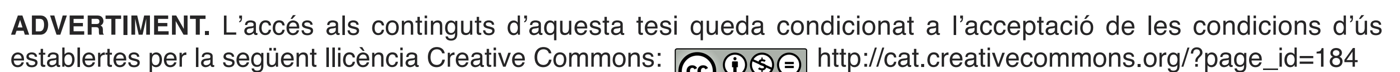
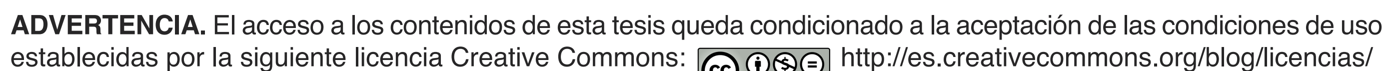
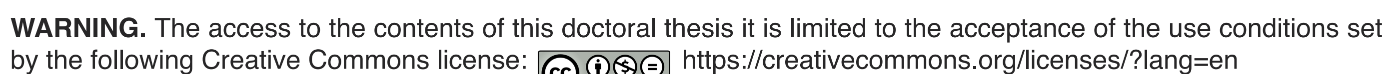
ADVERTIMENT. L'accés als continguts d'aquesta tesi queda condicionat a l'acceptació de les condicions d'ús establertes per la següent llicència Creative Commons:  http://cat.creativecommons.org/?page_id=184

ADVERTENCIA. El acceso a los contenidos de esta tesis queda condicionado a la aceptación de las condiciones de uso establecidas por la siguiente licencia Creative Commons:  http://es.creativecommons.org/blog/licencias/

WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

Autochthonous and invasive mosquitoes of Catalonia as vectors of zoonotic arboviruses

Marco Brustolin

PhD Thesis

Bellaterra, 2016

Universitat Autònoma de Barcelona

Autochthonous and invasive mosquitoes of Catalonia as vectors of zoonotic arboviruses

Tesis doctoral presentada por **Marco Brustolin** para acceder al grado de Doctor en el marco del programa de Doctorado en Medicina y Sanidad Animal de la Facultat de Veterinaria de la *Universitat Autònoma de Barcelona,* bajo la dirección de la Dra. **Núria Busquets Martí** y del Dr. **Nonito Pagès Martínez,** y la tutoría de la Dra. **Natàlia Majó i Masferrer.**

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Que la memoria titulada, **"Autochthonous and invasive mosquitoes of Catalonia as vectors of zoonotic arboviruses"** presentada por **Marco Brustolin** para la obtención del grado de Doctor en Veterinaria, se ha realizado bajo su supervisión y tutoría, y autorizan su presentación para que sea valorada por la comisión establecida.

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Doctorando

Alla cara nonna "Mariù"

perché ci hai insegnato come affrontare la vita

con un indomabile forza di volontá.

A mí querida abuela "Mariù"

porque nos enseñaste como afrontar la vida

con una indomable fuerza de voluntad.

一念岩をも通す

"La forza di volontá attraversa anche le rocce"

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LIST OF ABBREVIATION

ABD: Arthropod-Borne Disease

BL: Basal Lamina

CHIKV: Chikungunya Virus

CxFV: Culex Flavivirus

DENV: Dengue Virus

dpi: Days post infection

EI: Extrinsic Factor

EIP: Extrinsic Incubation Period

ER: Endoplasmic Reticulum

FEF: full engorged female

IF: Intrinsic Factor

IHC: immuno-histochemical

IMD: immune deficiency

ISF: Insect Specific Flavivirus

JAK-STAT: Janus kinase/signal transducers and activator of transcription

JEV: Japanese Encephalitis Virus

KUNV: Kunjin Virus

LACV: La Crosse Virus

MEB: Midgut Escape Barrier

MIB: Midgut Infection Barrier

MBD: Mosquito-Borne Disease

MVEV: Murray Valley Encephalitis Virus

nts: nucleotides

PFU/ml: Plaque Forming Unit per milliliter

PM: Peritrophic Matrix

ORF: Open Reading Frame

RNAi: RNA interference

RdRp: RNA-dependent RNA polymerase

RVF: Rift Valley Fever

RVFV: Rift Valley Fever Virus

SGEB: Salivary Gland Escape Barrier

SGIB: Salivary Gland Infection Barrier

SINV: Sindbis virus

SLEV: St. Luis Encephalitis Virus

USD: US dollar

VC: Vector Competence

VBD: Vector-Borne Disease

WNF: West Nile Fever

WNE: West Nile Encephalitis

WNM: West Nile Meningitis

WNP: West Nile Poliomyelitis

WNV: West Nile Virus

WHO: World Health Organization

YFV: Yellow Fever Virus

ZIKV: Zika Virus

ABSTRACT

Mosquito-borne diseases (MBDs) are an increasing global public health concern. The increase of international human activities and the global warming, have allowed the rapid spread and establishment of invasive mosquito species in new geographical areas. Moreover, the globalization also cause the introduction of exotic arboviruses in naïve areas where the mosquito vector is present, enhancing MBDs outbreaks.

