 (0)	0/30 (0)	0/30 (0)	0/0 (0)	0/0 (0)	0/0 (0)	0/0 (0)
	Ae. *aegypti*	19/20 (95)	19/20 (95)	16/19 (84.2)	19/19 (100)	1/16 (6.2)	17/19 (89.4)	1/19 (5.2)	1/19 (5.2)	0/1 (0)	0/1 (0)	0/0 (0)	0/0 (0)

Abbreviations: CE, cytopathic effect; IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; dpe, days post-exposure. *Note*: Data are given as number of positives/number of tested samples (percentage).

DISCUSSION

The present study demonstrates that the assessed population of *Ae*. *caspius* from Catalonia was unable to transmit the Suriname and MR766 ZIKV strains, belonging to the Asian and African I phylogenetic lineages, respectively. Our results indicate that the *Ae*. *caspius* population has a strong midgut escape barrier (MEB) since at 7 dpe we found infected bodies of MR766 ZIKV strain by RT-qPCR as well as at 14 and 21 dpe to the ZIKV Asian strain but neither the disseminated infection nor the transmission were detected at any of the time points analyzed. These findings are in agreement with the hypothesis that the MEB can limit virus dissemination from the midgut to the hemocoel or secondary organs as reported for other arbovirus-mosquito species combinations (Black et al. 2002, Franz et al. 2015). As the viral load detected by RT-qPCR was low in infected bodies (Fig. 1), we suggest that the virus is replicating at a very low level. Therefore, the virus would not be able to cross the MEB and disseminate through the mosquito hemocoel to reach the salivary glands making it unable to transmit the virus. In addition, these results are in accordance with a recent study in which another *Ae*. *caspius* population was assayed for a ZIKV strain of the Asian lineage (Puerto Rico, 2015) under constant environmental conditions (Gutiérrez-López et al. 2019). It should be noted that in our experiment, apart from testing a ZIKV strain of Asian lineage, we also assayed a ZIKV strain of the African I lineage (MR766) with cycled environmental conditions (26/22°C day/night). Both studies indicate that despite the anthropophilic behavior of *Ae*. *caspius*, its role in the transmission of ZIKV seems unlikely.

Aedes aegypti from PAEA (French Polynesia), selected as a positive control, was able to transmit the Suriname strain (Asian lineage). Suriname ZIKV infection, dissemination and transmission were observed at 14 and 21 dpe. These results are in agreement with other experiments reported on *Ae*. *aegypti* vector competence for ZIKV

(Li et al. 2012, Weger-Lucarelli et al. 2016). However, the MR766 strain (African I lineage; the historical strain isolated in 1947 in Uganda [Dick et al. 1952]) was unable to disseminate and be transmitted in this mosquito species. This ZIKV strain is an old strain that has suffered several passages in mice and cells from various laboratory sources that we assume may have influenced the vector competence assays in both assayed mosquito species. Furthermore, the inefficient dissemination and transmission in *Ae*. *aegypti* (PAEA strain) exposed to the MR766 ZIKV strain were in agreement with previous results reported by Diagne *et al*. (2015). The differences observed between our results and other vector competence experiments in which dissemination and transmission of the MR766 ZIKV strain were reported for *Ae*. *aegypti* (Li et al. 2012, Wong et al. 2013, Hall-Mendelin et al. 2016, Weger-Lucarelli et al. 2016, Calvez et al. 2018), could be explained by the genetic variability of *Ae*. *aegypti* populations as mentioned by Diagne *et al*. (2015). Furthermore, it is known that temperature can influence vector competence as described for several mosquito species infected with other arboviruses (*e.g*., dengue virus (DENV), CHIKV (Ciota et al. 2018) or WNV [Brustolin et al. 2016, Vogels et al. 2017, Veronesi et al. 2018]). A recent study showed that temperature may directly affect vector competence for ZIKV rendering the *Ae*. *aegypti* population tested at low temperature (18 °C) unable to transmit the virus (Heitmann et al. 2017). Therefore, the environmental conditions used in the present study could have also influenced our results; in earlier studies where higher dissemination and transmission were observed in the *Ae.aegypti*-MR766 ZIKV pairing, the assays were performed at a temperature of 28 °C in contrast to the 26/22 °C (day/night) used in our experiment (which we used to mimic summer environmental conditions in the area where the *Ae*. *caspius* population was captured).

Finally, with respect to the techniques used for virus detection in our vector competence assays, the RT-qPCR results had a slightly better sensitivity than those obtained by cytopathic effect in all

mosquito-virus pairings. However, although RT-qPCR was more sensitive, the cytopathic effect caused by the virus allows better knowledge of its viability which is more useful for a better estimation of the transmission efficiency of a mosquito population. Therefore, we strongly recommend that for the vector competence studies viable viruses in the saliva should be taken into account to determine the transmission efficacy.

CONCLUSIONS

Given the high risk of ZIKV introduction in Europe *via* infected travelers coming from endemic areas, it is important to know if anthropophilic European mosquito populations are able to transmit this virus and sustain a ZIKV outbreak. Our results indicate that it is unlikely that *Ae*. *caspius* mosquitoes from Spain, particularly from Catalonia, could be involved in the transmission of ZIKV if it was introduced. Therefore, *Ae*. *caspius* is not a relevant species to be monitored and controlled in case of ZIKV introduction. This is useful and crucial information for the health authorities with respect to the establishment of efficient surveillance and vector control programs for ZIKV. Moreover, our study highlights the importance of performing vector competence assays for each arbovirus-vector mosquito species in specific environmental conditions to provide information for more accurate predictions of the risk of arbovirus transmission in a specific area.

ACKNOWLEDGEMENTS

The authors are very grateful to BSL3 facilities staff for their excellent technical contribution and to Dr. Francesc Xavier Abad for his excellent BSL3 facilities management at IRTA-CReSA.

Chapter II

Chapter II. Evidence of Zika virus horizontal and vertical transmission in *Aedes albopictus* and *Culex pipiens* resistance, Spain

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ABSTRACT

Zika virus (ZIKV) was isolated for the first time in 1947 in the Zika forest (Uganda). It spread from the African continent to Asia, the Pacific Islands and the Americas. In 2015, after a great ZIKV outbreak in Brazil, it spread throughout the American continent affecting more than 45 countries. In Africa, ZIKV is maintained in a zoonotic cycle between mosquitoes and non-human primates. European *Aedes* mosquitoes such as *Aedes albopictus*, has been experimentally demonstrated to be a competent vector for ZIKV, but recent studies on Spanish *Ae*. *albopictus* populations showed contradictory results for ZIKV transmission. *Culex pipiens*, a widely distributed mosquito species in Mediterranean countries that plays a critical role in the transmission of several arboviruses, do not seems to be able to transmit ZIKV. Herein, the horizontal transmission of ZIKV in two field-collected *Ae*. *albopictus* and one *Cx*. *pipiens* population, and the vertical transmission (VT) of ZIKV in field-collected *Ae*. *albopictus* mosquitoes were evaluated for ZIKV strains of the African I and Asian ZIKV lineages in order to better estimate the risk of ZIKV introduction and transmission in Spain. Both *Ae*. *albopictus* populations assayed were infected by all tested ZIKV strains. Moreover, differences in vector competence between Spanish field-collected *Ae*. *albopictus* populations for ZIKV were detected and a higher susceptibility to the African I lineage strain than to the Asian ZIKV lineage strain was observed. On the other hand, ZIKV was unable to infect, replicate and be transmitted by the tested *Cx*. *pipiens* mosquito population. On the other hand, vertical transmission was demonstrated for both ZIKV lineages; the virus was detected in both males and females of the progeny of infected females. However, it is important to highlight that ZIKV dissemination was not detected in the infected females from the offspring. The results of the present study demonstrate that *Ae*. *albopictus* mosquito populations established in Spain could sustain virus transmission in case of ZIKV introduction and confirm that, it is unlikely that *Cx*. *pipiens* mosquitoes could be involved in ZIKV

transmission. In addition, the results showed that the progeny of ZIKV infected females were not able to disseminate ZIKV, suggesting the poor role of the VT in the ZIKV epidemiology. Our overall results provide helpful information to health authorities in order to establish efficient surveillance and vector control programs for ZIKV.

Keywords: Zika virus, *Aedes albopictus*, *Culex pipiens*, vector competence, vertical transmission.

INTRODUCTION

Zika virus (ZIKV) is an emergent arthropod-borne virus that belongs to *Flavivirus* genus within *Flaviviridae* family (Boyer et al. 2018). It was isolated for the first time in 1947 from a rhesus monkey in the Zika forest (Uganda) (Dick et al. 1952), and afterwards in an *Aedes africanus* pool from the same localization in 1948 (Zanluca and dos Santos 2016). Hereafter, ZIKV was sporadically found outside the African continent since the 1960s. In 2007, ZIKV emerged in the Yap Island (Micronesia) (Duffy et al. 2009) causing the first large outbreak. Then, it spread to the Pacific islands in 2013 (Musso et al. 2014) and reached the American continent in 2015 (Zanluca et al. 2015), where finally affected more than 45 countries (Boyer et al. 2018, WHO 2019). All phylogenetic analyses performed by now, confirm that ZIKV was originated in the African continent and then spread to Asia, Pacific Islands and the Americas (Li et al. 2017). Currently, ZIKV is classified into three lineages: Asian, African I, and African II (Saiz et al. 2017). The Asian lineage caused the spread of ZIKV into the Americas (Boyer et al. 2018). Due to the recent outbreaks in the Americas and since it has been demonstrated to cause microcephaly in neonates, the virus has become a threat to the public health (Boyer et al. 2018).

Zika virus is maintained in a sylvatic zoonotic cycle among zoophilic mosquitoes and non-human primates and other mammals (*e.g., Eidolon helvum* and *Capra aegagrus*) (Gutiérrez-Bugallo et al. 2019). The epidemic cycle occurs between humans and anthropophilic mosquitoes maintaining the virus in the urban sites (Boyer et al. 2018). The viral infection in humans generally occurs by the bite of infected females, but sexual (Mansuy et al. 2016) and perinatal (Besnard et al. 2014) ZIKV transmission have also been reported in humans. In this sense, ZIKV vertical transmission (VT) has also been shown in mosquitoes; ZIKV was detected in adult mosquitoes, which came from reared field-eggs collected of *Aedes albopictus*, *Aedes unilineatus* and *Aedes vittatus* mosquitoes (Diallo et al. 2014, Boyer et al. 2018, Costa

et al. 2018). In addition, ZIKV was detected in field collected adult males of *Aedes furcifer* (Diallo et al. 2014), *Aedes aegypti* and *Culex quinquefasciatus* mosquitoes indicating venereal or vertical transmission in the field (Elizondo-Quiroga et al. 2018, Gutiérrez-Bugallo et al. 2019). Moreover, ZIKV VT has also been experimentally demonstrated in *Ae*. *aegypti* (Thangamani et al. 2016, Ciota, Bialosuknia, Ehrbar, et al. 2017) and *Ae*. *albopictus* mosquitoes (Ciota, Bialosuknia, Ehrbar, et al. 2017); and venereal transmission, in *Ae*. *aegypti* mosquitoes (Campos et al. 2017). The epidemiological importance of ZIKV VT in mosquitoes in nature and the transmission by the progeny of infected females is still unknown. Zika virus VT has been suggested as an important mechanism to maintain the virus in the environment during hostile conditions (Thangamani et al. 2016). However, to better-understand the ZIKV ecology and epidemiology, further studies are needed to confirm the relevance of VT in the preservation of ZIKV, testing if the infected mosquito progeny is able to transmit the virus, since it has not been demonstrated yet.

ZIKV has been isolated from several African mosquito species in the field (Zanluca and dos Santos 2016) but *Ae*. *aegypti* is considered the main vector of ZIKV in urban areas (Boyer et al. 2018). Recently, numerous *Aedes* mosquitoes from all continents have been shown to be experimentally susceptible to transmit the virus, such as *Ae*. *vittatus*, *Aedes vexans* (Boyer et al. 2018), *Aedes polynesiensis* (Richard et al. 2016) and *Ae*. *albopictus* (Wong et al. 2013, Di Luca et al. 2016, Ciota, Bialosuknia, Zink, et al. 2017, Pompon et al. 2017, Kumar et al. 2018, Vazeille et al. 2019). The Asian tiger mosquito species, *Ae*. *albopictus* (Skuse, 1894), which was originated in the forests of Southeastern Asia, is widely distributed in tropical and subtropical regions. Nowadays, due to its global expansion, including Europe, it has been catalogued as an invasive mosquito species (Bonizzoni et al. 2013). This mosquito species, which is an anthropophilic and day-time feeder (Boyer et al. 2018), is known to be a competent vector of several arboviruses, such as chikungunya virus (CHIKV), West Nile virus

(WNV) or dengue virus (DENV) (Jolyon M. Medlock et al. 2012). Its current expansion and establishment in Eastern Spain since 2004 (Aranda et al. 2006) along the Mediterranean coast and Balearic Islands, and its anthropophilic feeding behavior, threat seriously the public health of the country. It has been experimentally demonstrated that Mediterranean *Ae*. *albopictus* mosquitoes are competent vectors for ZIKV (Di Luca et al. 2016, Heitmann et al. 2017, Gutiérrez-López et al. 2019, Vazeille et al. 2019). However, differences in vector competence (VC) among *Ae*. *albopictus* populations have been observed for ZIKV (Chouin-Carneiro et al. 2016). Moreover, recent studies with Spanish *Ae*. *albopictus* populations have shown controversial results in terms of ZIKV susceptibility (González et al. 2019, Gutiérrez-López et al. 2019, Hernández-Triana et al. 2019). On the other hand, *Culex pipiens* (Linnaeus, 1758), a member of the *Cx*. *pipiens* complex, which is a common mosquito extensively distributed across Mediterranean countries, included Spain, has been involved in the transmission of zoonotic pathogens, such as WNV, St. Louis encephalitis virus (SLEV) and Rift Valley fever phlebovirus (RVFV) (Brugman et al. 2018). However, there is no evidence to point that it is a potential ZIKV vector. Previous studies performed with *Cx pipiens* populations from Italy (Boccolini et al. 2016) and Germany (Heitmann et al. 2017) did not detect viral dissemination or transmission. However, its medical importance and feeding behavior highlighted the relevance to test Spanish *Cx*. *pipiens* populations to confirm that they could not be able to transmit ZIKV.

Recently, three cases of ZIKV autochthonous transmission have been reported in France (Giron et al. 2019), emphasizing the importance to determine if Spanish field-collected *Ae*. *albopictus* and *Cx*. *pipiens* mosquito populations are able to transmit the virus in order to develop an efficient vector surveillance and control programs by the competent authorities. For all the above-mentioned reasons, in the present study, we evaluated the VC of two field-collected Spanish *Ae*. *albopictus* mosquito populations for the African I and Asian ZIKV lineages and

tested one *Cx*. *pipiens* mosquito population for the African ZIKV lineage. In addition, VT and viral dissemination of the offspring from the first and second ovipositions (gonotrophic cycles E1 and E2) of intrathoracically inoculated *Ae*. *albopictus* females were assessed for the African I and Asian ZIKV lineages to better understand ZIKV VT and its importance in the ZIKV epidemiology. These studies provide relevant and helpful information to health authorities for both local mosquito species to design the strategies of the ZIKV surveillance programs.

MATERIAL AND METHODS

Mosquito rearing

Pupae from one field population of *Cx*. *pipiens* from Cerdanyola del Vallés (Catalonia, Spain), and eggs from two populations of *Ae*. *albopictus* from El Prat de Llobregat and Rubí (Catalonia, Spain), were collected to evaluate the VC of ZIKV. In addition, F0-F1 of field-collected *Ae*. *albopictus* females from Rubí municipality were also used for the VT assays. Larvae were reared in trays containing dechlorinated water supplemented with yeast (Gayelord- Hauser, Saint-Genis-Laval, France) until the adult stage. Emerging adults were maintained in the laboratory under controlled environmental conditions: 28ºC, 80 % of relative humidity and a light/dark cycle of 12:12 hours. Adult mosquitoes were provided with 10 % sucrose solution *ad libitum*.

Zika virus strains

Two ZIKV strains (Dak84 and Martinique) were used in the present study to evaluate the VC of local mosquito populations. The Dak84 strain (African I lineage, passage 4, Genbank reference: KU955592) was isolated from an *Aedes taylori* mosquito species in Senegal in 1984 and the Martinique strain (MRS_OPY_Martinique_ PaRi_2015, Asian lineage, passage 3, Genbank reference: KU647676) was isolated from human serum in 2015. Both ZIKV strains were propagated and titrated

in Vero cells (ATCC, ref. CCL-81) to obtain the 50 % tissue culture infective dose per mL ($TCID_{50}$/ml).

Oral infection of mosquitoes for vector competence assays

The VC experiment was performed using the same protocol recently reported by Vazeille *et al*. (2019). Briefly, a total of 381 and 879 *Cx*. *pipiens* and *Ae*. *albopictus* adult females of 10-12 and 7-9 days old, respectively, were artificially fed with washed rabbit erythrocytes mixed with adenosine 5'-triphosphate (ATP) ($5x10^{-3}$ M) (Sigma-Aldrich, St. Louis, MO) and containing ZIKV (final concentration: 7 log_{10} TCDI $_{50}$/mL of Dak84 and Martinique strains) (Table 1). The infectious blood was provided to mosquitoes by an artificial Hemotek feeding system set at 37.5 ºC ± 0.5 (Discovery Workshop, Accrington, UK). Females were fed during 30 min. Then, blood-engorged females were anaesthetized on ice, selected and maintained in groups of 10 in cardboard cages (Watkins & Doncaster, Leominster, UK) under the above-mentioned rearing conditions. Throughout the experiment, mosquitoes were maintained with 10% sucrose solution *ad libitum*. After a period of 7, 14 and 21 days post exposure (dpe) to infectious blood, females were anaesthetized on ice, the legs and wings were dissected and the saliva was extracted in 5 µl of FBS and collected in 45 µl of Dulbecco's modified Eagle's medium (DMEM) (Gibco, MA, USA). The head, body and saliva samples were stored in 300 µl of DMEM supplemented with 2 % FCS and 1 X anti-anti (Gibco Life Technologies 100 ml 15240-062 100X) at -80ºC until virus isolation. The infection (IR), disseminated infection (DIR), and transmission rates (TR), and transmission efficiency (TE) were assessed to evaluate the VC as described previously (Núñez et al. 2019). The experimental infections were carried out at IRTA-CReSA BLS3 facilities.

Intrathoracical ZIKV inoculation of mosquitoes for vertical transmission assays

Aedes albopictus (F0-F1) females from Rubí municipality were used for the VT assays. A total of 69, 29 and 38 females of 5-7 days old were intrathoracically-inoculated with the Dak84 (African I lineage) and Martinique (Asian lineage) ZIKV strains at 7.5 $log_{10}TCID_{50}$/ml and with the Asian (Martinique) strain at 8.2 log $log_{10}TCID_{50}$/ml, respectively. All intrathoracically inoculated females were maintained at the rearing conditions mentioned above. All females were fed with fresh rabbit blood at 7- and 14-days post-inoculation (dpi) to obtain the eggs of the first (E1) and second (E2) ovipositions. The eggs were dried during 7 days at rearing conditions and then hatched for subsequent mosquito rearing. Finally, adult males from the offspring of the inoculated females were sacrificed at 7 days old and whole bodies were kept at -80 ºC. Meanwhile, adult females from the same generation were anaesthetized on ice and dissected at 14 days old. Legs and wings were detached from the body to allow saliva extraction. All female mosquito samples (heads, bodies and saliva samples) were stored at -80 ºC until viral isolation following the same protocol described above. Finally, the filial infection rate (FIR) was used to estimate the proportion of infected progeny among all eggs produced by the inoculated females and the infection and disseminated infection of the progeny of the females inoculated with ZIKV were determined.

ZIKV detection

The head and body samples obtained from the experimental infections were thawed and homogenized in 0.5 ml of DMEM at 30 Hz for 1 min using TissueLyser II (Qiagen GmbH, Hilden, Germany). Virus detection in head and body samples was performed by inoculating on 96 well plates with Vero CCL-81 cells, as described by Valleize *et al*. (2019). To summarize briefly, Vero cells inoculated with the samples were maintained using DMEM, which was supplemented with 2 % FCS and 1 X anti-anti, during seven days at 37 ºC and 5 % CO_2. After

this period, to observe the cytopathic effect (CPE), cells were stained with a 0.2 % crystal violet solution (C.I. 42555, Merck KGaA, Darmstadt, Germany) and 2 % of formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) during 30 min. Saliva samples were titrated on six well plates with Vero CCL-81 cells using a method described previously by Arias-Goeta *et al*. (2014). After seven days of incubation under agarose overlay, the agar was removed, and the cells were stained with crystal violet solution for 30 min. Viral titers from saliva samples were expressed as plaque forming units per volume (PFUs/ml).

Table 1. Summary of vector competence assays.

				Number of mosquitoes tested per time point			
Mosquito species tested	**Population**	**ZIKV strains tested**	**Titer of ZIKV (TCID 50/ml)**	**7 dpe***	**14 dpe***	**21 dpe***	**Total**
Ae. albopictus	El Prat de Llobregat	Martinique	7 log10 TCID50/ml	20	20	17	57
	El Prat de Llobregat	Dak84	7 log10 TCID50/ml	11	13	12	36
Ae. albopictus	Rubí	Martinique	7 log10 TCID50/ml	28	27	22	77
	Rubí	Dak84	7 log10 TCID50/ml	28	27	25	80
Cx. pipiens	Cerdanyola del Vallés	Dak84	7 log10 TCID50/ml	17	16	15	48

*Dpe: days post-exposure

Statistical analyses

The statistical differences between mosquito populations, virus strains and dpe were analyzed using a generalized linear model with a binomial family distribution (logistic regression). In this model, we analyzed both fixed effect and all factor interactions. Post-hoc analyses to compare between mosquito populations among ZIKV lineages at each time point were performed with a χ^2 test using Monte Carlo permutations to determine the p-value. The statistical analyses were conducted using R 3.6.

RESULTS

Evidence of differences in the vector competence between Spanish field-collected *Ae. albopictus* populations for ZIKV

The feeding rates were 58.5 % (277/473) and 27.3 % (111/406) for *Ae. albopictus* from Rubí and El Prat de Llobregat populations, respectively. Infected bodies with ZIKV were detected from both field-collected *Ae. albopictus* populations exposed orally to infectious blood with both ZIKV strains tested at all-time points (7, 14 and 21 dpe) (Fig. 1, Table 2). Throughout the infection, population factor significantly modulated the viral infection depending on the strain (*i.e.*, dpe*population*strain interaction was significant, see Table 3). Particularly, the IR for Martinique ZIKV strain in el Prat de Llobregat population was higher at early stage of the infection (7 dpe) than at 14 and 21 dpe (p = 0.006) whereas it was homogenous for Dak84 ZIKV strain at all time-points. Conversely, both tested strains showed similar IR trends across dpe in Rubí population, both presenting significantly higher values at 14 dpe (p = 0.015). In addition, infection differences were observed between both populations for Martinique ZIKV strain since IR was significantly higher at 7 dpe in El Prat de Llobregat population than in Rubí population (p = 0.0009). Our results suggest that El Prat de Llobregat population was more susceptible to be infected earlier by the Asian lineage than Rubí population. However,

IR was significantly lower at 14 dpe in El Prat de Llobregat population than in Rubí population for the same strain (p = 0.02). Concerning the DIR and TR for Dak84 ZIKV strain, an earlier dissemination and transmission of the African I lineage (Dak84 ZIKV strain) could only be observed at 7 dpe in the Rubí population.

On the other hand, the proportion of females with infectious saliva among the total tested females (TE) was higher at 21 dpe in El Prat de Llobregat population than in Rubí population (p = 0.02) for Dak84 ZIKV strain. Regarding Martinique ZIKV strain, Rubí population was able to transmit it at 14 and 21 dpe; being the TE slightly higher at 21 dpe (TE= 4.5 %) than at 14 dpe (TE= 3.7 %) (Fig 1). No viral particles of the Martinique ZIKV strain were isolated in mosquito saliva samples from El Prat de Llobregat population probably due to the small sample size.

Spanish *Ae. albopictus* populations were more competent to the African I lineage than to the Asian ZIKV lineage

Apart from the above-mentioned infection differences between Dak84 and Martinique ZIKV strains in El Prat de Llobregat at 7 dpe, which showed a higher infection for Martinique ZIKV strain (Asian lineage), viral dissemination was higher for Dak84 ZIKV strain (African I lineage) than for Martinique ZIKV strain (Asian lineage) in both assayed mosquito populations along the infection (p = 1.18E-06) (Fig 1, Table 2 and 3). Moreover, during the infection, the virus dissemination for both ZIKV strains was increasing linearly (p = 6.31E-07). These results indicated that the midgut infection barrier was similar for both ZIKV strains throughout the infection, but the viral dissemination varied depending on the virus strain. The midgut escape barrier was stronger for the Martinique ZIKV strain (Asian lineage) than for the Dak84 ZIKV strain (African I lineage) in both populations due to its low dissemination at all tested time-points.

Concerning the TE (positive saliva samples among all blood feed mosquitoes), Dak84 ZIKV strain (African I lineage) showed 23 % and 58.3 % in El Prat de Llobregat population at 14 and 21 dpe, respectively, while Martinique ZIKV strain (Asian lineage) was not detected in any saliva sample. Moreover, Dak84 ZIKV strain (African I lineage) presented 18.5 % and 20 % of positive saliva among the assayed mosquito samples at 14 and 21 dpe in Rubí population while Martinique ZIKV strain (Asian lineage) presented 3.7 % and 4.5 % at 14 and 21 dpe, respectively. Furthermore, Dak84 ZIKV strain (African I lineage) TE for both populations was higher at 21 dpe than at 14 dpe.

Table 2. Infection, dissemination and transmission for two field *Ae*. *albopictus* populations infected with Dak84 and Martinique ZIKV strains.

Specie	Population	ZIKV strain	Dpe								
			7 dpe			14 dpe			21 dpe		
			IR (%)	DIR (%)	TR (%)	IR (%)	DIR (%)	TR (%)	IR (%)	DIR (%)	TR (%)
Ae. *albopictus*	El Prat de Llobregat	Martinique	17/20 (85%)	0/17 (0%)	-	8/20 (40%)	2/8 (25%)	0/2 (0%)	5/17 (29.4%)	1/5 (20%)	0/1 (0%)
		Dak84	6/11 (54.5%)	0/6 (0%)	-	8/13 (61.5%)	5/8 (62.5%)	3/5 (60%)	7/12 (58.3%)	7/12 (58.3%)	7/7 (100%)
Ae. *albopictus*	Rubí	Martinique	9/28 (32.12%)	0/9 (0%)	-	18/27 (66.6%)	2/18 (11.1%)	1/2 (50%)	10/22 (45.4%)	5/10 (50%)	1/5 (20%)
		Dak84	14/28 (50%)	2/14 (14.2%)	1/2 (50%)	17/27 (70%)	14/17 (82.3%)	5/14 (35.7%)	9/25 (36%)	7/9 (77.7%)	5/7 (71.4%)
Cx. *pipiens*	Cerdanyola del Vallés	Dak84	0/17 (0%)	-	-	0/16 (0%)	-	-	0/15 (0%)	-	-

IR= infection rate; DIR= dissemination rate. TR= transmission rate; Dpe: days post-exposure.

Table 3. Comparison of infection and dissemination rates between mosquito population, dpe and viral strains using a generalized linear model with a binomial family distribution (logistic regression).

	IR				DIR			
	d.f.	χ^2	***p* value**		**d.f.**	χ^2	***p* value**	
Dpe	2	4.2598	0.11885		2	28.551	**6.31E-07**	***
Strain	1	0.4988	4.80E-01		1	23.6072	**1.18E-06**	***
Population	1	0.4481	0.503233		1	0.0524	0.8189	
Dpe*Strain	2	0.8891	0.641128		2	2.482	0.2891	
Dpe*Population	2	12.4313	**0.001998**	**	2	1.7889	0.4088	
Strain*Population	1	0.4097	5.22E-01		1	1.4643	0.2262	
Dpe*Strain*Population	2	9.9645	**0.006859**	**	2	4.2258	0.1209	

** p-value <0.001 *** p-value <0.0001; Dpe: days post-exposure; d.f.: degrees of freedom

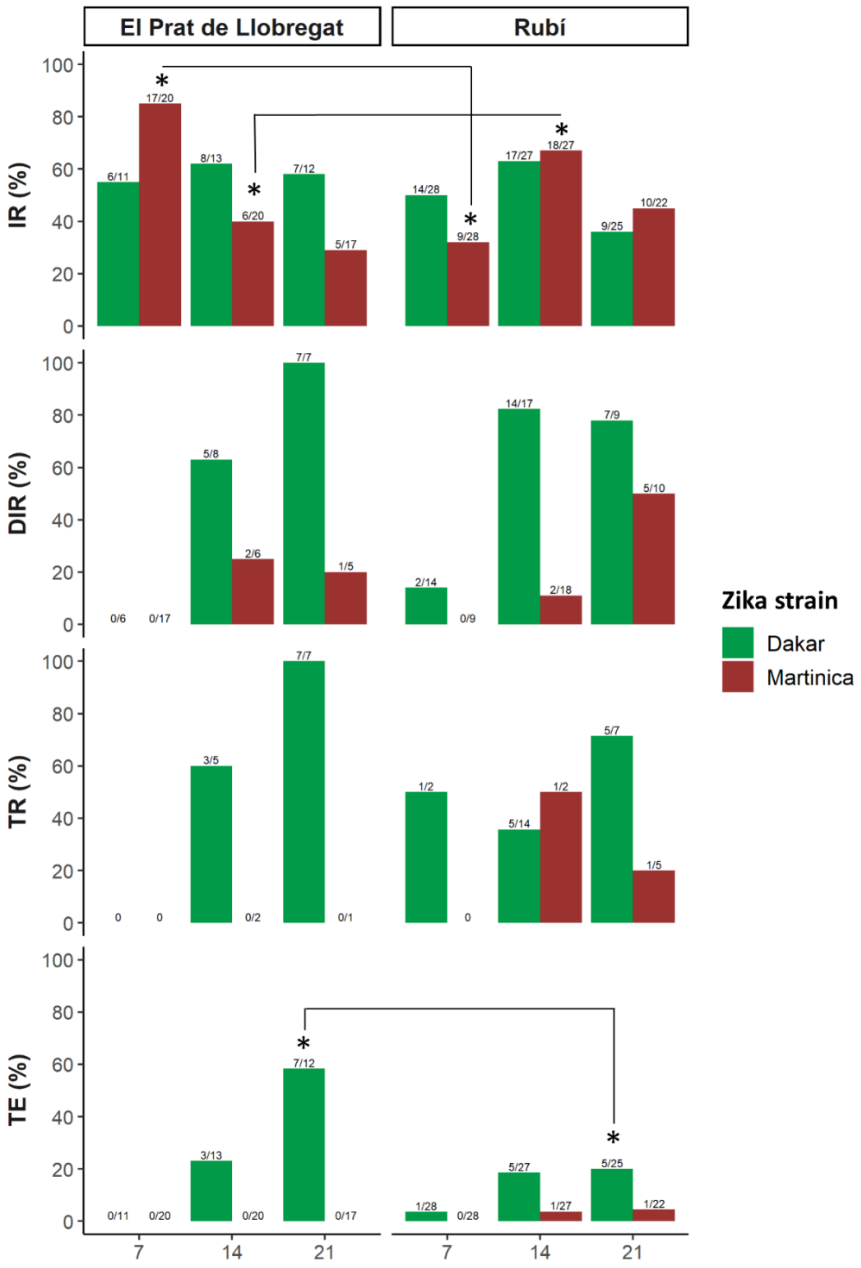

Figure 1. Infection, dissemination and transmission rate and transmission efficiency of two field *Ae*. *albopictus* populations exposed to Dak84 and Martinique ZIKV strains, which are represented with red and green colors, respectively. The * in the figures shows the statically significant results between populations. IR= infection rate; DIR= dissemination rate. TR= transmission rate; TE: transmission efficiency.

Regarding to viral titers in saliva of those female mosquitoes that were able to expectorate ZIKV within the saliva, both *Ae*. *albopictus* populations which were tested, showed higher viral titers in saliva samples for the Dak84 ZIKV strain (mean = 1712.4 PFU/ml) than for the Martinique ZIKV strain (mean = 186.8 PFU/ml), in which the size of the positive samples was low (2). The viral loads in the saliva of positive mosquitoes are shown in the Fig 2.

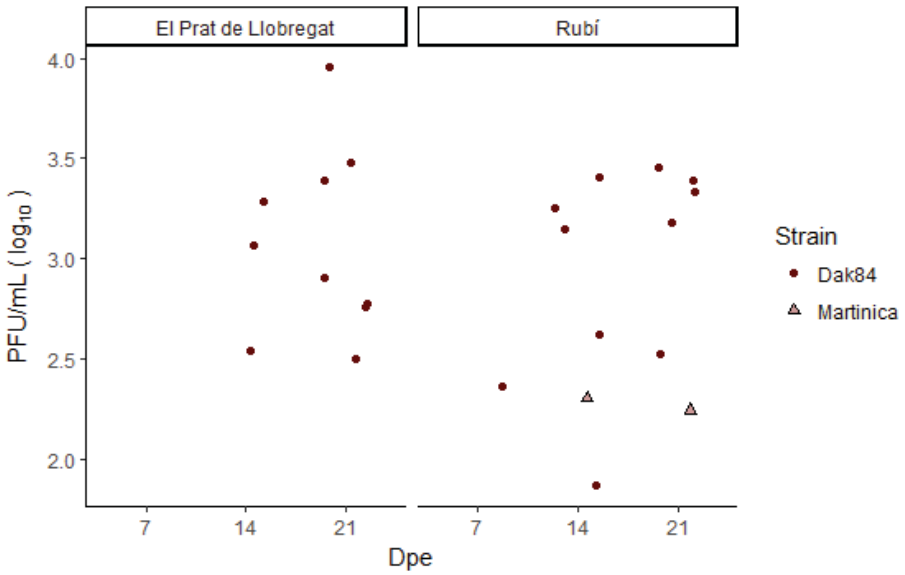

Figure 2. ZIKV load in saliva (Plaque forming units (PFU/mL)) of infected mosquitoes of two field *Ae*. *albopictus* mosquito populations (El Prat del Llobregat and Rubí) exposed to Dak84 and Martinique ZIKV strains. *Dpe: days post-exposure to the virus

Spanish *Cx. pipiens* was refractory to the African ZIKV lineage

The feeding rate for *Cx*. *pipiens* mosquitoes was 17.3 % (66/381), much lower than for the above-mentioned *Ae*. *albopictus* populations. Regarding the VC study of *Cx*. *pipiens*, Dak84 ZIKV strain (African I lineage) was unable to infect, replicate and be transmitted by *Cx*. *pipiens* mosquitoes at any tested time point (Table 2).

Spanish *Ae. albopictus* progeny of inoculated females were not able to disseminate ZIKV

Infection and dissemination of the inoculated females were confirmed at 7 dpi. The eggs obtained from the E1 and E2 of the gonotrophic cycles of inoculated females and FIR are shown in Table 4. Zika virus was detected in bodies of both males and females from the progeny of the E1 of inoculated females for both tested ZIKV lineages (Table 5). In addition, infected bodies were detected in males from the E2 of inoculated females for the Asian lineage. However, all heads from positive bodies were negative for both ZIKV lineages.

Table 4. Number of eggs obtained from ZIKV inoculated females during the first and second gonotrophic cycle and filial infection rates (including males and females).

Specie	Generation	Strains	Viral doses	FEF females	Egg E1	Filial infection rate	Egg E2	Filial infection rate
Ae. albopictus	F0-F1	Dak84	7.5 $\log_{10}$ $TCID_{50}$/ml	34	559	28: 559 (5 %)	322	<1: 322 (0 %)
Ae. albopictus	F0-F1	Martinique	7.5 $\log_{10}$ $TCID_{50}$/ml	19	246	7: 246 (2.8 %)	94	4: 94 (4.2 %)
Ae. albopictus	F1	Martinique	8.2 $\log_{10}$ $TCID_{50}$/ml	12	140	5: 140 (3.5 %)	133	-

FEF: full-engorged inoculated females; E1: first gonotrophic cycle; E2: second gonotrophic cycle; -: No data

Table 5. Infection and disseminated infection rates of the offspring of the *Ae*. *albopictus* females from Rubí intrathoracically inoculated with Dak84 and Martinique ZIKV strains

			Males		**Females**			
			E1	**E2**	**E1**		**E2**	
Species	**Strain**	**Viral dose**	**IR (%)**	**IR (%)**	**IR (%)**	**DIR (%)**	**IR (%)**	**DIR (%)**
Ae. albopictus	Dak84	7.5 $\log_{10}$ $TCID_{50}$/ml	23/74 (31%)	0/6 (0%)	5/87 (5.7%)	0/5 (0%)	0/5 (0%)	0/0 (0%)
	Martinique	7.5 $\log_{10}$ $TCID_{50}$/ml	5/24 (20.8%)	4/25 (16%)	2/17 (11.7%)	0/2 (0%)	0/30 (0%)	0/0 (0%)
	Martinique	8.2 $\log_{10}$ $TCID_{50}$/ml	5/14 (35.7%)	0/4 (0%)	0/6 (0%)	0/0 (0%)	-	-

IR= infection rate; DIR= disseminated infection rate; E1: first gonotrophic cycle; E2: second gonotrophic cycle; -: No data.

DISCUSSION

Due to the ZIKV outbreak in 2015 in Brazil, the amount of scientific research about this virus has recently increased because of its medical importance in human health. Consequently, the number of VC studies for ZIKV has also augmented in the last years since virus transmission via mosquito bite plays a crucial role to determine the risk of introduction of a pathogenic agent in a non-endemic country. Our present study shows the infection susceptibility of two field-collected *Ae*. *albopictus* populations from Spain for two ZIKV lineages (African I and Asian) and the refractoriness of a Spanish *Cx*. *pipiens* population to African I ZIKV lineage.