The outbreaks of MBDs, especially those caused by arbovirus like Dengue virus, Zika virus (ZIKV), Rift Valley fever virus (RVFV) or West Nile virus (WNV), have severe consequences for public and/or veterinary health. The economic losses linked to the zoonotic MBDs can severely compromise the economy of the affected country. This is particularly relevant in the developing countries, where diseases such as Rift Valley fever can provoke the death of a huge number of domestic and wild animals. For these reasons a deep knowledge of which mosquito species are present in a country and which of them are competent vectors of different pathogen agents is needed in order to set up appropriate surveillance programs and entomological control strategies.

The present thesis is divided in three parts and each one is further divided in chapters.

The first part consists of two sections: *Chapter 1- General Introduction* and C*hapter 2- Objectives*. The aim of this first part is to give a general overview on the importance of MBDs, the role of the vectors and the concept of vector competence. The descriptions of WNV and RVFV are also provided. Afterwards, the objectives of the thesis are exposed. So far, the population dynamics and the genetic structure of invasive *Aedes albopictus,* the Asian tiger mosquito, were unknown in Spain. Moreover, no studies on the vector competence of Spanish populations of *Culex pipiens* and *Ae.*

albopictus for zoonotic arboviruses (WNV and RVFV) have been performed until now. The aim of the present thesis is to clarify all these points.
The second part is formed by three chapters. Each of them is an original study aimed to answer the objectives.
In the first study (*Chapter 3*), the genetic structure of *Ae. albopictus* in Spain has been analyzed with the use of different genetic tools. Based on the results obtained, two different hypotheses have been postulated to explain the introduction and the spread of this species across the country. Moreover, a global analysis about the worldwide spread of *Ae. albopictus* has been performed. The results of both analyses confirmed the importance of human activities for the local and global dispersion of *Ae. albopictus*.
The second and third studies (*Chapter 4* and *Chapter 5*, respectively), demonstrated how Spanish *Cx. pipiens* and *Ae. albopictus* are competent vectors for WNV and RVFV. In both studies the importance of using variable cycling temperature during the extrinsic period of incubation have been verified. The results suggested that variable cycling temperature allows to estimate a more realistic vector competence. Moreover, different approaches have been used for the detection of viral particles in the saliva of infected mosquitoes.
The third includes *Chapter 6- General discussion* and *Chapter 7- Conclusions.* In this part all the findings are discussed, further studies are proposed and the major conclusions are detailed.
At the end of each chapter all the references used are listed.

Resumen

Las enfermedades transmitidas por mosquitos (ETM) son una creciente preocupación para la salud pública mundial. El aumento de las actividades humanas a escala internacional y el calentamiento global han permitido la rápida difusión y establecimiento de las especies invasoras de mosquitos en nuevas áreas geográficas. Además, la globalización facilita la introducción de arbovirus exóticos en áreas *naïve,* donde el mosquito está presente, incrementando la probabilidad de los brotes de ETM.

Los brotes de ETM, especialmente aquellos causados por arbovirus como el virus del Dengue, el virus del Zika, el virus de la fiebre del valle del Rift (VFVR) o el virus de la fiebre del Nilo Occidental (VFNO), tienen graves consecuencias para la salud pública y/o veterinaria. Las pérdidas económicas debidas a las ETM zoonóticas pueden comprometer severamente la economía del país afectado. Este aspecto es particularmente relevante en los países en desarrollo, donde enfermedades como la fiebre del valle del Rift pueden provocar la muerte de un número extremadamente elevado de animales, tanto domésticos como salvajes. Por estos motivos, es necesario adquirir un profundo conocimiento de las especies de mosquito presentes en un país y cuáles de ellas pueden ser vectores de los distintos agentes patógenos, con el fin de realizar eficientes planes de vigilancia y controles entomológicos.

La presente tesis está dividida en tres partes y cada una de ellas está subdividida en capítulos.

La primera parte se compone de dos apartados: *Chapter 1- General Introduction* y C*hapter 2- Objectives*. El objetivo de esta primera parte es ofrecer una visión general sobre la importancia de las ETM, el rol de los vectores y el concepto de competencia vectorial. Además se proporciona una descripción del VFNO y del VFVR. A continuación, se exponen los

objetivos de la tesis. Hasta ahora se desconoce la dinámica poblacional y la estructura genética de la especie invasora *Aedes albopictus* en España.
Además, no se ha realizado ningún estudio de competencia vectorial de poblaciones españolas de *Cx. pipiens* y *Ae. albopictus* para arbovirus zoonóticos (VFNO y VFVR).
El objetivo de esta tesis fue investigar estos dos puntos.
La segunda parte está formada por tres capítulos. Cada uno es un estudio original, cuyo objetivo es responder a las preguntas propuestas en el segundo capítulo.
En el primer estudio (*Chapter 3*-) se analizó la estructura genética de *Ae. albopictus* en España usando distintas técnicas de análisis genético. En base a los resultados obtenidos se formularon dos hipótesis para explicar la introducción y la difusión de esta especie en el país. Además, se realizó un estudio sobre la dispersión global de *Ae. albopictus.* Los resultados confirman la importancia de las actividades humanas en la dispersión local y global de *Ae. albopictus.*
El segundo y el tercer estudio (respectivamente, *Chapter 4*- y *Chapter 5*-) se demostró que las especies *Cx. pipiens* y *Ae. albopictus* de España son vectores competentes para VFNO y VFVR. En ambos estudios se demostró también la importancia de usar temperaturas variables durante el periodo extrínseco de incubación. Los resultados indican que la utilización de temperaturas variables permite estimar con más precisión la competencia vectorial. Además, se utilizaron distintos métodos para la detección de partículas virales en la saliva de los mosquitos infectados.
La tercera parte incluye *Chapter 6- General discussion* y *Chapter 7- Conclusions.* En esta parte, se discutieron todos los resultados, se propusieron futuros estudios y se enumeraron las conclusiones.
Al final de cada capítulo, se han enumerados todas las referencias usadas.