Aedes albopictus plays a major threat for the global transmission of arboviruses due to its anthropophilic behavior, and its physiological and ecological plasticity to colonize new areas (Paupy et al. 2009). In the last years, European *Ae*. *albopictus* mosquitoes have been demonstrated to be competent vectors for ZIKV although showing heterogeneous results (Di Luca et al. 2016, Jupille et al. 2016, Heitmann et al. 2017, Gutiérrez-López et al. 2019, Vazeille et al. 2019). Our results are in agreement with the above-mentioned studies, particularly with Vazeille *et al*. (2019) since they performed the experiment using the same protocol than in the present study, which was established in the frame of the European ZIKAlliance project. Vazeille *et al*. (2019) demonstrated that *Ae*. *albopictus* populations from France (Montpellier and Corsica) were more susceptible to Dak84 ZIKV strain (African I lineage) than to Martinique and Cambodia ZIKV strains (Asian lineage), which is in agreement with our findings. Both tested populations in the present study showed significant differences in transmission at 21 dpe for the African I lineage, as also previously presented by Vazeille *et al*. (2019). Moreover, our results showed an early transmission of the African I lineage at 7 dpe in *Ae*. *albopictus* population from Rubí as also previously demonstrated by Vazeille *et al*. (2019) for the African I lineage in an *Ae*. *albopictus*

population from France and Gutiérrez-López *et al*. (2019) for the Asian lineage in a Spanish *Ae*. *albopictus* population. This early transmission reported for *Ae*. *albopictus* mosquitoes could increase their vectorial capacity in case of ZIKV introduction into the Mediterranean countries. Besides, our results showed differences in terms of VC for ZIKV between the two tested mosquito populations. These differences could also be observed when we compared our results with those obtained for French *Ae*. *albopictus* mosquitoes. They seem to be better vectors for Dak84 ZIKV strain (African I lineage) than Spanish populations at 14 dpe. The TR obtained was around 80 % in both assayed French mosquito populations, and our transmission results were 60 % and 35 % in El Prat de Llobregat and in Rubí population respectively; although the transmission was similar at 21 dpe for all French (around 80 %) and Spanish mosquito populations (100 % and 71 %, in El Prat de Llobregat and Rubí, respectively). In addition, Spanish *Ae*. *albopictus* were less competent for the Asian lineage than French *Ae*. *albopictus*, where the TR was around 60 % for Cambodia strain and 40 % for Martinique strain. In our study, transmission was only detected in Rubí population for Martinique ZIKV strain at 14 and 21 dpe in a low number of tested mosquitoes (TR was 50 % and 20 %, respectively). Regarding the viral titer in saliva samples, Rubí mosquito population showed higher viral titer for the African I lineage than for the Asian lineage as previously showed Vazeille *et al*. (2019) and the viral titers obtained from French mosquito populations were similar to our results (Vazeille et al. 2019). European results show VC differences between mosquito populations as it was previously pointed out in American mosquito populations (Chouin-Carneiro et al. 2016) and as other studies have previously described for DENV (Lambrechts et al. 2009).

The vector competence of Spanish *Ae*. *albopictus* populations remained controversial considering that two recent studies (González et al. 2019, Hernández-Triana et al. 2019) showed that the virus was not detected in the expectorated saliva in contrast to another recent study (Gutiérrez-López et al. 2019), which tested an *Ae*. *albopictus*

population from the same region of Spain (Barcelona province) and showed ZIKV transmission. Our results, which are in agreement with the latest study, suggest that these contradictory results could be explained by at least four main reasons. Firstly, the rearing temperature, which is an extrinsic factor that affects direct and indirectly the *Ae*. *albopictus* susceptibility to arbovirus (Westbrook et al. 2010, Ciota, Bialosuknia, Zink, et al. 2017, Ciota and Keyel 2019). The environmental factors in which mosquitoes were reared, may have played a crucial role for ZIKV transmission of Spanish *Ae*. *albopictus* populations. The VC study performed by Hernández-Triana e*t al*. (2019), where the rearing temperature was low (20 ºC/ 25 ºC [night/day]), did not detect transmission for ZIKV, in contrast with the results of other VC studies that showed ZIKV transmission, where the experiments were performed under temperature conditions that ranged from 26 ºC to 28 ºC. Secondly, the viral concentration used to artificially feed the females could have also influenced the VC of Spanish *Ae*. *albopictus* mosquitoes for ZIKV. The low titer (1.8×10^6 PFU/ml; *cf*. the above-mentioned European mosquito studies and even our assay) used in the study of González *et al*. (2019) could explain their negative results in terms of ZIKV transmission. Thirdly, the VC of *Ae*. *albopictus* females for ZIKV depends on mosquito population and virus lineage interactions (McKenzie et al. 2019) as previously demonstrated in *Ae*. *aegypti* and *Ae*. *albopictus* mosquitoes for DENV and ZIKV (Lambrechts et al. 2009, Chouin-Carneiro et al. 2016). According with it, our results also showed the genetic variances between Spanish mosquito populations and ZIKV strains. Lastly, besides mosquito-virus interaction, the number of generations of laboratory colony affects VC since it is thought that the susceptibility to the virus increases for the colonized mosquitoes over the years, it had been previously reported for DENV (Vazeille et al. 2003). However, the fact that Hernández-Triana *et al*. (2019) performed the VC experiments using an old laboratory colony (from 2009) and was not able to transmit the ZIKV would seem to be contradictory. It is important to take into account that the laboratory colonies would not be

the best option to perform VC experiments since the long-term colonization may cause genotypic and phenotypic changes in mosquitoes (Azar and Weaver 2019).

The African I lineage tested in the present study, was not able to infect the midgut, disseminate to secondary organs and be transmitted by a *Culex pipiens* population from Cerdanyola del Vallès despite it was the most infectious for *Ae*. *albopictus*. This results suggest that this mosquito population has a strong MIB. Previous studies performed with European *Cx*. *pipiens* populations demonstrated that this specie has a strong MEB, since only infection was detected at 3 dpe, and at 14 and 21 dpe in Italian and German *Cx*. *pipiens*, respectively (Boccolini et al. 2016, Heitmann et al. 2017). Furthermore, no viral dissemination neither transmission was detected in European *Cx*. *pipiens* in any study performed until now (Boccolini et al. 2016, Heitmann et al. 2017).

To know how ZIKV could be maintained in nature during periods without vectors and available vertebrate hosts is essential to understand the ZIKV ecology. In this regard, the VT has been suggested as an alternative mechanism to preserve the virus in the environment (Thangamani et al. 2016). Arboviral VT takes place through two mechanisms: i) by transovarial transmission, whereby the virus infects germinal tissues and ii) by trans-ovum transmission, which occurs through viral contamination in the egg surface during oviposition or at the time of the fertilization (Rosen 1987a, Higgs and Beaty 2005, Sánchez-Vargas et al. 2018). It is thought that the VT of flaviviruses (specifically for DENV) generally occurs by trans-ovum transmission rather than by transovarial transmission (Rosen et al. 1983, Ciota, Bialosuknia, Ehrbar, et al. 2017). The ZIKV VT has been previously reported for *Ae*. *albopictus* (Ciota, Bialosuknia, Ehrbar, et al. 2017, Guo et al. 2020), but the viral dissemination in the progeny of infected females had not been tested until now. Our results confirmed VT, since infected males and females of the E1 progeny of intrathoracically inoculated *Ae*. *albopictus* collected from the field for

both African I and Asian lineages were detected. These results are in agreement with a recent study performed in *Ae*. *albopictus* mosquitoes (Guo et al. 2020), in which they detected the Asian lineage of ZIKV in males and females from the first oviposition progeny and with the results obtained by Ciota *et al*. (2017) that showed ZIKV in the larvae from the second oviposition of *Ae*. *albopictus* progeny after a blood-meal infection. The low infection rate of the progeny, which may suggest that the VT would not be linked to transovarial transmission and the contrasting results on the presence of ZIKV in E1 *cf*. E2 progeny, having higher prevalence in E1 progeny, reinforce the need to perform further studies to define the VT mechanism of this virus. Moreover, it is important to conduct further studies of ZIKV VT in eggs in diapause or desiccated as a possible method of ZIKV persistence, as it was suggested for DENV (Grunnill and Boots 2016). Furthermore, our results contrast with previous results obtained by Thangamani *et al*. (2016) and Phumee *et al*. (2019), where VT in *Ae*. *albopictus* was not demonstrated for the Asian lineage. These contrasting results could be explained because of the viral load within the infected mosquitoes, since they used a lower viral dose and the specimens were orally infected (Phumee et al. 2019), and as it was discussed above, viral dose may affect ZIKV infection in *Ae*. *albopictus* mosquitoes. In addition, negative results of Thangamani *et al*. (2016) for ZIKV VT after intrathoracic inoculation of the virus could be due to the specific pairing of vector population-virus strain. To our knowledge, this is the first time that the viral dissemination in the infected progeny was tested for ZIKV; the obtained results indicated that all infected females of the progeny were not able to disseminate the virus through hemolymph to reach secondary organs (*e*.*g*., salivary glands) suggesting that ZIKV VT in *Ae*. *albopictus* is unlikely to be relevant for ZIKV epidemiology.

Another important issue to be considered in future studies, is that arboviruses (*e*.*g*. La Crosse virus and dengue virus) could be amplified in a mosquito population by a venereal transmission

mechanism (Thompson and Beaty 1977, Rosen 1987b). In this case, male mosquitoes would obtain arboviruses by VT from an infected female and thereby, could transmit the virus horizontally to a non-infected female during mating as demonstrated in experimental studies for La Crosse virus in *Aedes triseriatus* (Thompson and Beaty 1977). After that, the females may develop infected oocytes, generating infected progeny (Thompson and Beaty 1977, Tesh et al. 2016, Thangamani et al. 2016). In this regard, the ZIKV detection in *Ae*. *albopictus* males in our experiment suggests that they could play a role in the preservation of ZIKV in nature due to its polygamous behavior (Choochote et al. 2001) as it was previously suggested for DENV infection, although the virus transmission by the females infected with DENV during mating has also not been demonstrated yet (Sánchez-Vargas et al. 2018). Venereal transmission for ZIKV was first described in *Ae*. *aegypti* (Campos et al. 2017). However, there is still a lack of knowledge about the epidemiological relevance of venereal transmission in ZIKV transmission by *Ae*. *albopictus*. Further studies are required to confirm the role of males in the transmission cycle of ZIKV.

CONCLUSIONS

To sum up, our study demonstrated that both evaluated Spanish *Ae*. *albopictus* populations were competent vectors for the African I and Asian ZIKV lineages since infectious ZIKV was isolated from saliva of experimentally infected mosquitoes, although with some differences in terms of viral susceptibility and transmission depending on mosquito population and virus strain. Therefore, *Ae*. *albopictus* established in the Barcelona province (Spain) may sustain virus transmission when the ZIKV is introduced. This is of major importance as most of the invaded areas by this species present anthropophilic dense populations, relevant factor in the vectorial capacity. On the other hand, one field-collected *Cx*. *pipiens* population from Spain was not able to transmit experimentally the African I ZIKV lineage, so it is unlikely that could

be a potential vector of ZIKV when the virus is introduced Europe. Vertical transmission of ZIKV was proved in Spanish *Ae*. *albopictus* but the obtained results suggest that viral transmission from females of the progeny of ZIKV infected females is unlikely. Further studies are needed to know if infected males of the progeny of ZIKV infected females could contribute to maintain the virus in the nature by venereal transmission. Overall, our results provide supportive information to the health authorities to apply efficient surveillance and vector control programs for ZIKV.

ACKNOWLEDGEMENTS

The authors are very grateful to BSL3 facilities staff for their excellent technical contribution, to Dr. Francesc Xavier Abad for his excellent BSL3 facilities management at IRTA-CReSA, to the Núria Busquets's family, since they allowed us to collect mosquito eggs in their orchard (Rubí municipality) and to Miguel Berdugo for their advices and contributions to develop the statistical analysis.

Chapter III

Alteration in the *Culex pipiens* transcriptome reveals diverse mechanisms of the mosquito immune system implicated during Rift Valley fever phlebovirus infection

Short tittle: ***Culex pipiens*** **molecular responses to Rift Valley fever phlebovirus infection**

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ABSTRACT

Rift Valley fever phlebovirus (RVFV) causes an emerging zoonotic disease and is mainly transmitted by *Culex* and *Aedes* mosquitoes. While *Aedes aegypti*-dengue virus (DENV) is the most studied model, less is known about the genes involved in infection-responses in other mosquito-arboviruses pairing. The main objective was to investigate the molecular responses of *Cx. pipiens* to RVFV infection focusing mainly on genes implicated in innate immune responses. Mosquitoes were fed with blood spiked with RVFV. The fully-engorged females were pooled at 3 different time points: 2 hours post-exposure (hpe), 3- and 14-days post-exposure (dpe). Pools of mosquitoes fed with non-infected blood were also collected for comparisons. Total RNA from each mosquito pool was subjected to RNA-seq analysis and a *de novo* transcriptome was constructed.

A total of 451 differentially expressed genes (DEG) were identified. Most of the transcriptomic alterations were found at an early infection stage after RVFV exposure. Forty-eight DEG related to immune infection-response were characterized. The majority of them were related with the Toll and IMD pathways, the RNAi system, ubiquitination pathway and apoptosis.

Our findings provide for the first time a comprehensive view on *Cx. pipiens*-RVFV interactions at the molecular level. While genes from the Toll and IMD immune pathways were altered in response to RVFV none of the DEG were related to the JAK/STAT pathway. The early depletion of RNAi pathway genes at the onset of the RVFV infection would allow viral replication in mosquitoes. The fact that most of the DEG involved in the Ubiquitin-proteasome pathway (UPP) or apoptosis were found at an early stage of infection would suggest that apoptosis plays a regulatory role in infected *Cx. pipiens* midguts. This study provides several target genes that could be used to identify new molecular targets for vector control.

AUTHOR SUMMARY

Rift valley fever (RVF) is an emerging zoonotic disease and it is caused by RVFV. This virus is commonly transmitted in endemic areas between wild ruminants and mosquitoes, mainly by mosquitoes of *Culex* and *Aedes* genus. Starting from the year 2000, several outbreaks have been reported outside Sub Saharan Africa, in countries facing the Mediterranean Sea (Egypt), or Yemen and Saudi Arabia. Available vaccines for ruminants present limited efficacy or residual pathogenic effects. Consequently, new strategies are urgently required to limit the expansion of this zoonotic virus. The main objective of this work is to investigate transcriptional alterations of *Cx. pipiens* to RVFV focusing mainly on genes implicated in conventional innate immunity pathways, RNAi mechanisms and the apoptotic process in order to evaluate the involvement of these genes in viral infection. The immune altered genes here described could be potential targets to control RVFV infection in mosquitoes. Some of the genes related to the immune defense response were previously described in others mosquito-arbovirus models, as well as in *Drosophila* and human. To our knowledge, this study highlights for the first time the *Cx. pipiens*-RVFV interactions in terms of defense infection-response and provides information for developing in the future new approaches to prevent and control the expansion of the virus.

INTRODUCTION

Rift Valley fever (RVF) is an emerging arthropod-borne zoonotic disease caused by an enveloped RNA segmented phlebovirus, RVFV. Beside wild ruminants, RVFV affects mainly domestic ruminants and humans (Pepin et al. 2010). It was first described in 1930 in the Rift Valley (Kenya), after observing high abortion and mortality rates in sheep near Lake Naivasha (Daubney et al. 1931). In 1948, mosquitoes of the *Aedes* genus were identified as RVFV vectors in Uganda (Smithburn et al. 1948). Since then, the virus has been isolated from at least 53 mosquito species covering 8 genera, mainly *Aedes* and *Culex* genus, and belonging to the Culicidae family (Linthicum et al. 2016). After successive outbreaks in eastern and southern Africa, RVFV was detected in Egypt in 1977, causing a major human outbreak involving *Cx. pipiens* species as the main potential vector (Linthicum et al. 2016). RVFV was identified for the first-time outside Africa in 2000, in Saudi Arabia and Yemen. During this outbreak, the impact on the economy and public health was very high, reaching a total of 882 confirmed cases with 124 deaths (Balkhy and Memish 2003). The spread of RVFV outside the African continent and its presence in Egypt, which faces the Mediterranean Sea, highlighted the possibility of RVFV introduction in Europe (Mansfield et al. 2015). Indeed, European autochthonous vectors, such as *Cx. pipiens* or the invasive Asian tiger mosquito, *Aedes albopictus* have been proven to be competent vectors under laboratory condition (Moutailler et al. 2008, Brustolin et al. 2017). Currently, RVFV is circulating in several African countries causing sporadic outbreaks (WHO 2013) and according to WHO, it is a prioritized emerging infectious disease.

Mosquitoes within the *Cx. pipiens* complex are known to be competent vectors for RVFV (Turell et al. 2008, 2014, Brustolin et al. 2017). The capacity of the mosquitoes to acquire, replicate and transmit an arbovirus, known as vector competence, depends on: i) extrinsic factors, such as environmental conditions (Brubaker and Turell 1998)

or genetic variations of the pathogens (Moutailler et al. 2008), and ii) intrinsic factors, such as the genetics of vector populations (Kumar et al. 2018), and the immune system of each mosquito strain (Sim et al. 2013). The immune response of vectors leads to a stable arbovirus infection with low vector fitness costs and a sustained viral infection necessary for transmission. Multiple immune effector mechanisms in insect are implicated in the defense against microorganisms: apoptosis, encapsulation, melanization, phagocytosis of the pathogens and the production of antimicrobial peptides (AMP) (Lemaitre and Hoffmann 2007, Sim et al. 2014, Mussabekova et al. 2017). Immune effectors are triggered mainly by three innate immunity pathways: Toll, Immune deficiency (IMD) and Janus kinase/signal transducer and activator of transcription (JAK/STAT). All of them are involved in the defense against arbovirus infection (Lemaitre and Hoffmann 2007). The Toll pathway is activated after the interaction between the antigen and the Toll-like receptors. This eventually induces the expression of antiviral effectors (Sim et al. 2014). Several functional assays have shown that the Toll signaling pathway plays a key role for DENV control in infected *Ae*. *aegypti* mosquitoes (Xi et al. 2008, Ramirez and Dimopoulos 2010, Sim et al. 2014). Moreover, activation of the Toll pathway in the midgut of *Ae*. *aegypti* infected with the Sindbis virus (SINV) and Zika virus (ZIKV) has also been suggested (Sanders et al. 2005, Angleró-Rodríguez et al. 2017). Additionally, there is mounting evidence for the antiviral function of the IMD signaling pathway in mosquitoes (Sim et al. 2013). The IMD pathway is triggered upon virus infection recognition by the adaptor IMP protein, resulting in the transcription of IMD effector genes (Sim et al. 2014). Upon DENV infection, the salivary glands of *Ae*. *aegypti* secrete an IMD dependent antimicrobial peptide (AMP) with antiviral activity against DENV and Chikungunya virus (CHIKV) (Luplertlop et al. 2011). The invertebrate JAK/STAT pathway is similar to the mammalian interferon response. The JAK/STAT pathway is also involved in *Ae*. *aegypti* defense responses against DENV and ZIKV infection (Sim et al. 2013, Angleró-Rodríguez et al. 2017). Elements of different immune

pathways may act synergistically and interact with components of apoptosis and other metabolic pathways. Apoptosis is a defense mechanism that insects use against viral infections by killing infected or neighboring cells. Programmed cell death is involved, at the midgut level, in the control of DENV and West Nile virus (WNV) infections in *Ae*. *aegypti* (Olson and Blair 2015) and *Cx*. *pipiens* (Vaidyanathan and Scott 2006) respectively. On the other hand, Patel *et al*. (Patel and Hardy 2012), reported that the phosphatidylinositol 3-kinase (PI3K)-Akt-TOR signaling pathway promotes viral replication in insect cells during SINV infection and causes inhibition of apoptosis. Although the above-mentioned transduction pathways are relevant for controlling arbovirus infections, the RNA interference (RNAi) pathway is considered to be the major innate immune response against viral infections (Olson and Blair 2015). The small interfering RNA (siRNA) pathway is the best known regarding its role in restricting arbovirus infections (i.e. DENV, SINV and CHIKV) in *Ae*. *aegypti* (Campbell et al. 2008, Sánchez-Vargas et al. 2009, McFarlane et al. 2014), but it can also generate virus diversification during WNV infection in *Cx*. *quinquefasciatus* (Brackney et al. 2009). Additionally, the P-element-induced Wimpy testes gene (PIWI)-interacting RNA (piRNA) pathway, which is involved in maintaining genomic integrity, has also been suggested to be implicated in antiviral defense (Vodovar et al. 2012).

The importance of the mosquito innate immune system against different flaviviruses, specifically against DENV has been studied in detail. However, less is known on the interaction of bunyaviruses such as RVFV with the *Cx*. *pipiens* immune system. In the present study, the effect of the RVFV in infected *Cx*. *pipiens* has been studied using RNA-seq analysis. Differentially expressed genes related to the mosquito's immune system response have been identified after RVFV exposure. This is the first study that analyzes the *Cx*. *pipiens* transcriptome during RVFV infection in order to better understand interactions between *Cx*. *pipiens* mosquitoes and RVFV and potential mechanisms involved in the control of RVFV infection.

MATERIALS AND METHODS

Mosquitoes rearing

Culex pipiens hybrid form mosquitoes used for RVFV infection were reared from a laboratory colony originally collected in Gavà in 2012 (Spain). The molecular genetic characterization of the mosquitoes was performed in a previous study (Brustolin et al. 2017). Mosquitoes were reared at 26 ºC during the day and 22 ºC at night at 80 % of relative humidity (HR). The photoperiod consisted in 14 h: 10 h (light: dark) with two crepuscular cycles of 30 min to simulate dawn and dusk. Mosquitoes were fed using 10 % sucrose solution.

Virus production and titration

A virulent RVFV strain (RVF 56/74), kindly provided by Dr. Alejandro Brun (CISA-INIA) and originally isolated from cattle in 1974 (Barnard and Botha 1977), was used for mosquito experimental infection. RVF 56/74 was propagated in C6/36 cells and titrated in Vero cells as previously described (Brustolin et al. 2017).

Experimental RVFV infection in *Culex pipiens* mosquitoes

Mosquitoes were kept in 500 ml volume plastic cages with mesh screening and fed *ad libitum* on a 10 % sucrose solution. The sucrose solution was removed 30 hours before feeding the mosquitoes with infectious blood. Three hundred adult females of 7-10- days-old were used for the present study. One hundred fifty adult females were exposed to infectious blood and 150 females were exposed to non-infectious blood. The heparinized bovine blood mixed with adenosine 5'-triphosphate (ATP) ($5x10^{-3}$ M) (Sigma-Aldrich, St. Louis, MO) was extracted from bovine (medial caudal vein) and was provided to mosquitoes using the Hemotek artificial system (Discovery Workshop, Accrington, UK) at 37.5 °C ± 0.5. Aliquots of RVFV-spiked blood were collected after feeding and used to calculate the viral titer using the $TCID_{50}$ assay in Vero cell (RVFV titer blood 7.46 $\log_{10}$ $TCID_{50}$/ml).

The fully-engorged females were anaesthetized using CO_2, selected and maintained in individual cardboard cages (Watkins & Doncaster, Leominster, UK) sealed with a net on top and stored inside a climatic chamber under rearing environmental conditions. Throughout the experiment, the mosquitoes were maintained with permanent access to 10% sucrose solution. Twelve fully-engorged females from each group (infectious and non-infectious blood) were removed at 2 hours post-exposure (hpe), and at 3 and 14 days post-exposure (dpe). These females were anaesthetized using CO_2 and stored at -80 °C until use. The experimental infection was performed in Biosafety Level 3 facilities at the *Centre de Recerca en Sanitat Animal* (IRTA-CReSA).

Total RNA extraction and RVFV detection

Fully-engorged females were pooled in groups of four. Three pools were used for each time point (2 hpe, 3 dpe and 14 dpe) and condition: exposed to infectious (hereafter referred to as RVFV) and non-infectious blood (hereafter referred to as control). In total, 18 pools of four females were obtained. The samples were homogenized with glass beads for 2 min at 30 Hz using TissueLyser II (Qiagen GmbH, Hilden, Germany) and total RNA was extracted from each pool using RNeasy Mini Kit (250) (Qiagen GmbH, Hilden, Germany). Total RNA was eluted using 50 µl of RNase-free water.

RVFV RNA was detected by reverse transcription quantitative PCR (RT-qPCR) using primers defined previously (Drosten et al. 2002) and AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, Inc., Foster City, CA, U.S.A.) following the amplification protocol previously described (Brustolin et al. 2017). The limit of sensitivity was 0.09 $TCID_{50}$ per reaction.

RNA library preparation and sequencing

Total RNA from *Cx*. *pipiens* samples was assayed for quantity and quality using Qubit® RNA HS Assay (Life Technologies) and RNA 6000 Nano Assay on a Bioanalyzer 2100 (Agilent).

The RNASeq libraries were prepared from total RNA using the TruSeq™ RNA Sample Prep Kit v2 (Illumina Inc., Rev. E, October 2013). Briefly, after poly-A based mRNA enrichment with oligo-dT magnetic beads of at least 0.52 μg of total RNA as input material, the mRNA was fragmented to 80-250 nt, with the major peak at 130 nt. The second strand cDNA synthesis was performed in the presence of dUTP instead of dTTP, to achieve the strand specificity. The blunt-ended double stranded cDNA was 3´adenylated and Illumina indexed adapters were ligated. The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay.

The libraries were sequenced on HiSeq2000 (Illumina, Inc) in paired-end mode with a read length of 2x76 bp using the TruSeq SBS Kit v4 (Illumina). Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 1.18.66.3) and followed by generation of FASTQ sequence files by CASAVA.

De novo transcriptome assembly and annotation

Transcriptome assemblies were performed for each time point (2 hpe, 3 dpe or 14 dpe) with Trinity v2.2.0 (Grabherr et al. 2011), with read trimming and normalization options activated. Afterwards, the Rapclust (Trapnell et al. 2013) approach was followed on each of the Trinity assemblies in order to reduce redundancy before merging the three resulting transcriptomes together with Rapclust. In this process, a pseudoalignment is first performed with Sailfish v0.10.0 (Li et al. 2010) and then Rapclust v0.1 is used to cluster the assembled sequences into contained isoforms, in order to reduce redundancy and

to cluster together all the isoforms that are likely to belong to the same gene.

After obtaining the reference transcriptome, Open Reading Frames were annotated in the assembled transcripts with Transdecoder (Haas et al. 2013). Next, we used Trinotate v3.0.1 (Bryant et al. 2017) to functionally annotate the protein-coding transcripts. First, BLAST (Altschul et al. 1990) searches were performed against the Swiss-prot database (last accessed Nov 2016). Moreover, the program HMMER and Signalp (Petersen et al. 2011) were also used to detect protein domains in the predicted ORFs. Finally, the outputs of all these programs were combined into an SQL database by using Trinotate.

Transcript quantification and differential expression analysis

RNA-seq reads were mapped against the *de novo* assembled transcriptome with the program rsem-extract-reference from RSEM (Li and Dewey 2011) using STAR (Dobin et al. 2013) indices and the option ***-transcript-to-gene-map*** to map transcripts (contigs) to genes (clusters). Genes were quantified with RSEM using our custom gene annotation. As exploratory analysis, a principal component analysis with the top 500 most variable genes was performed with the 'prcomp' function and 'ggplot2'. Differential expression analysis was performed with DESeq2 (Love et al. 2014). Genes with adjusted P-value <0.05 were considered significant. Gene ontology enrichment of the differentially expressed genes was performed with TopGO (Alexa A 2019) with a cut-off of 'classicFisher' P-value <0.05.

The workflow of the experimental infection in *Cx. pipiens* mosquitoes exposed to RVFV and transcriptome analysis is shown in the Fig 1.

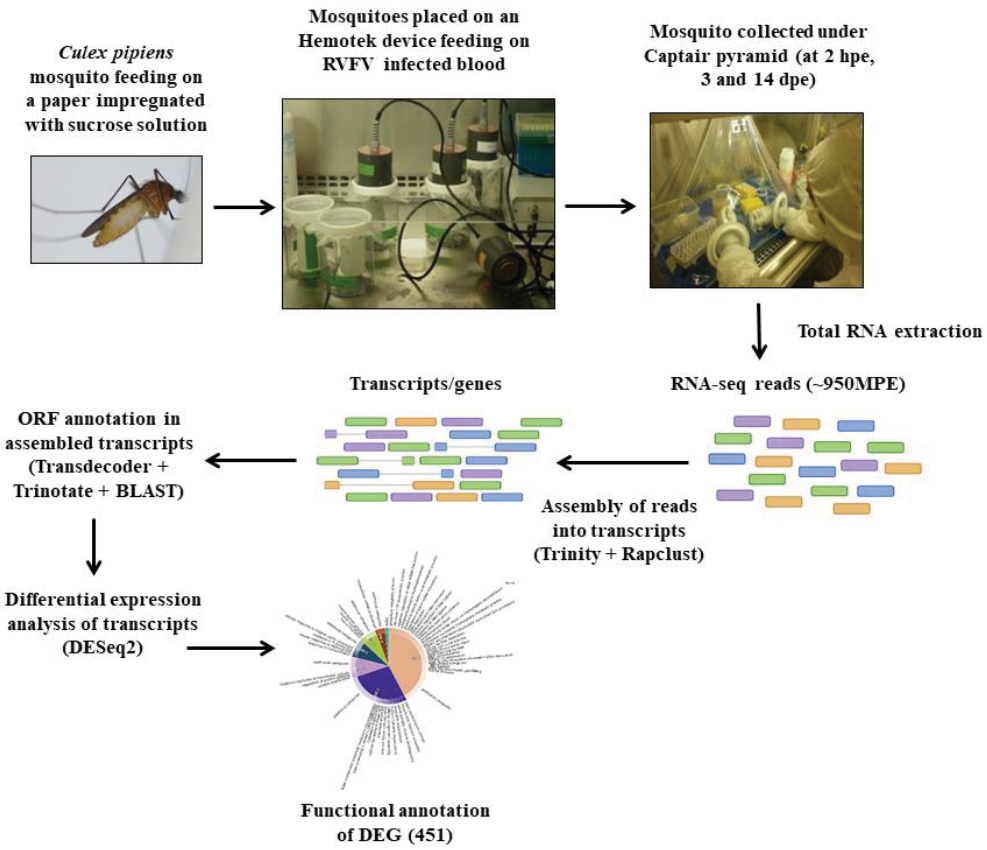

Figure 1. The workflow of the experimental infection in *Cx*. *pipiens* mosquitoes exposed to RVFV and transcriptome analysis.

Identification of differentially expressed genes related to immunity

Differentially expressed genes (hereafter DEG) coding for proteins involved in mosquito innate immunity were identified by screening two databases. First, a local database of *Cx*. *quinquefasciatus* proteins involved in immunity was created by retrieving the *ad hoc* entries from the ImmuneDB data base (Waterhouse et al. 2007) for mosquito species. BLASTX BioEdit (Altschul et al. 1990, Hall 1999) was used to compare *Cx*. *quinquefasciatus* immunity-related proteins with our database of DEG of *Cx*. *pipiens*. Second, *Cx*. *pipiens* DEG related to antiviral/immunity responses were retrieved from our *Cx*. *pipiens de*

novo assembled transcriptome annotated with the Gene Ontology database. All *Cx. pipiens* DEG identified by both methods were further curated by performing a BLASTX search of the nr protein database at the National Center for Biology Information (NCBI). The online NCBI BLASTX search also provided information on conserved protein domains by screening the CDD and pFAM databases for homologies (Finn et al. 2016) and UniProt was used to obtain information about the protein characteristics (The UniProt Consortium 2017).

Accession numbers:

The transcriptome assembly has been deposited in the European Nucleotide Archive (ENA) under the project accession number PRJEB35657 and the RNA-seq expression was submitted to the Gene Expression Omnibus (GEO) database with the accession number GSE142339.

RESULTS

RVFV infection in *Culex pipiens* mosquitoes

The three RVFV pools were positive for RVFV by RT-qPCR at the three selected time points (2 hpe, 3 dpe and 14 dpe) of study. As expected, all the control pools were negative by RT-qPCR (Table 1). These results demonstrated that each pool had at least one RVFV infected mosquito female after exposure to infectious blood and that the infection was sustained for the entire 14 days of the experiment (with the exception of RVFV pool 3 at 14 dpe).

Table 1. Viral load (Ct values) in mosquito pools tested by RT-qPCR specific to RVFV. Pools were composed of four mosquito females.

	Mosquito pool	2 hpe	3 dpe	14 dpe
Control	1	0	0	0
	2	0	0	0
	3	0	0	0
RVFV	1	23.13	27.09	24.3
	2	26.15	28.84	25.32
	3	21.9	25.47	33.88

Transcriptome *de novo* assembly

To obtain a good reference transcriptome assembly to be used in further analysis we first assembled the reads that belonged to each time-point independently and then clustered all the transcriptomes. A total of approximately 950 million reads were detected. The final assembly is composed of 355,773 assembled contigs with a contig N50 of 1716 nt. The transcripts clustered into 106,771 clusters, hereafter referred to and treated as "genes", although the number is inflated due to the usual transcript fragmentation and duplication exhibited by transcriptome assemblies. Finally, 46,635 contigs are considered protein-coding. The gene completeness of the reference transcriptome was estimated with Benchmarking Universal Single-Copy Orthologs (BUSCO) v1.1b1 (Simão et al. 2015), using an arthropod specific dataset made of 2675 genes. According to BUSCO, our reference transcriptome has 92% of complete genes, 5.1% fragmented genes and only a 2.6% of missing genes. In total, 141,669 transcripts have been annotated as protein coding, of which 113,513 (80%) have been functionally annotated with protein descriptions and 97,283 (69%) with gene ontology (GO) terms.

Comparisons of transcripts accumulation level in RVFV infected and non-infected mosquitoes

The abundance of genes in RVFV-infected *versus* non-infected *Cx. pipiens* mosquitoes were evaluated for each of the three different time points (2 hpe, 3 dpe and 14 dpe). Genes with adjusted p-value ≤ 0.05

and Fold Change (FC) ≥ + 1.5 or ≤ - 1.5 were considered significantly up-regulated or down-regulated. A total of 451 DEG were identified (Fig 2 and S1 Table) and the top 50 DEG are represented in S1-S3 Fig. From the 451 DEG, 176 genes were up-regulated and 263 were down-regulated in RVFV infected mosquitoes. In addition, six DEG were up or down-regulated depending on the time-point. In infected mosquitoes, 122, 33 and 27 genes were found to be significantly up-regulated at 2 hpe, 3 dpe and 14 dpe, respectively. Conversely, the exposure to RVFV resulted in the down-regulation of 217, 27 and 31 genes at 2 hpe, 3 dpe and 14 dpe, respectively. When compared to non-infected mosquitoes, most of the DEG (336 genes) were transcriptionally altered at the earliest time point (2 hpe) (Fig 2).

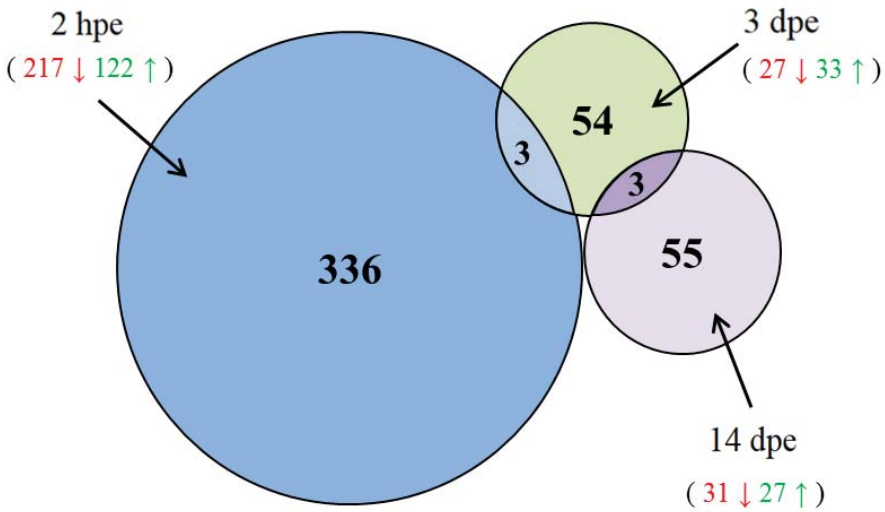

Fig 2. The Venn diagram represents the number of genes differentially expressed in response to RVFV infection at different time-points (2 hpe, 3 dpe and 14 dpe). In red, the number of down regulated genes, in green the number of up regulated genes.

Functional enrichment of the differentially expressed genes

A functional enrichment analysis of the DEG between the RVFV and control groups was performed using TopGO (Gotz et al. 2008) (S2 – S3 Tables) and Revigo (Supek et al. 2011) was used to make the charts of the most representative GO categories at each time point tested (S1 –

S3 Fig). At all-time points evaluated, the GO categories within biological processes related to cellular and metabolic mechanisms were the most affected categories. Within down-regulated DEG, the subcategories related to cellular process, cellular component organization or biogenesis, cellular metabolic process, immune system, multi-organism process or developmental process were more prominent. However, the response to stimulus, cell death, localization, immune system process and developmental process were the most represented GO subcategories within up-regulated DEG.

Out of the 451 DEG, a total of 297 (65.9 %) genes were similar to proteins with a functional annotation in the nr NCBI database, as determined by BLASTX; 22.1 % were annotated as conserved or hypothetical protein, and 12% did not match with the nr NCBI data base.