PUBLICATIONS

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

Brustolin M, Soler-Membrives A, Talavera S, Aranda C, Delacour S, Marquès E, Torrell A, Pagès N. *Phylogeography of Stegomyia albopicta (Aedes albopictus): deciphering the introduction event in Spain and insights of global patterns.* Submitted for publication

Brustolin M, Talavera S, Santamaria C, Rivas R, Pujol N, Aranda C, Marques E, Valle M, Verdun M, Pages N, Busquets N. *Culex pipiens and Stegomyia albopicta (= Aedes albopictus) populations as vectors for lineage 1 and 2 West Nile virus in Europe.* Med Vet Entomol. 2016 Feb 18.
doi: 10.1111/mve.12164.

Brustolin M, Talavera S, Nuñez A, Santamaria C, Rivas R, Pujol N, Valle M, Verdun M, Brun A,Pages N, Busquets N. *Rift Valley fever virus and European mosquitoes: vector competence of Culex pipiens and Aedes albopictus.* Submitted for publication.

PART I

General introduction and Objectives

"Science has explained nothing; the more we know the more fantastic the world becomes and the profounder the surrounding darkness"

Aldous Huxley

Chapter 1

General introduction

1.1 VECTOR-BORNE DISEASES

The general term Vector-Borne Diseases (VBDs) defines all that diseases caused by pathogens transmitted by vectors. Thus, a vector is a living organism that can transmit a pathogenic agent to a sensible host. VBDs include diseases caused by pathogens from four taxons: nematodes, protozoa, bacteria and virus (Gubler, 2009). According to the World Health Organization (WHO), VBDs are responsible for more than 17% of the infectious diseases, with more than 1 million human death cases/year (WHO, 2016). *Jones et al.* estimated that VBDs are responsible for 22.8% of emerging infectious disease events. In this context, an event is defined "as the first temporal emergence of a pathogen in a human population which was related to the increase in distribution, incidence or virulence, or eventually any other factor which led to that pathogen being classed as an emerging disease" (Jones *et al.*, 2008).

The *phylum* arthropoda is one of the most diverse. Arthropods can be found almost everywhere in the world. Arthropods include many different vectors in particular mosquitoes, ticks and sandflies. Hematophagy in arthropod vectors is closely linked to the transmission of pathogen agents. In many insect species, the blood meal is essential for biological processes: mainly growth, development and the synthesis of yolk material in the eggs. Bloodmeals also represents a source of energy for mobility and body maintenance (Beaty *et al.*, 1996). The hematophagy in arthropods is used by pathogen agents to find new hosts, making arthropods highly efficient biological vectors.

The diseases caused by pathogen agents transmitted by arthropods are known as Arthropod-borne diseases (ABDs). ABDs can cause severe morbility and mortality, and the burden of many of these diseases is borne largely by developing countries (Hill *et al.*, 2005). The acronym Arbovirus (Arthropod-borne-virus) refers to a group of viruses transmitted by

arthropods. Viruses from six different families are included among arboviruses (Figure 1-1; (Go *et al.*, 2014)). Many of them, especially from *Flavivirus*/*Alphavirus* genus and *Bunyaviridae* family, are responsible for zoonotic disease with severe implications for public and veterinary health, like West Nile virus (WNV), Eastern Equine Encephalitis virus and Rift Valley fever (RVFV) (Hollidge *et al.*, 2010; Go *et al.*, 2014).

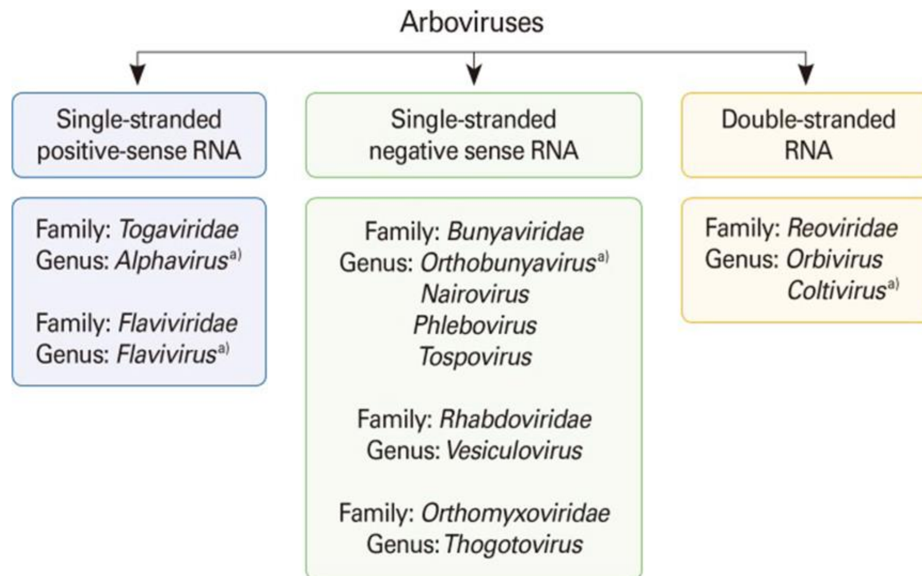

Figure 1-1. Classification of arboviruses. Arboviruses are included in six different taxonomic virus families. [a)]Arboviruses that cause human encephalitis belong to four genera in four virus families. From: (Go *et al.*, 2014).