Functional prediction of differentially expressed genes related to immunity in response to RVFV infection

A total of 20 DEG were similar to genes involved in the *Cx. quinquefasciatus* mosquito immune system as assessed by the ImmunoDB database. Additionally, another 28 DEG involved in immunity processes were identified using the GO database. Thirteen genes were determined by using both methods. In total, 48 DEG related to antiviral and immune system functions were identified and analyzed in depth (Tables 2, 3 and 4).

- *DEG at 2 hpe to RVFV.*

A total of 16 DEG related to immunity were down-regulated at 2 hpe (Table 2). These included genes encoding two antimicrobial peptides (AMP) (Cpip1A009744 and Cpip1A014968), one C-type lectin (CTL) (Cpip1A029893), one leucine-rich repeats (LRR) protein (Cpip1A005512), three small regulatory RNA pathway (SRRPs) members (Cpip1A091918, Cpip1A031059 and Cpip1A025203), two

proteins involved in RNAi pathway (Cpip1A088100 and Cpip1A027127), two proteins implicated in the proteasome assembly pathway (Cpip1A057434 and Cpip1A053625), one protein related to the ubiquitination pathway (Cpip1A079710), and one protein involved in apoptotic process (Cpip1A073928). Finally, one nuclear pore complex protein Nup155 (Cpip1A048037) (Le Sage and Mouland 2013), and two CTP synthases (Cpip1A002183 and Cpip1A075565) (Martin et al. 2014), similar to genes putatively related to antiviral/immune response processes, and with no previous description in insects were identified.

A total of 18 DEG related to immunity were up-regulated at 2 hpe. Four of their putative protein products had homology with three members of myeloid differentiation 2-related lipid recognition (MLs) receptors (MD2-like) (Cpip1A012593, Cpip1A019311 and Cpip1A039204) and one member of fibrinogen-related proteins (FREPs) (Cpip1A010903). Whereas MD2-like proteins are associated with the Toll pathway, FREPs, which are involved in pathogen recognition, have not been linked to any specific immune pathway (Angleró-Rodríguez et al. 2017). Moreover, DEG coding for two fork head (FKH) transcription factors involved in AMP regulation via the TOR pathway (Cpip1A000929 and Cpip1A014760), one lipin-3 (Cpip1A058921) involved in PI3K-Akt-TOR signaling pathway, one protein related to proteasome assembly (Cpip1A101513) and three proteins involved in apoptotic process (Cpip1A000671, Cpip1A035296, and Cpip1A018924) were also upregulated. The gene Cpip1A018924 had a best match with *Cx. quinquefasciatus* Croquemort (SCRBQ3), a scavenger receptor B member of a multigene family harboring the CD36 domain. When screening the whole *Cx. pipiens* transcriptome, six other genes homologous to Cpip1A018924 were found; all coded for the CD36 domain (result not shown). Three additional up-regulated DEG were identified as trypsins (Cpip1A06169, Cpip1A037043 and Cpip1A083737). Finally, the putative products of Cpip1A054937, Cpip1A065392, Cpip1A059920

and Cpip1A087985, which are homologous to proteins involved in diverse responses to viral and antimicrobial infections were also up-regulated.

Table 2. DEG related to the immune infection-defense response at 2 hpe.

Cx. pipiens SEQ ID	Padj	FC	Sequence length	Blastx results: Best match [Species]*	Conserved domain	E value	IDB.ID accession number	Predicted process/activity	References
Cpip1A029893	4.12E-04	-53.8	581 NT	XP_001842114.1 serine protease [*Culex quinquefasciatus*]**	Lectin_C (pfam00059); CLECT (cd00037)	1.48E-08; 5.21E-10	Cpip: CTL24	Pathogen recognition. C-type lectin (CTL24)	(Cheng et al. 2010, Liu et al. 2014, Xia et al. 2017)
Cpip1A025203	6.06E-04	-39.5	1877 NT	XP_001846442.1 tar RNA binding protein [*Culex quinquefasciatus*]	No pfam; DSRM (cd00048)	3.82E-05	Cpip: SRRP7	miRNA pathway; SRRPs: Small Regulatory RNA Pathway Members (LOQS (R3D1))	(Haac et al. 2015)
Cpip1A088100	2.34E-03	-23.9	512 NT	XP_001854407.1 gawky *[Culex quinquefasciatus]*	Not found		-	RNAi	(Li et al. 2013)
Cpip1A057434	2.00E-91	-19.26	674 NT	XP_001846424.1 proteasome maturation protein [*Culex quinquefasciatus*]	UMP1 (pfam05348)	1.09E-19	-	Proteasome assembly;	(Matias et al. 2010, Choy et al. 2015)
Cpip1A048037	1.31E-02	-17.6	1416 NT	XP_001842183.1 nuclear pore complex protein Nup155 [*Culex quinquefasciatus*]	nucleoporin N superfamily (pfam08801)	2.51E-38	-	Nuclear transport of transcription factors	(Le Sage and Mouland 2013)
Cpip1A079710	1.59E-05	-14.1	2229 NT	XP_001866714.1 UBX domain-containing protein 8 [*Culex quinquefasciatus*]	UBX (pfam00789); UAS_ETEA (cd02991); Faf1_like2_UBX (cd01774); UBA_FAF2 (cd14414)	0.000121; 7.96E-41; 2.59E-23; 1.08E-10	-	Ubiquitination pathway	(Buchberger 2002)
Cpip1A031059	8.51E-03	-12.3	3014 NT	XP_001843599.1 ribonuclease 3 [*Culex quinquefasciatus*]	Ribonuclease_3 (pfam00636); RIBOc (cd00593)	4.41E-16; 5.19E-20	Cpip: SRRP6	miRNA pathway; SRRPs: Small Regulatory RNA Pathway Members (DROSHA)	(Shapiro et al. 2014)

Cpip1A009744	1.54E-02	-11.6	574 NT	XP_001842945.1 defensin-A [*Culex quinquefasciatus*]	Defensin_2 (pfam01097)	5.39E-12	Cpip: AMP6	Toll/ IMD Pathways. AMP: Antimicrobial Peptide (DEFA)	(Zhao et al. 2018)
Cpip1A014968	4.31E-02	-11.2	487 NT	AAEL003832-PA [*Aedes aegypti*]	Defensin_2 (pfam01097)	1.12E-06	Cpip: AMP6	Toll/ IMD Pathways. AMP: Antimicrobial Peptide (DEFC)	(Zhao et al. 2018)
Cpip1A005512	3.00E-69	-10.4	1499 NT	XP_021702392.1 leucine-rich repeats and immunoglobulin-like domains protein 3 isoform X2 [*Aedes aegypti*]	LRR_8 (pfam13855); LRR_RI (cd00116)	2.54E-05; 5.67E-04	-	Toll/ IMD Pathways/ Pattern Recognition Receptor	(Cirimotich et al. 2010, Zhao et al. 2019)
Cpip1A075565	5.55E-06	-8.3	3034 NT	XP_001866729.1 CTP synthase [*Culex quinquefasciatus*]	CTP_synth_N (pfam06418); CTGs (cd03113)	4.91E-85; 2.45E-80	-	Sustainability of activated immune cell proliferation	(Martin et al. 2014)
Cpip1A027127	2.00E-169	-8.1	1378 NT	XP_001846690.1 tnf receptor associated factor [*Culex quinquefasciatus*]	zf-TRAF (pfam02176)	3.87e-10	-	RNAi; Apoptosis	(Paradkar et al. 2014, Dhillon et al. 2019)
Cpip1A053625	1.00E-175	-5.0	1238 NT	XP_001842520.1 proteasome subunit alpha type 1 [*Culex quinquefasciatus*]	Proteasome (pfam00227); proteasome_alpha_type_1 (cd03749)	9.77e-37; 8.16e-109	-	Proteasome assembly	(Gupta et al. 2018)
Cpip1A073928	0.00E+00	-4.6	2802 NT	XP_001846422.1 apoptosis inhibitor [*Culex quinquefasciatus*]	API5 (pfam05918)	1.89E-130	-	Apoptosis	(Mayank et al. 2015)
Cpip1A002183	8.04E-03	-4.5	2885 NT	XP_001866729.1 CTP synthase [*Culex quinquefasciatus*]	CTP_synth_N (pfam06418); CTGs (cd03113)	2.54E-85; 1.35E-80	-	Sustainability of activated immune cell proliferation	(Martin et al. 2014)
Cpip1A091918	5.20E-03	-3.4	2066 NT	XP_001862491.1 piwi [*Culex quinquefasciatus*]	Piwi (pfam02171); Piwi_piwi-like_Euk (cd04658)	2.83E-58; 1.55E-124	Cpip: SRRP33	RNAi; SRRPs: Small Regulatory RNA Pathway Members (PIWI4 transcript 1)	(Dietrich et al. 2017, Varjak et al. 2017)

Cpip1A087985	2.99E-02	4.0	2946 NT	KXJ72819.1 hypothetical protein RP20_CCG017164 [*Aedes albopictus*]	PBD (pfam00786); Pkinase (pfam00069); STKc_PAK3 (cd06656)	4.91E-11; 3.92E-47; 7.99E-116	-	Not found immunity function reference. PKC-dependent signaling is a negative regulator of the midgut epithelial barrier	(Pakpour et al. 2013)
Cpip1A058921	2.23E-02	8.3	3952 NT	XP_001843328.1 lipin-3 [*Culex quinquefasciatus*]	LNS2 (pfam08235); Lipin_N (pfam04571); Lipin_mid (pfam16876)	3.40E-100; 6.24E-43; 2.32E-20	-	PI3K-Akt-TOR signaling pathway	(Schmitt et al. 2015)
Cpip1A000671	2.78E-05	10.3	1398 NT	XP_001844338.1 conserved hypothetical protein [*Culex quinquefasciatus*]	No pfam; P53 (cd08367)	2.70E-21	-	Apoptosis (P53)	(Popova et al. 2010, Chen et al. 2017)
Cpip1A039204	1.99E-02	11.1	689 NT	XP_001844330.1 conserved hypothetical protein [*Culex quinquefasciatus*]	E1_DerP2_DerF2 (pfam02221); Npc2_like (cd00916)	3.13E-13; 8.65E-19	Cpip:ML12	Toll/ IMD pathways; MLs: MD2-Like Receptors (ML22)	(Inohara and Nuez 2002, Dong et al. 2006, Shi et al. 2012, Jupatanakul et al. 2014)
Cpip1A010903	3.96E-02	15.5	1221 NT	XP_001862507.1 ficolin-2 [*Culex quinquefasciatus*]	Fibrinogen_C (pfam00147); FReD (cd00087)	8.77E-28; 5.02E-43	Cpip: FREP46	Pattern Recognition Receptor	(Dong and Dimopoulos 2009)
Cpip1A101513	8.00E-92	18.5	661 NT	XP_001846424.1 proteasome maturation protein [*Culex quinquefasciatus*]	UMP1 (pfam05348)	8.49E-20	-	Proteasome assembly; Apoptosis	(Matias et al. 2010, Choy et al. 2015)
Cpip1A065392	1.83E-02	25.4	1135 NT	XP_001843329.1 conserved hypothetical protein [*Culex quinquefasciatus*]	Prefoldin (pfam02996); Prefoldin_alpha (cd00584)	1.21E-06; 6.11E-05	-	RNAi; Virus infection control	(Zhang et al. 2015)

Cpip1A059920	2.64E-02	25.7	1673 NT	XP_001841680.1 chitinase [*Culex quinquefasciatus*]	Glyco_hydro_18 (pfam00704); CBM_14 (pfam01607); GH18_chitolectin _chitotriosidase (cd02872)	2.79E-64; 1.13E-10; 7.50E-127	-		(Kucerova et al. 2016)
Cpip1A019311	3.37E-02	27.5	668 NT	XP_001844320.1 MPA2 allergen [*Culex quinquefasciatus*]	E1_DerP2_DerF2 (pfam02221); Npc2_like (cd00916)	2.99E-19; 2.94E-26	Cpip:ML8	Toll/ IMD pathways; MLs: MD2-Like Receptors (ML6)	(Inohara and Nuez 2002, Dong et al. 2006, Shi et al. 2012, Jupatanakul et al. 2014)
Cpip1A012593	5.05E-03	27.6	933 NT	XP_001844321.1 conserved hypothetical protein [*Culex quinquefasciatus*]	E1_DerP2_DerF2 (pfam02221); Npc2_like (cd00916)	3.72E-11; 5.23E-09	Cpip:ML16	Toll/ IMD pathways; MLs: MD2-Like Receptors (ML26)	(Inohara and Nuez 2002, Dong et al. 2006, Shi et al. 2012, Jupatanakul et al. 2014)
Cpip1A000929	4.04E-02	28.4	3386 NT	XP_001843517.1 fork head [*Culex quinquefasciatus*]	Forkhead (pfam00250); FH (cd00059)	1.34E-29; 3.47E-31	-	TOR (target of rapamycin); Toll and IMD pathways	(Varma et al. 2014)
Cpip1A054937	3.90E-02	31.3	1894 NT	XP_001865865.1 tetraspanin 97e [*Culex quinquefasciatus*]	Not found			Virus cell-cell spreading. Apoptosis	(Luo et al. 2013, Yang et al. 2015)
Cpip1A037043	1.68E-02	37.3	1131 NT	XP_001846625.1 serine protease1/2 [*Culex quinquefasciatus*]	Trypsin (pfam00089); Tryp_SPc (cd00190)	6.46e-46; 2.42e-50	-	Blood digestion	(Molina-Cruz et al. 2005)
Cpip1A035296	1.43E-05	37.3	1509 NT	XP_001844339.1 conserved hypothetical protein [*Culex quinquefasciatus*]	No pfam; P53 (cd08367)	3.22E-21	-	Apoptosis (P53)	(Popova et al. 2010, Chen et

									al. 2017)
Cpip1A014760	7.78E-03	48.3	4632 NT	XP_001843517.1 fork head [*Culex quinquefasciatus*]	Forkhead (pfam00250); FH (cd00059)	1.85E-29; 4.78E-31	-	TOR (target of rapamycin); Toll and IMD pathways	(Varma et al. 2014)
Cpip1A061691	4.61E-06	74.2	939 NT	XP_001846625.1 serine protease1/2 [*Culex quinquefasciatus*]	Trypsin (pfam00089); Tryp_SPc (cd00190)	1.66e-46; 2.04e-51	-	Blood digestion	(Molina-Cruz et al. 2005)
Cpip1A083737	2.16E-03	79.7	898 NT	XP_001849502.1 chymotrypsin-2 [*Culex quinquefasciatus*]	Trypsin (pfam00089); Tryp_SPc (cd00190)	5.28e-51; 7.84e-54	-	Blood digestion	(Molina-Cruz et al. 2005)
Cpip1A018924	2.19E-03	83.1	448 NT	XP_001862840.1 croquemort [*Culex quinquefasciatus*]	CD36 (pfam01130)	1.05E-28	Cpip:SCR17	Apoptosis/Phagocytosis. SCRs: Scavenger Receptors, Class B (SCRBQ3)	(González-Lázaro et al. 2009)

* First match resulting from the BLASTX search; FC: Fold change; -: no accession number.
**XP_001842114.1 is not a serine protease but a C-type lectin, probable annotation mistake.

- *DEG at 3 dpe to RVFV*.

A total of four DEG involved in insect immunity were downregulated at 3 dpe (Table 3): one encoding for a protein related to the ubiquitination pathway (Cpip1A095413), one related to alternative antiviral response, with a SH2_Grb2_like protein domain (Cpip1A069141) and two DEG encoding for chymotrypsin-like proteins (Cpip1A089245 and Cpip1A065153). The SH2 domains are implicated in signal transduction and the Grb2 is supposed to play a role in apoptosis (Oda et al. 2010, Marchler-Bauer et al. 2017). In the opposite direction, two DEG implicated in ubiquitination and proteasome assembly pathways were upregulated at 3 dpe (Cpip1A038039 and Cpip1A080538, respectively).

Table 3. DEG related to the immune infection-defense response at 3 dpe.

Cx. pipiens SEQ ID	Padj	FC	Sequence length	Blastx results: Best match [Species]*	Conserved domain	E value	IDB.ID accession number	Predicted process/activity	References
Cpip1A095413	4.06E-06	-153.2	1948 NT	XP_001864599.1 ubiquitin-conjugating enzyme [*Culex quinquefasciatus*]	UQ_con (pfam00179); UBCc (cd00195)	4.01E7; 2.22E-09	-	Ubiquitination pathway (E2)	(Hoffmann 1996, Gupta et al. 2018)
Cpip1A089245	1.15E-02	-81.9	994 NT	AAL78376.1 putative chymotrypsin-like protein [*Culex pipiens pallens*]	Trypsin (pfam00089); Tryp_SPc (cd00190)	8.84E-29; 0E	-	Blood digestion	(Molina-Cruz et al. 2005)
Cpip1A065153	4.18E-02	-17.6	1354 NT	AAX59051.1 chymotrypsin-like [*Culex pipiens*]	Trypsin (pfam00089); Tryp_SPc (cd00190)	1.62E-30; 5.74E-29	-	Blood digestion	(Molina-Cruz et al. 2005)
Cpip1A069141	3.37E-02	-5.2	967 NT	XP_001862989.1 conserved hypothetical protein *[Culex quinquefasciatus*]	No pfam; SH2_Grb2_like (cd09941)	6.56E-05	-	Insect cytokine signaling in hemocytes. SH2, domain-binding motifs in adaptor protein (P77).	(Oda et al. 2010, Marchler-Bauer et al. 2017)
Cpip1A038039	2.75E-02	20.9	655 NT	XP_001844072.1 mlo2 [*Culex quinquefasciatus*]	zf-UBR (pfam02207)	5.63E-05	-	Ubiquitination pathway (E3)	(Bergmann and Steller 2010, Choy et al. 2013)
Cpip1A080538	1.72E-02	27.8	839 NT	XP_001844753.1 proteasome activator complex subunit 3 [*Culex quinquefasciatus*]	PA28_beta (pfam02252); PA28_alpha (pfam02251)	1.72E-32; 2.87E-12	-	Proteasome assembly (PA28)	(Respondek et al. 2017, Gupta et al. 2018)

* First match resulting from the BLASTX search; FC: Fold change; -: no accession number

- *DEG at 14 dpe to RVFV*.

A total of six DEG related to immunity were down-regulated at 14 dpe (Table 4). These DEG included genes encoding one CTL protein (Cpip1A086629), one protein involved in the ubiquitination pathway (Cpip1A075447), two proteins related to melanization (Cpip1A099497 and Cpip1A046835), one heat shock protein 70 (Hsp70) (Cpip1A097556), which has been described previously in mosquito immune response (Pujhari et al. 2019), and finally, one protein (Cpip1A034914) that inhibits a virus sensor described in humans (Ubol et al. 2010). In the other direction, two DEG related to immunity response were up-regulated at 14 dpe. These genes encode one FREP member (Cpip1A019555) and the nuclear pore complex protein Nup88-like (Cpip1A005797) related to antiviral/immune response process in insects.