Mosquitoes act as vectors of several human and animal diseases. Mosquitoes of the genus *Aedes*, *Anopheles* and *Culex* are the best known disease vectors. Mosquito-Borne Diseases (MBDs) owe their notoriety to the high impact on the public and animal health. According to the WHO, in 2015, 214 million of malaria cases were estimated worldwide, with 438.000 deaths associated (WHO, 2015). Moreover, MBDs can cause severe economic loses. The outbreak of Rift Valley fever (RVF) in Egypt 1977 caused more than 115 million USD of economic losses (Bird *et al.*, 2009).

MBDs, and especially those caused by arboviruses, are increasing their global burden (Gubler, 2002; LaBeaud *et al.*, 2011a). Several factors influence this positive trend (Gould *et al.*, 2009; Tabachnick, 2010) however two of them are particularly important: global warming and globalization. The global warming has a direct effect on the density of the mosquito population due to the modification of environmental conditions (mainly temperature and average precipitation), which affect the presence of possible vector (Paz, 2015). Global trade allows the rapid spread of mosquitoes from different regions of the world. It has been certainly confirmed as a way of introduction for invasive species, as is the case of the Asian tiger mosquito, *Aedes albopictus* (Reiter, 1998; Gratz, 2004). Commercial trade is also known to be responsible for disease importation. A common way of disease introduction is related to the importation of infected animals from epidemic areas, as was the case of RVFV in Egypt 1977 (Ahmed Kamal, 2011). As a result of all these factors, the number of arboviral outbreaks is increasing, especially in areas where the disease has never been described before. Recent examples are the outbreaks of Chikungunya virus (CHIKV) in Europe (Rezza *et al.*, 2007; Grandadam M *et al.*, 2011; Delisle E, 2015) and the Caribbean (Cassadou *et al.*, 2014; Cauchemez *et al.*, 2014; Van Bortel *et al.*, 2014) and the Zika epidemics in South America and in the Caribbean (Gubio *et al.*, 2015; Chang *et al.*, 2016).

1.1.1 Mosquito biological cycle

Mosquitoes are classified in the family of *Culicidae,* order Diptera (from the Greek *di*= two and *ptera*= wings). Three subfamilies can be evidenced: *Anophelinae*, *Culicinae* and *Toxorhynchitinae.* Mosquitoes are the most important group of arthropods of medical and veterinary importance and

over than 3500 species and subspecies are widely distributed everywhere in the world, except in the Antarctic (Eldridge, 2005).

Mosquitoes are ectothermic insects. Their body temperature is variable and depends on ambient temperature. This affects different aspects of mosquito life, as for example the length of the immature stages of development or the extrinsic period of incubation during arboviral infection.

Mosquitoes have four development stages: eggs stage, larval stage, pupal stage and the adult or *imago* stage (Figure 1-2). With the exclusion of adult stage, the others stages are aquatic. Eggs are generally deposited on the surface of water, with the exception of *Aedini* mosquitoes which lay the eggs on moist substrates subject to later flooding. Larvae molt three times and become a fourth-stage larva. After a variable time, the larva molts again into pupa. Finally, after a complete metamorphosis, the pupa molt into adult form. Different factors modify the length of the development period. Aquatic temperature and availability of food are inversely related to time of development (Eldridge, 2005).