Table 4. DEG related to the immune infection-defense response at 14 dpe.

Cx. pipiens SEQ ID	Padj	FC	Sequence length	Blastx results: Best match [Species]*	Conserved domain	E value	IDB.ID accession number	Predicted process/activity	References
Cpip1A034914	3.23E-03	-102.5	1416 NT	XP_001848307.1 dihydroxyacetone kinase [*Culex quinquefasciatus*]	Dak1 (pfam02733)	5,72E-23	-	Virus sensor inhibition	(Ubol et al. 2010)
Cpip1A097556	4.71E-02	-29.5	3834 NT	XP_019527564.1 PREDICTED: heat shock 70 kDa protein 4-like isoform X2 [*Aedes albopictus*]	HSP70 (pfam00012); NAD-GH (pfam10712); HSPA4_like_NDB (cd10228)	2.33E-100; 2.58E-08; 4.11E-149	-	RNAi; Virus infection control	(Das et al. 2009, Taguwa et al. 2015, Pujhari et al. 2019)
Cpip1A099497	2.24E-02	-29.0	3556 NT	XP_001851487.1 lamin [*Culex quinquefasciatus*]	Treacle (pfam03546); Filament (pfam00038); LTD (pfam00932); Treacle (pfam03546); BAR_SNX (cd07596)	1.65E-39; 5.15E-04; 5.02E-03; 1.13E-06	-	Melanization	(Markovic et al. 2009)
Cpip1A075447	1.01E-02	-20.5	4348 NT	XP_001652595.1 cullin-3 isoform X2 [*Aedes aegypti*]	Cullin (pfam00888); Cullin_Nedd8 (pfam10557)	1.21E-168; 2.36E-17	-	Ubiquitination pathway (E3)	(Bergmann 2010, Choy et al. 2013, Paradkar et al. 2015)
Cpip1A046835	3.02E-03	-12.2	3441 NT	XP_001851487.1 lamin [*Culex quinquefasciatus*]	Treacle (pfam03546); Filament (pfam00038); BAR_SNX (cd07596); LTD (pfam00932)	4.84E-03; 1.58E-39; 1.09E-06; 4.97E-04	-	Melanization	(Markovic et al. 2009)
Cpip1A086629	2.01E-03	-10.7	301 NT	XP_001848473.1 salivary C-type lectin [*Culex quinquefasciatus*]	CLECT (cd00037)	8.03E-10	Cpip: CTL27	Pathogen recognition. C-Type Lectins (CTL27)	(Cheng et al. 2010, Liu et al. 2014, Xia et al. 2017)
Cpip1A019555	1.05E-02	23.3	1224 NT	XP_001847908.1 microfibril-associated glycoprotein 4 [*Culex quinquefasciatus*]	Fibrinogen_C (pfam00147); FReD (cd00087)	4.42E-54; 1.33E-71	Cpip: FREP18	FREPs: Fibrinogen-Related Proteins (FREP18). Pattern Recognition Receptor	(Dong and Dimopoulos 2009)

Cpip1A005797	4.40E-03	78.9	1051 NT	XP_019551901.1 PREDICTED: nuclear pore complex protein Nup88-like [*Aedes albopictus*]	Nup88 (pfam10168)	2.94E-52	-	Nuclear transport of transcription factors; Toll pathway	(Wiermer et al. 2010)

* First match resulting from the BLASTX search; FC: Fold change; -: no accession number

Evolution of the immune response during the course of RVFV infection in *Culex pipiens* mosquitoes

All of the above detailed DEG during RVFV-infection in mosquitoes were involved in the conventional immune Toll/IMD pathways, RNAi mechanism, the ubiquitination pathway, apoptosis and other inducible antiviral immune genes (Fig 3). Most of the transcriptional changes related to immunity occurred at the early RVFV-infection stage (2 hpe) in all the defense mechanisms. It is worth mentioning that all DEG related to the RNAi system were downregulated at this time point (Fig 3).

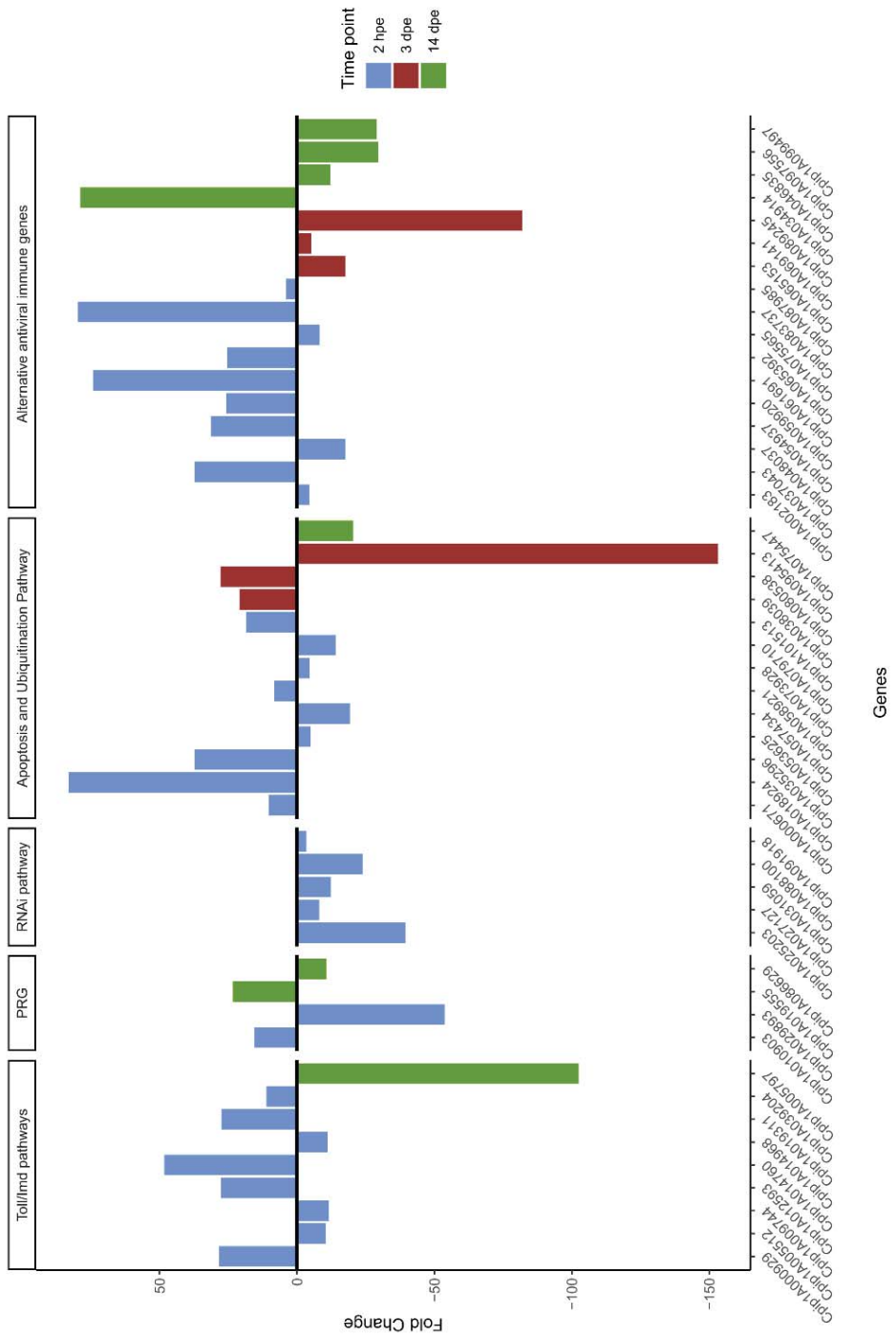

Figure 3. Evolution of the immune infection-response during RVFV infection in *Cx. pipiens* mosquitoes. The immune DEG altered at 2 hpe, 3 and 14 dpe are represented in blue, red and green colors, respectively. PRG: Pathogen recognition genes.

DISCUSSION

The full genome sequence of *Cx. pipiens* is not available, therefore, in the present study we constructed a *de novo* transcriptome using adult *Cx. pipiens* females fed on RVFV infectious or non-infectious blood allowing us to perform an accurate comparative transcriptome analysis between RVFV exposed and unexposed female mosquitoes.

Most of the transcriptomic alterations were found within 2 hpe, at an early stage of infection. Most of the DEG identified at this stage were enriched in GO categories corresponding to metabolic and cellular processes most likely at a time when the mosquito midgut is primed with RVFV. These results are in line with previous findings obtained with ZIKV- infected *Ae. aegypti* mosquitoes (Etebari et al. 2017), midguts of DENV-infected *Ae. aegypti,* and midguts of CHIKV-infected *Ae. albopictus* (Chauhan et al. 2012, Vedururu et al. 2019), where most of the altered genes were detected at the beginning of the infection. Among all DEG, we focused specifically on genes putatively involved in the mosquito immune system since antiviral mechanisms very often do not entirely clear the virus but allow the establishment of a persistent viral infection in vectors. In the present study, 48 DEG belonging to several infection defense gene families have been identified upon RVFV exposure. These genes are related to Toll, IMD and RNAi pathways as well as infection-responsive pathogen recognition genes (PRG), apoptotic processes, and other alternative inducible antiviral immune genes.

RVFV alters *Cx. pipiens* immune responses, mainly Toll and IMD pathways

Genes belonging to the Toll and IMD pathways coding for defensin-A (DEFA), defensin-C (DEFC) and ML proteins (ML6, ML22, ML26) were significantly positively or negatively altered throughout the course of RVFV infection in *Cx. pipiens* mosquitoes. Conversely, none of the DEG were related to the JAK/STAT pathway, contrary to what

has been reported in several studies performed in *Ae*. *aegypti* with other arboviruses (ZIKV and DENV) (Souza-Neto et al. 2009, Angleró-Rodríguez et al. 2017) or *Cx*. *quinquefasciatus* with WNV (Paradkar et al. 2012). However, our results agree with two previous studies performed in *Ae*. *albopictus* infected with CHIKV (Vedururu et al. 2019) and in *Ae*. *aegypti* infected with ZIKV (Etebari et al. 2017) where the JAK/STAT pathway was not involved in virus-mosquito interactions. These apparent contradictory data suggest that the implication of the JAK/STAT pathway in arbovirus defense depends on the pairing combination of mosquito and arbovirus and is influenced by the viral genotype - host genotype interaction.

The AMPs are induced following activation of Toll, IMD and JAK/STAT pathways. They are potent immune effectors whose ultimate function is to clear pathogens (Lee et al. 2019). Their mode of action is better understood for bacteria and fungi, but how they interact with viruses remains unclear. Their modes of action vary among mosquito species depending on the type of pathogen that triggers the response (Lee et al. 2019). In the present study, two AMPs, *defensin-A* (*DEFA*) and *defensin-C* (*DEFC*), were under-expressed in RVFV exposed females at the beginning of the infection (at 2 hpe). Defensins (Zhao et al. 2018) are mainly known to disrupt the membrane permeability of Gram- positive bacteria (Bartholomay et al. 2004). In contrast with our results, a previous study performed in *Ae*. *aegypti* infected with ZIKV or CHIKV showed that *DEFA* and *DEFC* were over-expressed at 3 hpe to both viruses. However, at 10 dpe, in comparison with uninfected controls, *DEFA* and *DEFC* were significantly downregulated in CHIKV and upregulated in ZIKV infected females, respectively (Zhao et al. 2018). Regulation of defensins seems to vary depending on the virus ingested and the progression of the infection. RVFV probably modifies the expression of both defensins in order to allow initiation of the infection in *Cx*. *pipiens*, but returns to steady state levels when the virus crosses the midgut barrier. According to results obtained in *Drosophila*, up-

regulation of the transcription factor FKH, which acts downstream of TOR signaling, inhibits production of defensins while increasing other AMPs mainly diptericin and metchnikowin (Varma et al. 2014). However, no other AMP genes were affected in RVFV-infected females. Furthermore, members of the ML family (*ML22*, *ML6* and *ML26* genes) were up-regulated in RVFV-infected mosquitoes at 2 hpe. *ML* genes, which encode lipid-binding proteins, have been implicated in host-pathogen interactions. Currently, the role of the ML protein family in mosquito immunity is still not well understood. *Anopheles gambiae* ML1 (AgMDL1) has been associated with immune responses against both bacteria and *Plasmodium falciparum* infections (Dong et al. 2006) and a *Drosophila* ML protein has been implicated in activation of the IMD pathway against bacterial infection in S2 cells (Shi et al. 2012). However, Jupatanakul *et al*. (2014) showed that when the *ML33* gene is silenced in DENV infected *Ae*. *aegypti* females, virus titers in midguts are reduced, indicating that the *ML33* gene facilitates viral infection in the mosquito midgut (Jupatanakul et al. 2014b). Our results may also suggest that *ML22*, *ML6* and *ML26* could act as agonists of RVFV infection in *Cx*. *pipiens* females since they were up-regulated at an early stage of infection. Also, a gene coding for a LRR-containing domain protein was down-regulated in RVFV infected females at 2 hpe. Beside the fact that LRR immune proteins (LRIMs) play a key role against *Plasmodium* infection in mosquitoes (Cirimotich et al. 2010), a recent study by Zhao *et al*. (2019) has shown that the expression of *AaeLRIM1* and *AaeAPL1* (coding for proteins containing LRR) were also downregulated in ZIKV infected *Ae*. *aegypti* at 7 dpi (Zhao et al. 2019), which is in agreement with our results. Currently, it is unknown how these proteins affect the outcome of arbovirus infection in mosquitoes (Zhao et al. 2019) and functional studies are required to better understand the role of LRR immune proteins against arboviruses.

Additionally, the nuclear pore complex protein Nup88 seems to promote nuclear retention of immune regulators, such as the

transcription factors Dif and Dorsal, contributing to an efficient control of the immune response when the Toll pathway is engaged upon microbial infection (Wiermer et al. 2010). However, at this time point (14 dpe) no other members of the Toll pathway were altered in *Cx. pipiens* RVFV-infected females.

RVFV alters *Cx. pipiens* infection-responsive pathogen recognition genes

The FREPs and CTLs have not been directly classified within any of the conventional immune pathways but are described as infection-responsive pathogen recognition proteins and as such play a role in mosquito immunity response (Dong and Dimopoulos 2009, Angleró-Rodríguez et al. 2017). A study performed in *Ae. aegypti* mosquitoes infected with ZIKV showed that the *FREP37* gene was downregulated at 7 dpe (Angleró-Rodríguez et al. 2017). Our study indicates over-expression of *FREP46* and *FREP18* genes at 2 hpe and 14 dpe, respectively. However, we cannot assess whether the regulation of FREP genes is affected one week after infection since no intermediary time point was performed between 3 dpe and 14 dpe. In addition, the *CTL24* and *CTL27* genes were depleted in RVFV-infected females at 2 hpe and at 14 dpe. The CTLs play an essential role as pattern recognition receptors to mediate immune responses, such as phagocytosis and melanization (Mayer et al. 2017, Xia et al. 2017). A previous study demonstrated that *Ae. aegypti* (*mosGCTL-1*) and *Cx. quinquefasciatus* (*Culex mosGCTL-1*) CTLs were overexpressed and facilitated WNV infection in adult mosquitoes at six days post-inoculation (dpi) (Cheng et al. 2010). The same results were obtained on DENV infected *Ae. aegypti* mosquitoes (Liu et al. 2014). Accordingly, our results suggest that CTL24 and CTL27 may mitigate the RVFV replication in *Cx. pipiens* mosquitoes at different stages of the infection.

Early depletion of the RNAi pathway by RVFV infection

The RNAi pathway seems suppressed at the onset of the RVFV infection in *Cx*. *pipiens* since crucial SRRP members such as *Piwi4*, *Loquacious* (Loqs or R3D1), *ribonuclease 3* (Drosha), *tnf receptor associated factor* (zf-TRAF) and *Gawky* were down-regulated at 2 hpe.

The Piwi4 protein, which belongs to the PIWI family, interacts with key components of the siRNA (Ago2 and Dicer-2 proteins) and piRNA (Ago3, Piwi5 and Piwi6 proteins) pathways, although it has been proposed that its antiviral role is independent of either pathway (Varjak et al. 2017). Piwi4 has been involved in the control of Semliki Forest virus and Bunyamwera virus in *Ae*. *aegypti* and *Ae*. *albopictus*-infected mosquito cells, respectively (Schnettler et al. 2013, Dietrich, Shi, et al. 2017). Moreover, Dietrich *et al*. (2017) also demonstrated the antiviral activity of Piwi4 in Aag2 cells infected with RVFV since its silencing led to an increase of viral replication (Dietrich, Jansen, et al. 2017). According to these studies, the depletion of Piwi4 mRNA could indicate that RVFV suppresses the piRNA pathway allowing its replication in *Cx*. *pipiens* mosquitoes at an early stage of infection. Additionally, the mRNA coding for the Loqs protein, which is a dsRNA-binding protein belonging to the microRNA (miRNA) pathway, was also downregulated. Haac *et al*. (2015) proposed that in dipterans, including mosquitoes, Loqs-PA and Loqs-PB isoforms are involved in the control of the miRNA pathway (Haac et al. 2015). Therefore, RVFV may also alter the miRNA pathway in *Cx*. *pipiens* via *Loqs* downregulation early after RVFV exposure.

Other components of the miRNA and RNAi pathways were downregulated: *Drosha* gene, which codes for an enzyme that generate small RNAs in cooperation with Dicer to control viral infections (Shapiro et al. 2014), and *Gawky*, which codifies for an Argonaute-associating protein whose role in the miRNA pathway repression is still not clear (Li et al. 2013). Finally, ending the list of down-regulated genes at 2 hpe, TRAFs has been implicated in the activation of the

Culex Vago protein, which is a secreted peptide with antiviral functions (Deddouche et al. 2008) in response to WNV in infected mosquito cells (Paradkar et al. 2014).

In summary, the depletion of *Piwi4*, *Loqs*, *Drosha*, *Gawky* and *TRAF*, all involved in several RNAi pathways, would suggest a severe alteration of this pathway at the onset of the RVFV infection to allow viral replication and further virus dissemination. However, in addition to the detection and quantification of siRNAs and piRNAs, further functional studies will be required to better understand the role of the RNAi pathway in RVFV-infected *Cx. pipiens* mosquitoes to evidence the presence or absence of a RVFV potential inhibitor of this pathway. Nonetheless, our results highlight the importance of early events preceding RVFV dissemination and probably the crucial role of the intestinal barrier in altering the RNAi pathway. On the other hand, transcription of genes involved in the RNAi pathway were not affected in the later sampled time points (at 3 and 14 dpe), suggesting that, once the initial RVFV infection is established, steady state levels of these genes allows proper dissemination of the virus without causing lethal tissue damages in *Cx. pipiens*.

RVFV infection alters expression of genes related to the ubiquitination pathway and apoptosis

The UPP, which recognizes and degrades polyubiquitylated proteins modified via ubiquitination, contributes to multiple biological processes including apoptosis (Gupta et al. 2018), an important immune mechanism that modulates viral infections in vertebrates and invertebrates (Clem 2016). The ubiquitin-proteasome pathway in *Drosophila* can repress the IMD pathway by degrading Relish (Rel), a *Drosophila* NF-κB-like protein (Khush et al. 2002). Furthermore, it was observed that *in vivo*, silencing of some proteasome catalytic β sub-units reduced the number of DENV infected mosquitoes, and upregulation of UPP-related genes were required in *Ae. aegypti* midguts for the production of infectious DENV (Choy et al. 2015).

Therefore, it appears that a functional proteasome is required to generate viral particles.

In the present study, at the beginning of the infection (2 hpe), three DEG and one DEG related to proteasome assembly were downregulated and upregulated respectively, in mosquitoes exposed to RVFV. Deprivation of the proteasome subunit alpha and reduced dimerization of precursor complexes containing β subunits, mediated by ubiquitin-mediated proteolysis (UMP1a) (Matias et al. 2010), combined with a deficiency of ubiquitin-activating enzymes (UBX/UBA or E1), which initiate the ubiquitination pathway cascade (Buchberger 2002), would alter the formation and function of proteasomes in *Cx. pipiens* infected by RVFV. However, the up-regulation of a gene coding for UMP1b could counterbalance the effect of UMP1a. At the present state of knowledge, it is difficult to assess if *UMP1a* and *UMP1b* are two isoforms of the same gene or the products of duplicated genes. The fact that they are oppositely regulated argues for the existence in *Cx. pipiens* of two distinct *UMP1* genes. The involvement of E1 enzymes in infectious processes has also been previously described for DENV infected *Ae. aegypti* midguts, but in this case, up-regulation of these genes probably exerted a beneficial role for the development of the virus (Choy et al. 2015).

At 3 dpe one DEG coding for one ubiquitin-conjugating enzyme (UBC) or E2, belonging to the ubiquitination pathway in *Drosophila* (Hoffmann 1996, Gupta et al. 2018), was depleted. At this time point, the ubiquitin ligase containing the UBR box (zf-UBR) or E3, intervening at the last step of the ubiquitination cascade (Bergmann 2010, Choy et al. 2013), and the proteasome activator complex subunit 3 (PA28) were enriched in RVFV infected females. As far as we know, neither of these two proteins have thus far been implicated in arbovirus infectious processes in dipterans. However, in mammals, PA28, a regulatory complex protein induced by IFN-γ (Respondek et al. 2017), has been shown to interfere with the replication of coxsackievirus B3 in

human and murine cells by suppressing viral replication (Respondek et al. 2017). Once the RVFV infection was established in *Cx*. *pipiens* mosquitoes at 14 dpe, we found that a *cullin* gene, belonging to the ubiquitin ligase family E3, was down-regulated. Paradkar *et al*. (2015) demonstrated that in *Cx*. *quinquefasciatus* the *cullin* gene product Cul4, a member of the above ubiquitin ligase family (E3), is a pro-viral protein since silencing the corresponding gene resulted in decreased WNV titer in the mosquito saliva (Paradkar et al. 2015). Overall, results obtained in this study suggest that the UPP and the proteasome could affect the developmental cycle of RVFV. Six DEG would inhibit virus replication while one up-regulated DEG (the ubiquitin ligase of the E3 family) could enhance it. However, experimental proof is needed to ascertain the role of the above-mentioned genes during RVFV infection.

As mentioned above, the UPP and proteasome are tightly related to the apoptotic process. As soon as 2 hpe, RVFV-infected females exhibited five DEG related to apoptosis. The gene coding for the inhibitor of apoptosis API5 was down-regulated, while those coding for lipin-3, p53-1, p53-2 and the scavenger receptor B Croquemort were up-regulated. Suppression of API5 in mammalian cells during Influenza A infection led to apoptosis and increased viral production (Mayank et al. 2015). Furthermore, in *Drosophila*, the reduction of *Lipin* expression resulted in a drawdown of the PI3K-Akt-TOR signaling pathway (Schmitt et al. 2015), with which viruses interact to modify cellular functions (Patel and Hardy 2012). Additionally, the PI3K-Akt-TOR signaling pathway is involved in the control of SINV replication in mosquito cells, suggesting that it is a proviral pathway (Patel and Hardy 2012). Thereby, according to it, our results would indicate that lipin-3 might promote RVFV replication at the beginning of the infection. In addition, the *p53-2* gene (but not *p53-1*) was upregulated in response to DENV C6/36 infected cells at 24 hpe. When *p53-2* was knocked out, apoptosis was enhanced suggesting that the transcription factor p53-2 may protect infected cells from death thus

ensuring virus replication (Chen et al. 2017). However, in human airway epithelial cells infected by RVFV, phosphorylation of p53 did not induce genes necessary for initiation of apoptosis highlighting alternative mechanisms of down-regulation of apoptosis to protect cells from death (Popova et al. 2010). These contradictory results suggest different roles for p53 in insects and mammals and/or those different types of virus might trigger different apoptotic pathways. In our study, both, p53-1 and p53-2 were up-regulated suggesting that a hypothetical apoptotic regulatory pathway is engaged. Moreover, the Croquemort transcript, coding for a class B scavenger receptor essential for phagocytosis of apoptotic cells (González-Lázaro et al. 2009) and up-regulated in *Drosophila* during Flock House Virus infection (Go et al. 2006), was enriched in RVFV infected mosquitoes strengthening the above hypothesis that the apoptosis might be occurring in some mosquito cells after RVFV exposure. Furthermore, involvement of class B scavenger receptors in infectious processes have been documented for *A. gambiae* where the SCRBQ2 transcript was up-regulated in the midgut after ingestion of blood infected with *Plasmodium berghei* and silencing this gene resulted in decreased formation of oocysts (González-Lázaro et al. 2009). The role of apoptosis in arboviral infections is still subject to debate. For example, apoptosis occurring in the midgut epithelium and salivary glands of *Cx. pipiens* and *Cx. quinquefasciatus* respectively, induced resistance to WNV infection (Vaidyanathan and Scott 2006) and reduced transmissibility of WNV (Girard et al. 2007) while silencing the apoptosis inhibitor IAP1 in *Ae. aegypti* resulted in an increment of SINV infection (Trapnell et al. 2013).

In the present study, we do not know if apoptosis takes place; this will require specific experiments in different organs or tissues, such as midgut, fat body or salivary glands of RVFV infected females. However, the fact that most of the DEG implicated here in apoptosis or UPP were found at an early stage of infection would suggest, that if apoptosis does occur, then it will take place in *Cx. pipiens* midguts. It is

important to highlight the lack of understanding about the effect of apoptosis during arboviral infections. Further experiments will be necessary to clarify how apoptosis contributes to RVFV infection/tolerance in *Cx*. *pipiens* mosquitoes.

Alternative inducible antiviral immune genes

In the present study, some DEG with no conventional immune pathway attribution but which are involved in immune responses against pathogens were identified. Trypsins, Hsp70 and lamin transcripts belong in this last category. The trypsins, which are blood-digestive enzymes, were overexpressed within the first hours of infection and were down regulated at 3 dpe. Our results suggest that blood digestion and probably RVFV proteolytic processing via midgut trypsins may facilitate RVFV infection as previously reported by Molina-Cruz *et al*. (2005) in DENV-2 infected *Ae*. *aegypti*. Inhibition of trypsins prior RVFV infection will be required to test this hypothesis. At a stage when the RVFV infection is already established in *Cx*. *pipiens* mosquitoes (at 14 dpe), a gene coding for Hsp70 was under-expressed. The chaperone protein Hsp70 facilitates infection of multiple viruses in mammalian cells, such as DENV (Taguwa et al. 2015), Japanese encephalitis virus (Das et al. 2009) or ZIKV (Pujhari et al. 2019). Moreover, Pujhari *et al*. (2018) have recently demonstrated that the Hsp70 protein has an important role in the cell cycle during infection, as well as viral entry, replication and egress. Additionally, they showed that the inhibition of Hsp70 led to decreased ZIKV titer in infected Huh7.5 human cells (Pujhari et al. 2019). By analogy, our results indicate that lower levels of Hsp70 might limit but not abolish RVFV production to increase mosquito viability. Finally, two lamin genes were under-expressed at 14 dpe in RVFV infected mosquitoes. The lamin genes have been beforehand identified as involved in melanization in *Drosophila* (Markovic et al. 2009), but their potential antimicrobial activity in mosquitoes and arboviral infections need to be confirmed.

Transcriptomic studies are crucial for detecting genes implicated in mosquito vector competence for arboviruses, since new control strategies can be drawn. To date, no comprehensive transcriptomic analysis has been performed in mosquitoes with this particular zoonotic virus. The present study significantly expands our understanding of *Cx. pipiens* and RVFV interactions, proposing possible roles of immune response mechanisms (Toll, IMD, RNAi, UPP and apoptosis) in the control or exacerbation of the infection. Moreover, other inducible antiviral genes were modulated during the course of RVFV infection in *Cx. pipiens* females. At an early stage of infection, some crucial defense effectors are inhibited, providing an opportunity for RVFV to disseminate. Once the infection is established counteracting mechanisms mitigate virus replication allowing host survival. Thus, the present work provides a number of target genes and hypotheses on which to base future functional studies of the mechanisms inducing viral replication and resistance/tolerance to RVFV infection.

ACKNOWLEDGEMENTS

The authors are very grateful to Dr. Francesc Xavier Abad for his exceptional BSL3 facilities management and to BSL3 facilities staff for their brilliant technical contribution at IRTA-CReSA. Additionally, we are very grateful for the excellent technical contribution of Marta Verdún, Núria Pujols and Raquel Rivas from CReSA/IRTA.

SUPPORTING ONLINE INFORMATION

S1 Figure. Representation of Gene Ontology categories assigned to DEG at 2 hpe during RVFV infection in *Cx. pipiens*. A) Main GO categories of down-regulated DEG at 2 hpe. The legend shows the GO categories (GO1 to GO5). The most represented were cellular process and cellular component organization or biogenesis. B) Principal GO categories of up-regulated DEG at 2 hpe. In the legend the GO categories (GO1 to GO7) are shown. The most represented

corresponded to cellular process, response to stimulus, metabolic process and cell death.

S2 Figure. Representation of Gene Ontology categories assigned to DEG at 3 dpe during RVFV infection in *Cx. pipiens*. C) Main GO categories of down-regulated DEG at 3 dpe. The legend shows the GO categories (GO1 to GO4). The most represented corresponded to multi-organism process, cellular response to stimulus, cellular metabolic process and developmental process. D) Principal GO categories of up-regulated DEG at 3 hpe. In the legend the GO categories (GO1 to GO3) are shown. The most represented categories were cellular metabolic process, localization and multicellular organismal process.

S3 Figure. Representation of Gene Ontology categories assigned to DEG at 14 dpe during RVFV infection in *Cx. pipiens*. E) Principal GO categories of down-regulated DEG at 14 dpe. The legend shows the GO categories (GO1 to GO4). The most represented corresponded to immune system process, cellular component organization or biogenesis, development process and localization. F) Main GO categories of the up-regulated DEG at 14 dpe. In the legend the GO categories (GO1 to GO6) are shown. The most represented were cellular component organization or biogenesis, metabolic process, cellular metabolic process, immune system process and developmental process.

S1 Table. DEG during RVFV infection in *Cx. pipiens* mosquitoes. The transcripts differentially expressed at 2 hpe, 3 dpe and 14 dpe were listed with corresponding best matches in the protein nr database at NCBI. Sequences of the transcripts were subjected to BLASTX with a cut-off of P value < 0.0001. Open Reading Frame (ORF) annotations (*) were obtained by combining a search performed with the Pfam, Conserved Domain and Swiss-prot databases. The DEG highlighted in green colour corresponds to the immune related DEG identified in the present study.FC= fold change; nr = non-redundant; - = no significant

S2 Table. **Down-regulated DEG at 2 hpe and at 3 and 14 dpe with their respective GO categories in RVFV infected *Cx. pipiens***. DEG were assigned to GO functional categories for biological process using TopGO software with Fisher's exact test. The cut-off for 'classicFisher' was P-value <0.05.

S3 Table. Up-regulated DEG at 2 hpe and at 3 and 14 dpe with their respective GO categories in RVFV infected *Cx. pipiens*. DEG were assigned to GO functional categories for biological process using TopGO software with Fisher's exact test. The cut-off for 'classicFisher' was P-value <0.05

PART III

General section: General discussion, Conclusions and References

GENERAL DISCUSSION

Currently, arboviral diseases cause high global morbidity and mortality worldwide due to the emergence and re-emergence of several arboviruses such as DENV, YFV, WNV, ZIKV or CHIKV in the last decades (Wilder-Smith et al. 2017). The ongoing globalization and the ecological plasticity of vectors such as *Ae*. *aegypti* and *Ae*. *albopictus* have contributed to their geographical expansion and the spreading of arboviruses that they can transmit. Moreover, the capacity of several arboviruses to perform urban transmission cycles due to its efficient adaptation to anthropophilic vectors (*e.g.*, *Ae*. *aegypti* and *Ae*. *albopictus*), as well as the maintenance of some arboviruses within enzootic transmission cycles, comprising secondary vectors (*e.g.*, *Culex* mosquitoes) and urban hosts (*e.g.*, urban birds), represent a huge risk for human health (Weaver and Reisen 2010). In addition, there are no vaccines for most arboviral diseases and the vector resistance to insecticides has also increased in the last years. Thus, it is crucial to develop new strategies to ensure the control and prevention of arbovirus diseases.

Vector competence studies are key because they contribute to establish: i) the risk of arboviral transmission within a non-endemic country and ii) arbovirus surveillance programs to control and prevent arbovirus outbreaks. Developing of interdisciplinary frameworks by mixing the information provided by VC findings, and the distribution of mosquito species (*e.g.*, from epidemiological studies), are essential to design effective arbovirus surveillance programs that guarantee human health. Vector competence studies should be conducted under controlled environmental conditions simulating natural conditions, so that the results obtained can be used to estimate the risk of a successful arbovirus transmission in a specific area. On the other hand, from the point of view of producing engineered mosquitoes to control arbovirus transmission, nowadays two strategies are proposed: creating resistances in the vectors to the pathogens in order to avoid the virus

replication within mosquitoes or destabilizing the vector tolerance to pathogens, thus, increasing mosquito mortality when they are infected by the virus (Lambrechts and Saleh 2019, Oliveira et al. 2020). To achieve these goals molecular analyses of virus-vector interaction are needed. In the present thesis, three strategies have been performed to deep on the influence of arbovirus-vector on arbovirus transmission i) two VC studies of three Spanish mosquito populations for one emergent virus that affects humans (ZIKV) (Chapters I and II) ii) a ZIKV vertical transmission study in one *Ae*. *albopictus* population (Chapter II), and iii) one molecular study on the transcriptome of infected *Cx*. *pipiens* with a zoonotic virus (RVFV) (Chapter III).

After the recent ZIKV outbreaks in the American continent, we focused particularly on VC studies of *Ae*. *albopictus* (an invasive species) and *Cx*. *pipiens* and *Ae*. *caspius* mosquito populations (both autochthonous species from Spain) for different ZIKV strains to better estimate the risk of ZIKV transmission in our country. The studies concerning the ability of different mosquito species to transmit ZIKV have increased in the last years. However, since there is not a standardized protocol on how to perform vector competent assays it is difficult to carry out a meta-analysis with all the recent data generated. Therefore, it is essential to increase the reproducibility of VC studies by the arbovirologist community, as previously pointed out by Azar *et al*. (2019). To do that, it would be necessary to implement a protocol that standardizes the basic methodologies for the development of the VC experiments, such as, virus stock conditions, number of virus passages, the use of cloned virus, mosquito microbiome and virome, dose of virus in artificial blood-feeds, sequential blood meals, artificial blood meals elements (*e.g*., artificial blood method: membrane feeding system, pledgets or hanging blood drops) or extrinsic incubation temperatures (Azar and Weaver 2019). Doing so would allow to make results comparable among different laboratories. For this reason, within the European ZIKAlliance project, a protocol was developed for all partners to perform VC experiments for ZIKV. Thus, our results have

been able to be compared with recent studies performed by Hery *et al*. (2019) and Vazeille *et al*. (2019) to determine the VC of different *Ae*. *albopictus* mosquito populations. Our results showed that Spanish *Ae*. *albopictus* populations were more competent vectors for the African I lineage than for the Asian lineage, similarly as what was reported for the French mosquito populations (Vazeille et al. 2019). In addition, the VC differences found among *Ae*. *albopictus* populations remark the relevance of vector-virus pairing for arbovirus transmission studies. Based on our results and studies performed with other European *Ae*. *albopictus* populations (Di Luca et al. 2016, Heitmann et al. 2017, Gutiérrez-López et al. 2019, Vazeille et al. 2019), *Ae*. *albopictus* is currently the most relevant mosquito species in our country to be included in ZIKV surveillance programs. On the other hand, from our results we can conclude that it is not necessary to include *Cx*. *pipiens* and *Ae*. *caspius* mosquito species in ZIKV surveillance programs since it is unlikely that they are involved in the transmission of the ZIKV in our area, which agrees with previous studies (Boccolini et al. 2016, Gutiérrez-López et al. 2019).

Regarding the VT of ZIKV in mosquitoes, little is known about its contribution in the ZIKV epidemiology, which supposes an important gap of knowledge hindering our understanding of virus transmission within mosquito populations. The results obtained in the Chapter II confirmed the VT of both tested ZIKV strains (Asian and African I lineages) in Spanish *Ae*. *albopictus* mosquitoes, as previously demonstrated by Gutierrez *et al*. (2019) for the Asian lineage in larvae. We detected the virus in both adult males and females of the progeny of infected females, but with low FIR. Moreover, it was not possible to demonstrate that the progeny was able to disseminate ZIKV. Therefore, it may be unlikely that the VT plays an important role in the ZIKV epidemiology. On the other hand, it would be interesting to deepen on the ZIKV venereal transmission between *Ae*. *albopictus* mosquitoes, since we detected ZIKV in *Ae*. *albopictus* adult males. Venereal transmission of ZIKV in *Ae*. *aegypti* mosquitoes was previously shown

from males to females and vice versa (Campos et al. 2017), so it could be a mechanism that contribute to maintain the virus in nature, but further research studies on virus transmission of the potential infected *Ae*. *albopictus* mosquito females via infected males would be also necessary to know the importance of the venereal transmission of ZIKV in its epidemiology.