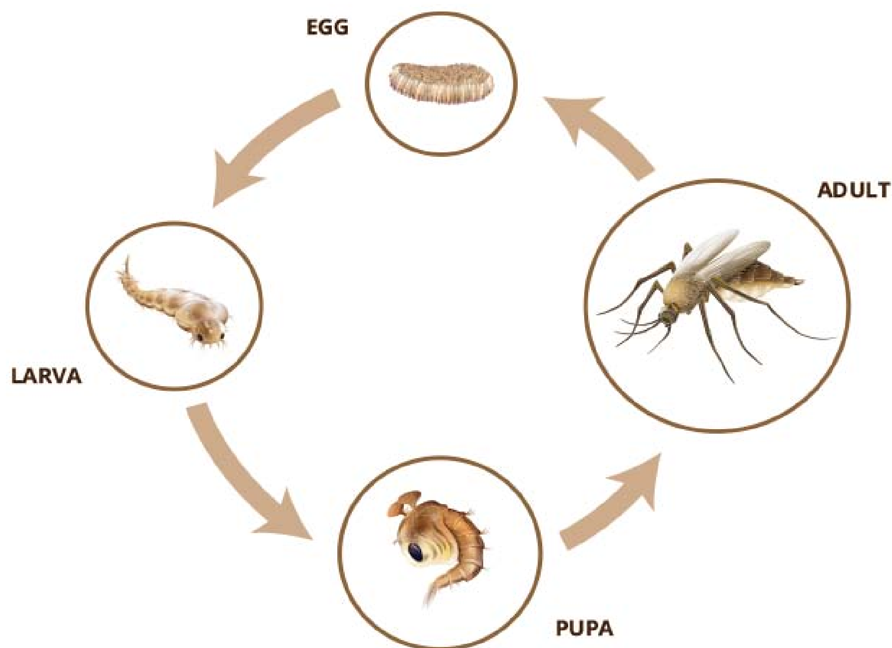

Figure 1-2. Mosquito lifecycle. From: http://www.mosquitoes.org/education/

1.1.2 Vector competence and vectorial capacity

Vector competence (VC) refers to the arthropod ability of acquiring, maintaining and transmitting a pathogen agent (Goddard, 2009). VC is the result of a complex balance between vector and pathogen and it is influenced by intrinsic factors (IFs) and extrinsic factors (EFs) (Hardy *et al.*, 1983).

VC is one of the parameters used to predict the course of an epidemic episode of an infectious disease. Others factors, such as vector density, vector survivability and host density, have to be analyzed. The index resulting from the integration of all of these factors is called vectorial capacity and is expressed by the modified equation of MacDonald done by Garret-Jones in 1964 (MacDonald, 1957; Garrett-Jones, 1964; Smith *et al.*, 2012):

$$\mathrm{V} = \frac{m \cdot a^2 \cdot\ p^n \cdot b}{-\ln p}$$

where m is vector density in relation to the host, a is the probability of the vector to feed on a host in 1 day, b is vector competence, p the probability of vector surviving through 1 day, n is the duration of Extrinsic Incubation Period (EIP, the period of time from the ingestion of infected blood to the transmission capability) and $1/(-\ln p)$ is the duration of the vector's life after surviving the EIP.

Therefore, vectorial capacity is defined as the average number of potentially infective bites that will be delivered by all vectors feeding from a single host in 1 day (Smith *et al.*, 2012).

1.1.2.1 Extrinsic factors of Vector Competence

EFs are all those factors that do not depend directly on the vector. The principal ones are the environmental conditions and the genetic variation of the pathogen. Temperature is the most important environmental parameters. Environmental temperature directly influences the development and growth of the mosquito, and the increase or decrease of vector density. Seasonal rains also affect the density of mosquito populations. The unusual strength of *El Niño* in 2015 caused abnormal rainfalls in all East African countries. The rainfalls enhanced the vector density thus increasing the risk of RVF outbreaks. (FAO *et al.*, 2015). Variation in the vector competence depending on the pathogen can be due to virus evolution (Tsetsarkin *et al.*, 2007) or different viral serotypes (da Moura *et al.*, 2015).

1.1.2.2 Intrinsic factors of Vector Competence

IFs depend directly on the vector. IFs influence vector hability to become infected and to transmit the pathogen, after the ingestion of infectious bloodmeal. The main IFs are the mosquito immunity and the tissue barriers that the virus has to overcome once it enters the mosquito's body.

Mosquitoes are exposed to a wide variety of microorganisms and their immune system has developed different immune signaling pathways against them. The principal ones are the Toll pathway, the immune deficiency pathway (IMD) and the Janus kinase/signal transducers and activator of transcription pathway (JAK-STAT). In addition to these pathways, the mechanism of the RNA interference (RNAi) plays a key role in antiviral defense (Sim *et al.*, 2014).

The insect microbiome is not traditionally considered an intrinsic factor, but reviewing the microbiome effect on the VC, the author of this thesis have considered to include microbiome in this paragraph. The relationship between the insect and its microbiome is complex and affect multiple aspects of insect biology, such as nutrition, digestion, metabolism, development and immunity (Jupatanakul *et al.*, 2014). A recent study shows how the titer of Dengue virus (DENV) serotype 2 in the midguts of antibiotic-treated mosquitoes was two times higher than that in the non-treated mosquitoes (Xi *et al.*, 2008). Contrarily, evidence of negative modulation of vector competence is show in the study by *Ramirez et al.*, 2014. The authors described that *Chromobacterium sp.* isolated from field-caught *Ae. aegypti* can reduce mosquito susceptibility to DENV when introduced to the mosquito midgut tissue (Ramirez *et al.*, 2014). These examples proved the important influence that the microbiome can have on vector competence. Bacteria not associated with midgut flora also play an important role in the biology and in the vector competence of insect (Jupatanakul *et al.*, 2014). Bacteria of the genus *Wolbachia* are obligate intracellular symbionts that have been estimated to infect 66% of insects (Hilgenboecker *et al.*, 2008). *Wolbachia* infects different organs, especially the reproductive system, and it is transmitted to the next generation from female adults to their eggs. The infection of *Wolbachia* interferes with the development of a wide range of pathogens in mosquitoes. Several studies showed that *Ae. aegypti* infected with *Wolbachia* had a high resistance to DENV, CHICK, Yellow fever virus (YFV) and *Plasmodium* infection (Moreira *et al.*, 2009; Walker *et al.*, 2011; van den Hurk *et al.*, 2012b). Moreover, *Wolbachia* can produce cytoplasmatic incompatibility in mosquitoes, which can cause reproductive alterations. For these reasons the use of *Wolbachia* has been proposed for mosquito vector and arbovirus spreading control (Baldacchino *et al.*, 2015).

1.1.2.3 Pathogens transmission

An arthropod may transmit disease agents from host to host in two different ways: mechanical transmission or biological transmission (Gubler, 2009).

- Mechanical transmission consists in simpler transfer of the pathogen agent on contaminated mouthparts or other body parts (Foil, 1989; Mullens, 2002). In this type of transmission neither multiplication nor developmental change of the pathogen are implicated (Mullens, 2002) and the arthropod is a mere vehicle.
- Biological transmission is the most important type of transmission. It can be divided in: i) Propagative transmission when the pathogen undergoes simple multiplication in the body of the vector i.e. arboviruses; ii) Cyclopropagative transmission when both developmental cycle and multiplication are carried out by the pathogen inside the vector i.e. plasmodium; iii) Cyclodevelopmental transmission when the pathogen undergoes developmental changes but does not multiply i.e. filarial worm; iv) Vertical transmission when the pathogen agents are transmitted from the female parent through the eggs to the offspring (trans-ovarian transmission) (Gubler, 2009).

1.1.2.4 Viral infection and tissues barriers

Four principal tissue barriers are present in the mosquito: Midgut Infection Barrier (MIB), Midgut Escape Barrier (MEB), Salivary Gland Infection Barrier (SGIB) and Salivary Gland Escape Barrier (SGEB). They could be virus dose-dependent or independent (Hardy *et al.*, 1983; Franz *et al.*, 2015).

The key steps of an arboviral infection in the mosquito are: a) ingestion of viraemic blood from a vertebrate host; b) infection of midgut epithelial cells, viral replication and subsequently escape from midgut; c) dissemination to hemocoel and secondary tissues; d) infection of the salivary glands (Hardy *et al.*, 1983). After ingestion, the bloodmeal reaches the posterior midgut (Figure 1-3) where the adsorption of blood compounds is carried out. Once in the posterior midgut region, arboviruses enter the epithelial cells via receptor-mediated endocytosis. Some *Alphavirus* have the capacity to enter by direct penetration. Once inside the cell, the virus does a first cycle of replication (Franz *et al.*, 2015).

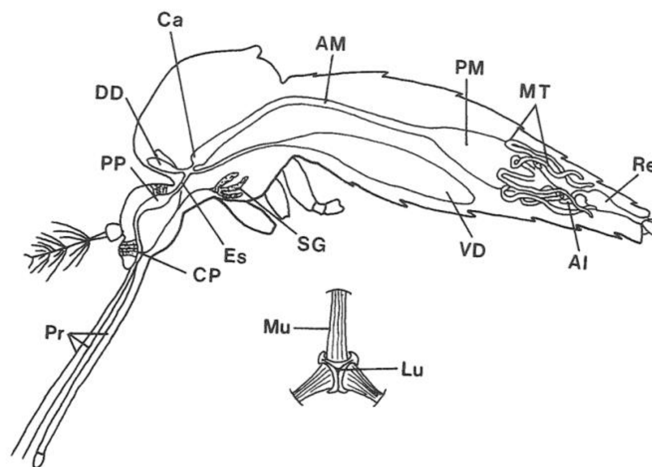

Figure 1-3. Mosquito alimentary canal. Below, cross-section of pharyngeal pump. AI= anterior intestine, AM= anterior midgut, Ca= cardia, CP= cibarial pump, DD= dorsal diventriculum, Es= esophagus, MT= Malpighian tubule, PM= posterior midgut, PP= pharyngeal pump, Pr= proboscis, Re= rectum, SG= salivary gland, VD ventral diventriculum (crop), Mu= muscle, Lu= lumen. The proboscis is the organ assign to suck the blood. Thanks to the cibarial pump and pharyngeal pump the blood is pumped throw the esophagus and the foregut to the posterior midgut for the digestion. The cardia regulates the passage of material from foregut to midgut and prevents the reflux from midgut to foregut. Dorsal and ventral diventriculum are not involved in the digestion of bloodmeal, but they are implicated in the storage and the digestion of sucrose solutions (water and/or nectar). From: (Beaty *et al.*, 1996).

Failure in the infection of the epithelium cells indicates the presence of MIB. The impossibility to enter inside the cell is generally related to an absence/modification of the receptor and represents a dose independent barrier. The peritrophic matrix (PM), a chitinous sac which involves the bloodmeal during the digestion, also prevents the entrance of pathogens in the midgut epithelial cells (Lehane, 1997). However, the formation and the maturation of PM requires until 12 hours in *Aedes aegypti* (Perrone et al., 1988). This lag of time between the ingestion of infectious blood and the formation of mature PM makes possible the infection of the mosquito epithelial midgut cells by some arboviruses (Lehane, 1997). The impossibility of some arboviruses to replicate inside the midgut epithelial cells is principally due to the RNAi pathway, a component of the innate immune response. This is a dose-dependent mechanism of immune defense that can be overcome by a sufficient titer of virus (Campbell *et al.*, 2008; Sánchez-Vargas *et al.*, 2009) .