As mentioned above, VC studies give us knowledge about the susceptibility of mosquitoes to arboviruses. It has been demonstrated the viral transmission dependence on interactions between mosquito genotype-virus genotype (G x G) (Lambrechts et al. 2009), so it is necessary to deepen our knowledge on these interactions at the molecular level to understand better the mechanisms involved in VC and to establish the bases for further engineered control. On this issue, transcriptome studies are key because they provide information about the high specificity between G x G interactions, revealing possible molecular strategies to control the arbovirus transmission. In the present thesis, we performed a transcriptome study on *Cx*. *pipiens* mosquitoes exposed to RVFV (Chapter III) to increase our knowledge on the *Cx*. *pipiens* -RVFV interaction. Our group had previously demonstrated that this mosquito species was able to transmit RVFV (Brustolin et al. 2017) and there were not previous studies on the genes involved in the *Cx*. *pipiens* response to RVFV exposition. A transcriptome *de novo* analysis was performed since the *Cx*. *pipiens* genome is not completely sequenced yet. Our transcriptomic results significantly increase the knowledge of *Cx*. *pipiens*-RVFV molecular interactions, providing potential novel target genes to develop future functional studies of resistance/tolerance mechanisms to RVFV infection. Our results offer possible genes involved in the immune response mechanisms (*e.g*., Toll, IMD, RNAi, UPP and apoptosis) that could be used for the infection control or exacerbation. Furthermore, other inducible antiviral genes were altered in the course of RVFV infection in *Cx*. *pipiens* mosquitoes, such as trypsins, *Hsp70* or *lamin*, which may have a key role in the RVFV infection. We found that some

crucial defense elements were inhibited at the beginning of the infection such as some members of the RNAi mechanism and some immune effectors triggered by the three classical immune pathways (*e.g.*, defensin-A), which may give an opportunity to RVFV dissemination. Our results also suggest that, when the infection was established, countering mechanisms (*e.g.*, RNAi pathway) moderated the viral replication, thus, inducing tolerance in the host by preventing high fitness costs. One limitation of our study was that, some DEG involved in response to RVFV infection suggested broad roles and it was difficult to disentangle whether the biological processes have an antiviral function or facilitate viral dissemination within mosquitoes. To shed light on this issue, functional studies and analysis in different tissues (*e.g.*, midgut, salivary glands or fat body), rather than processing mosquito pools, during the infection should be explored in future studies.

Future investigations to understand mosquito mechanisms to resist/tolerate arboviral infection should be performed to offer new opportunities to block arbovirus transmission. For this purpose, functional studies to know the role of the genes during arboviral infections and engineered control strategies based on mosquito resistance or tolerance to pathogens are key. With regards to our study, we would focus the first functional studies to demonstrate the role of the most relevant DEG that we have described for *Cx. pipiens* –RVFV pairing and have also been altered in other mosquito-virus pairings, such as *defensin-A*, or RNAi pathway members (*e.g.*, *Piwi4*, *LOQS* or *Drosha*). To perform functional studies, techniques such as interference RNA silencing can be used, such as Dietrich *et al*. (2017) performed with Piwi4 to demonstrate its antiviral function in *Ae. aegypti* cells. Moreover, information on mosquito immune mechanisms against arboviral infections must be expanded since there is still a great lack of knowledge in this field, and functional studies are also crucial to enlighten the relationship between the mosquito physiological functioning and resistance/tolerance mechanisms to arboviruses.

Additionally, it is also important to highlight the scarcity of data on the implications of apoptosis during arboviral infections. It is also key to know the tissues where apoptosis take place. A previous study on one WNV refractory *Cx*. *pipiens* colony showed that the apoptosis was produced in the midgut (Kean et al. 2015), which seems to be in accordance with our results. To confirm this, an apoptosis kinetic study should be performed during RVFV infection in the *Cx*. *pipiens* to verify the presence of apoptotic cells in the mosquito midgut after RVFV exposure. Regarding engineered control studies based on mosquito resistance or tolerance to pathogens, in recent years, the development of new strategies to control arbovirus expansion has focused on increasing mosquito resistance to arbovirus infection (Lambrechts and Saleh 2019) by natural and engineered control programs (Kean et al. 2015). Most strategies to increase the resistance to arboviruses would be designed by genetically enhancing RNAi pathway and other antiviral systems (*e.g*., Toll, IMD or JAK/STAT pathways) (Kean et al. 2015). For instance, some engineered studies have performed transgenic *Ae*. *aegypti* mosquitoes with increased or repressed antiviral defenses (such as RNAi, Toll and IMD pathways and its effectors, the AMPs [*e.g*., cecropin and defensin-A]) to study the mosquito immune responses during mosquito-pathogen interactions (Shin et al. 2003, Bian et al. 2005, Kokoza et al. 2010, Olmo et al. 2018). Regarding arboviruses, Olmo *et al*. (2018) showed that the ectopic expression of a component of the RNAi pathway (Loqs2) in the midgut of *Ae*. *aegypti* mosquitoes was enough to restrict DENV replication and dissemination (Olmo et al. 2018). However, engineered control studies have not yet been proved against arboviruses in the field. It is important to take in account, as previously pinpointed by Kean *et al*. (2015), that transgenic mosquitoes with a modification in their immune system for a specific arbovirus, should be evaluated to prove whether they are able to transmit other pathogens before implementing any control program in nature. The future challenges of these approaches could be the replacement of mosquito populations. In this case, each genetically modified mosquito must undergo a risk evaluation process to assess the

benefits and risks. It has not yet been determined how these mosquitoes could be released safely, effectively and efficiently, so semi-field test experiments will be required before implementing it (Kean et al. 2015). A previous theoretical study demonstrated that the replacement of mosquito population with genetically modified refractory mosquitoes would be easier to maintain and should lead to a greater long-term reduction in competent vectors compared to population reduction approaches (Robert et al. 2014).

When implementing control programs in nature based on resistance/tolerance of mosquitos to arboviruses, it must be taken careful consideration on the difference between resistance and tolerance strategies. As proposed recently by Lambrechts *et al*. (2019), increasing the resistance to arbovirus infection may select the most virulent virus strains, so this strategy may result less evolutionarily sustainable. Conversely, changing the strategies to disrupt the mosquito tolerance to arbovirus infection could be a more sustainable strategy that would focus on increasing fitness costs for mosquito (usually humble) to the viral infections (Lambrechts and Scott 2009). The tolerance of mosquitoes to arboviruses is a necessary condition to successful arboviral transmission into vertebrate host since mosquito survival and high viral loads are necessary (Lambrechts and Saleh 2019). In other words, the control of viral growing by mosquito defense mechanisms is essential for its survival against arboviruses (Lambrechts and Saleh 2019, Oliveira et al. 2020). Current knowledge of vector strategies to tolerate arboviruses and its variance between mosquito populations is limited. Understanding how mosquitoes tolerate arboviral infection is crucial to select target genes to reduce mosquito tolerance against arbovirus infection (Oliveira et al. 2020). Some mechanism known to be involved in mosquito tolerance are: i) homeostasis and cellular renovation, ii) viral pathology reduction, and iii) modulation of persistent infections of arboviruses (Oliveira et al. 2020). As it was discussed in the Chapter III, the response to arboviral infection consist in the activation of the immune system apart of a wide

range of physiological changes such as stress response, tissue repair and metabolic adaptations (Lambrechts and Saleh 2019). Our results showed several DEG related to metabolic processes. A revision of total DEG could be performed to analyze possible metabolic genes involved in tolerance modulation of *Cx. pipiens* females for RVFV, which is largely ignored. Some physiological responses that we found altered in the response of *Cx. pipiens* exposed to RVFV could be related to some of the mechanisms involved in mosquito tolerance above-mentioned. For instance, the chaperone protein Hsp70, which was down-regulated at 14 dpe, has previously involved in the stress response (Muturi et al. 2011). The down-regulation of this protein might suggest that low Hsp70 levels may limit RVFV production to increase mosquito viability. Therefore, a functional study of this protein is needed to better understand its role during RVFV infection in *Cx. pipiens* and for its potential use in engineered mosquitoes to decrease the tolerance to arboviruses. Moreover, a big battery of DEG related to apoptosis process were altered during the RVFV infection in *Cx. pipiens*, which could also be potential targets to study the arbovirus tolerance mechanisms.

To sum up, this thesis has increased the knowledge to better design efficient ZIKV surveillance programs to prevent and control the transmission of this virus, apart from pave the way to perform functional future studies about *Cx. pipiens*-RVFV interactions to a better control of RVFV transmission.

CONCLUSIONS

1. *Ae*. *caspius* and *Cx*. *pipiens* mosquitoes from Catalonia were refractory to ZIKV, suggesting that it is unlikely that these mosquito species could be involved in the ZIKV transmission. Therefore, *Ae*. *caspius* and *Cx*. *pipiens* mosquitoes should not be taken into account in arbovirus surveillance programs.
2. Catalonian *Ae*. *albopictus* populations were competent vectors for different ZIKV strains (African I and Asian lineages), suggesting that local mosquitoes could initiate a ZIKV autochthonous transmission when ZIKV is introduced. Moreover, the early ZIKV transmission founded in *Ae*. *albopictus* (at 7 dpe) would increase its vectorial capacity, thus enhancing the time of ZIKV horizontal transmission via mosquito.
3. The two tested *Ae*. *albopictus* populations were more competent for DAK84 ZIKV strain (African I lineage) than for Martinique ZIKV strain (Asian lineage) and showed differences between them in terms of VC. These results reinforce the relevance of studying the vector-virus pairing to better assess the risk of arbovirus transmission.
4. Zika virus VT was demonstrated in *Ae*. *albopictus* by the detection of the virus in adult males and females of the progeny of infected females. However, the low FIR and the absence of viral dissemination in the infected females of the progeny suggest that it is unlikely the VT plays an important role in the ZIKV epidemiology.
5. For the first time a transcriptome study on *Cx*. *pipiens* exposed to RVFV was performed, which has allowed to expand the understanding of *Cx*. *pipiens* and RVFV interactions at molecular level, and showed that while genes from the Toll, IMD and RNAi immune pathways were altered in response to

RVFV none of the deregulated genes were related to the JAK/STAT immune pathway.

6. The early depletion of RNAi pathway and AMP (*e.g.*, *defensin-A*) genes at the onset of the RVFV infection in *Cx. pipiens* would allow the initial viral replication in mosquitoes. Moreover, the fact that most altered genes involved in the Ubiquitin-proteasome pathway (UPP) or apoptosis were also found at an early stage of infection would suggest that apoptosis may play a regulatory role in infected midguts of *Cx. pipiens*.

7. Forty-eight genes involved in the immune response (Toll, IMD, RNAi, UPP and apoptosis) and antiviral mechanisms were identified during the course of RVFV infection in *Cx. pipiens* females. These findings pave the way for future functional studies that allow engineered control in *Cx. pipiens* mosquitoes by inducing resistance or decreasing tolerance to RVFV infection.

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Agradecimientos

Durante todos estos años en el CReSA he tenido la suerte de coincidir con personas maravillosas, que me han ayudado a madurar tanto en lo laboral como personal, no podría estar más agradecida. Vine a Barcelona para un contrato de 7 meses y hace unos cuatro meses cumplí aquí ¡¡5 años!!

En primer lugar, me gustaría agradecer al *Artropovir team* el gran esfuerzo que realizan cada día para que los proyectos salgan a delante. Núria muchísimas gracias por tu paciencia, perseverancia y motivación. Siempre has estado ahí para todo, al pie del cañón a cualquier hora del día (literalmente). Espero que no cambies nunca, tanto como persona como jefa, me considero muy afortunada de haberte tenido como mentora durante estos años de doctorado.

Muchas gracias, Sandra, por haber sido como una hermana mayor para mí, siempre tranquilizadora y apaciguadora, consigues que en los momentos de crisis uno vea las cosas de otro color. Cuando la situación lo permita, me debes como mínimo un día de vía ferrata hehehe.

Núria, Marta y Raquel, muchas gracias por todo lo que me habéis enseñado y por todos los momentos que hemos pasado en NBS3. Sin vuestra ayuda esta tesis no habría sido posible, y siempre con la mejor de las actitudes. Núria y Marte, ha sido muy divertido compartir parte de nuestra vida en el lab y compartir las experiencias diarias. Raquel, me has enseñado a ser precisa, metódica, ordenada y tranquila trabajando en condiciones de bioseguiridad, no te podría estar más agradecida. A día de hoy sé qué me podrían soltar en cualquier laboratorio y apañármelas como sea. Nunca olvidaré todos los consejos que me has dado durante las jornadas de laboratorio y, sobre todo, que con orden, calma y buena música de fondo al final siempre todo sale bien. Además, me llevo a una amiga en el corazón.

Lotty (o Malotty para los amigos) amiga, confidente y compañera, muchas gracias por haber sido mi mayor apoyo durante estos 5 años. No te imaginas lo que te añoré aquellos meses que

volviste a Ecuador, menos mal que conseguiste volver. Estos 3 años de tesis no hubiesen sido lo mismo sin tenerte a mi lado, ¡tú comprensión y capacidad para escuchar no tienen límites, gracias otra vez!

Por supuesto, gracias a Cris y Marco, que fueron los pioneros en poner a punto todos los protocolos con los que trabajamos a día de hoy para experimentos de VC. Y a María, reciente incorporación al grupo.

Cómo no, es imposible que me olvide de agradecerle a Albert todo el esfuerzo y desempeño que ha invertido conmigo leyendo y estudiando bibliografía sobre la inmunidad de los mosquitos y otros insectos. Ha sido un enorme placer aprender a tu lado, eres una fuente inagotable de conocimiento. Discutir contigo sobre política es, seguramente, de las cosas más divertidas que hemos hecho un viernes a última hora del día en el CReSA.

Muchas gracias también a todo el personal de NBS3 y estabulario por su apoyo técnico durante estos años, en especial a Claudia, María Ángeles y Valentí por tratarnos como parte de su familia y contagiarnos con sus sonrisas y humor cada larga jornada de trabajo. Iván, muchas gracias por toda tu paciencia y apoyo logístico durante nuestras experimentales, incluso cuando nos has tenido que echar la bronca. No me olvido de todo el equipo de gestión por el apoyo brindado durante este tiempo: PepeMari, Merche, Xavi, Samanta, Mónica A. y Raquel Maeso.

Por supuesto, estos años de tesis no habrían sido ni la mitad de buenos si no hubiese contado con la compañía y amistad de los tres mosqueperros, Carlos, Miguel y Álvaro. Desayunos, risas, meriendas, cafés, increpaciones, consejos de bioinformática, excursiones montañeras o a desayunar de *forquilla*, cenas, natación en el SAF y un largo etcétera de actividades que han contribuido a forjar un amistad única y maravillosa, que estoy segura durará muchos años. Gracias también a la amistad del "*falso* becario" (hehehe): Ponti, como bien dijo Raúl, ¿qué haríamos sin tus consejos, tu apoyo y tus ganas de

escucharnos relatar? Siempre estás ahí, da igual la hora, el día o el sitio, ¡muchas muchas gracias por todo! Os llevaré siempre conmigo (L).

Ha sido un enorme placer compartir despachito con esta increíble generación de becarios precarios: Uxi, Lou, Alejo, Jordi, Perlas, Laia, Te Nigeer, Sergi, Miaomiao, Claudia, Vivi, Yinja, Elena. Y con los que ya no están, Eli, Marta, Laura, Fra y Raúl, cuyas marchas dejaron un gran vacío. Gracias, gracias y mil gracias por los momentos tan divertidos que hemos pasado juntos, tanto las cenas y diversas festividades como los días de charla-terapia de despacho. De esta generación ha surgido el *Precarruners team*, contando con integrantes de la calidad de Marta Muñoz, que ha sido el espíritu de este equipo. Otra gran amiga que me llevo en el bolsillo, a parte es muy útil porque sirve de guía de viaje :P. Espero seguir yendo a un montón de carreras con vosotros, sufriendo en buena compañía, todo es más ameno hahaha. Muchas gracias también a Rosa y Mónica, por sus divertidas conversaciones en la sala de descanso y sus ánimos en momentos de terapia.

Gracias de corazón al Colectivo de *Docs en Lluita*, que se están desviviendo por mejorar nuestras condiciones laborales, tanto que casi les van a convalidar el Grado en Derecho hehehe. Ha sido un placer poder aportar mi granito de arena en vuestro movimiento, y espero poder seguir contribuyendo una vez acabada la tesis.

No me olvido de todos los amigos que han contribuido a que mi vida en Barcelona sea plena y muy divertida: Júlia y Salva, gracias por acogerme nada más caer en la ciudad y estar siempre cerca y disponible ante cualquier emergencia. Chris, Juan, Marc y Luis, o también conocidos como los Fumis, gracias por haberme recibido en el equipo con los brazos abiertos y haberme preparado para los campeonatos como cuando era joven hehe. Ir a entrenar ya no es lo mismo sin vosotros. Por último, gracias a Elena y Jofre, por ser parte de nuestra familia (y familia perruna también) en Barcelona y gracias a Óscar y Mari, por acogernos en Sant Feliu de Codines como si fuésemos

familia, ha sido un placer compartir tantas experiencias con vosotros (tanto en roca como en el agua), me habéis ayudado a desconectar los fines de semana y a superar algún que otro miedo hehehe, así como a Judit, Edu, y compañía del CESF. Momentos maravillosos que nunca olvidaremos, y ¡espero que se sigan sumando!

A pesar de la distancia, los amigos de Madrid siempre han estado ahí: los de siempre (Ángel, Carlos, Marta, Sandra y Viole), los biologuillos repartidos por el mundo (Darío, Nacho, Julia, Lu, Rober, Car, Clara, Felipe, Isa, Irene, María, Borja y Blanca), los socios (Elenita, Gonza, Piña y Manu), a mis sirénidas Ale y Clau, y a todo el equipo de C.D. Halegatos (Pau, Terín, Eli, Osu, Juan, Ibáñez, Kukis, etc…), que aunque este año nos hemos quedado sin campeonato vendrán muchos más los próximos años. Gracias a Nuria y a Pitu, que desde su viaje por el mundo siempre han estado enviando ánimos (y podcast, muchos podcats hehe) de su parte, y a Pascual, ese gran amigo que siempre está ahí. Y muchas gracias también a mi familia política, por estar siempre ahí para todo a pesar de la distancia (Javier, Maca, Ángela y Gaby). Especialmente a la artista de la familia, Ángela, que se ha currado el dibujo de la portada ni más ni menos que ¡en acuarela! Miles de gracias :)

Muchísimas gracias a mi familia, mis padres y mi hermano. También a la yaya, y a los que ya no están, el yayo y los abuelos, seguro que estarían muy felices de poder disfrutar este día con nosotros. Sin duda alguna, si estoy aquí es porque todos ellos se han esforzado en allanarme el camino y darme las facilidades para poder continuar estudiando y formándome. Pese a la distancia siempre os he sentido a mí a lado, animándome y motivándome cada día a seguir a delante. ¡Mil gracias, os quiero!

Por último, me gustaría darle las gracias a la persona que le ha dado sentido a la realización de esta tesis, Miguel. Gracias por estar siempre a mi lado, por transmitirme la pasión que sientes por la ciencia, recordándome en los momentos de crisis que hacer ciencia es *guay*. Me

has enseñado mucho a lo largo de estos años, sobre todo el manejo de datos y análisis estadístico durante el cual has demostrado una enorme paciencia. Me has enseñado a ser más profesional, mejor científica, abordar los problemas desde varias perspectivas y a convertir la búsqueda de las soluciones en un juego. Gran investigador y mejor persona, tengo mucha suerte de tenerte a mi lado. Te quiero, y me encanta saber que tenemos toda una vida por delante para seguir resonando juntos.