In order to disseminate from midgut to hemocoel and secondary target organs, the virus has to evade from midgut epithelial cells. For that, the virus has to overcome the Basal Lamina (BL). The BL is formed by a proteinaceous matrix which surrounds the basolateral surface of epithelial cells (Franz *et al.*, 2015). This densely packed matrix provides structure to epithelium and is a physical barrier for microorganisms and viruses. The BL has a porous nature. The pore size was investigated by *Reddy and Locke* (Reddy *et al.*, 1990) demonstrating how BL is a physical barrier to the arboviruses. The escaping from BL can be possible by transient transformation. During the blood feeding, the gut can expand its size several times causing extensive reorganization of BL and the formation of temporal gaps, which can be used by the virus to cross the BL into the hemocoel or into the tracheal system (Franz *et al.*, 2015). Several studies underline the importance of trachea system in the dissemination of the virus (Romoser *et al.*, 2004; Romoser *et al.*, 2005). Trachea and tracheoles

penetrate the BL and directly connect to the midgut epithelial cells, providing the virus with a way of evading the BL.
A schematic representation of different permissive and not permissive midgut epithelial cells is shown in figure 1-4.

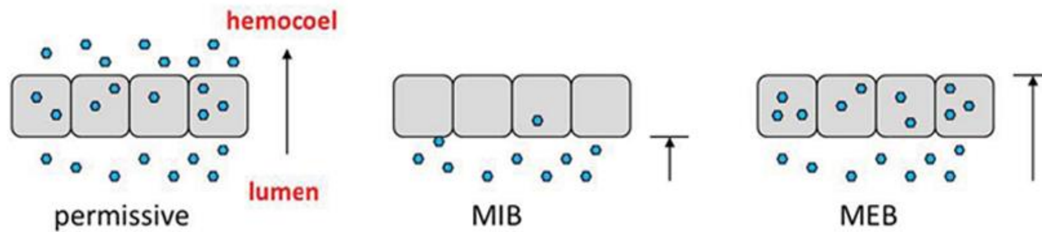

Figure 1-4. Schematic representation of midgut barrier. Midgut Infection Barrier (MIB), Midgut Escape Barrier (MEB). From (Franz *et al.*, 2015).

Once the virus overcomes the BL, it infects several tissues and organs. For some couple of mosquito-virus species, vertical transmission to the offspring is possible if the virus reaches and infects the ovarian, as in the case of *Aedes spp*. infected by RVFV (Linthicum *et al.*, 1985).
Infection of salivary glands is fundamental for the transmission of the virus to a sensible host. The SGIB is the first barrier that virus have to overcome in order to infect the cells of salivary glands. As in the case of midgut cells, the BL surrounding the salivary glands epithelial cells acts like a physical barrier (Romoser *et al.*, 2005). *Romoser et al.* also suggest that the structure of the BL could occasionally obscure the cell surface receptor necessary for cell entry. Finally, the presence of SGEB has been described for La Cross virus and Sindbis virus in *Aedes* and *Culex* mosquitoes (Grimstad *et al.*, 1985; Jupp, 1985). Molecular mechanisms that clearly explain SGIB or SGEB have not been described so far. However, the role of apoptosis as a possible mechanism used by the virus to destroy the acinar cells of the salivary gland and be released into the salivary ducts, has been hypothesized (Franz *et al.*, 2015).

1.1.2.5 Extrinsic incubation period & Extrinsic Incubation Temperature

The extrinsic incubation period (EIP) is the period of time between the ingestion of an infectious bloodmeal by a vector and the viral transmission to a sensible host while refeeding (Hardy *et al.*, 1983). EIP establishes how long a mosquito must survive before it can transmit a virus, influencing the vectorial capacity. EIP is a dynamic parameter, it varies in each arbovirus-mosquito system and is influenced by several factors, being the temperature one of the most important (Hardy *et al.*, 1983). The extrinsic incubation temperature (EIT) corresponds to the temperature at which the vector is exposed during the EIP. Under laboratory conditions, different studies demonstrate that EIP is normally inversely proportional to the EIT (Dohm *et al.*, 2002; Anderson *et al.*, 2008; Tjaden *et al.*, 2013; Xiao *et al.*, 2014). This relation between EIT and EIP is due to the effect of the temperature on the replication and on the dissemination of the virus, as showed by different studies (Brubaker *et al.*, 1998; Dohm *et al.*, 2002; Lambrechts *et al.*, 2011; Richards *et al.*, 2012; Xiao *et al.*, 2014).

1.1.3 Arboviruses and surveillance programs

Surveillance programs are essential to ensure an early detection of virus activity before an outbreak occurs (van den Hurk *et al.*, 2012a). Coordinated human, veterinary, entomological and environmental surveillance is needed for arbovirus surveillance (Zeller *et al.*, 2013). Abundance and spatio-temporal distribution of virus can be measured with sentinel animal surveillance, isolation virus from vectors, virus and viral antibody isolation from wild and domestic animal. However, it is important to note that isolation of virus from a mosquito or a pool of mosquitoes does not provide evidence of virus transmission capacity (Rutledge *et al.*, 2003).

Recently, the use of honey-baited FTA cards to rapidly detect circulation of arboviruses and set up a "mosquito-free" surveillance system has been improved (van den Hurk *et al.*, 2012a).
Entomological surveillance is essential to know which mosquito species are present in the territory and their seasonality, with special interest to invasive mosquito species. Then vector competence studies result indispensable to determine whether the mosquito populations present in the territory are competent for a specific pathogen.
Entomological surveillance is fundamental to estimate abundance and spatio-temporal distribution of mosquito populations present in a determined area. Both adult and immature mosquitoes (larvae or pupae) can be sampled and identified (Day *et al.*, 2011). The use of baited traps to collect adult mosquitoes is the most used technique. This technique is an efficient tool to understand the ecological assessment of vector populations and determine potential areas under risk.

1.1.3.1 Invasive vector species in Europe

The global warming and increasing globalization have favored the introduction and the establishment of invasive mosquito species (Baldacchino *et al.*, 2015). Some species of mosquitoes in the genus *Aedes* are particularly important and frequently found as invasive species (Baldacchino *et al.*, 2015). The success of invasive capacity of *Aedes* species is linked to their physiological plasticity that allows the mosquito to adapt to different environmental conditions (Paupy *et al.*, 2009). Moreover, their eggs can withstand desiccation for several months and survive long transportation time (Baldacchino *et al.*, 2015). The importance of invasive species is principally linked to their ability to be competent vectors for pathogens that cause exotic diseases (Medlock *et al.*, 2015). Moreover, they

are a serious nuisance for people reducing the quality of life (Aranda *et al.*, 2006). There are currently five invasive *Aedes* mosquito species known to be established in Europe, namely *Ae. albopictus*, *Ae. aegypti*, *Ae. japonicus*, *Ae. koreicus* and *Ae. atropalpus* (Medlock *et al.*, 2015). *Ae. albopictus* was first identified in Europe in Albania (1979) and its introduction has been linked to the international trade of used tyres with China. Since then, *Ae. albopictus* rapidly spread to 25 different countries of Europe, including Spain (Medlock *et al.*, 2015). *Ae. albopictus* is known to be a competent vector for more than 26 arbovirus (Moore *et al.*, 1997), including CHIKV or DENV. In 2007, it was the responsible for the first outbreak of CHIKV in Europe (Rezza *et al.*, 2007). *Ae. aegypti* is established in Madeira island (Portugal outermost region) since 2004/2005 (Almeida *et al.*, 2007) and more recently in Southern Russia and Georgia (Medlock *et al.*, 2015). The presence of *Ae. aegypti* increases the risk of transmission of Yellow fever virus (YFV) and DENV. However, its intolerance to temperate winter limits its capacity to establish in temperate regions (Medlock *et al.*, 2015). *Ae. japonicus* was first reported in Europe in 2010 and it is currently established in Austria, Belgium, Croatia, France, Germany, Hungary, Switzerland and Slovenia (Medlock *et al.*, 2015). *Ae. japonicus* is not considered an important disease vector, however, a recent study shows his vector competence for transmitting CHIKV and DENV (Schaffner *et al.*, 2011). *Ae. koreicus* was identified for the first time in Belgium in 2008 and later in Italy in 2011. Actually the species is established only in these two countries (Medlock *et al.*, 2015). *Ae. atropalpus* was introduced in Italy between 1996 and 1997, in France in 2003 and 2005, and in the Netherlands in 2009. However, immediate control measures prevented the establishment of mosquitoes and currently no population is known to persist in Europe (Medlock *et al.*, 2015).

1.1.3.2 Autochthonous vector species in Europe

The number of autochthonous mosquito species that can be potential vector of pathogens is very high. A detailed study to determine which mosquito species could be a competent vector should be done for every pathogen. For the purpose of this thesis, only the most important autochthonous species considerate vectors for WNV and RVFV will be mentioned.

Cx. pipiens, *Cx. perexiguus*, *Cx. modestus* and *Coquilletidia richiardii* are indicated as principal vector of WNV in Europe (Hubalek *et al.*, 1999; Rizolli *et al.*, 2015); similarly, *Cx. pipiens*, *Cx. perexiguus*, *Cx. theileri*, *Ae. vexans* and *Ochlerotatus caspius* are considerate the principal potential vector of RVFV in Europe (Chevalier *et al.*, 2010).

Different forms of *Cx. pipiens* are present in the territory: *Cx. pipiens* form *pipiens*, *Cx. pipiens* form *molestus* and its hybrid form. The different forms of *Cx. pipiens* present different behavior: *Cx. pipiens* form *pipiens* feed mainly from birds and *Cx. pipiens* form *molestus* feed mainly from mammals (Farajollahi *et al.*, 2011). The hybrid between pipiens and molestus forms present a duplex host pattern behavior. This make hybrid form a superior bridge vector for WNV.

1.2 WEST NILE FEVER VIRUS

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae*. WNV was first identified from the blood of a febrile women in the West Nile district of Uganda in 1937 (Smithburn *et al.*, 1940). WNV is the most widespread flavivirus in the world. It is maintained in nature in a mosquito-bird enzootic cycle but infections have been reported in a wide number of vertebrate species. In the last 20 years, numerous outbreaks of West Nile virus disease have been reported in humans and horses in Africa, Europe and the Americas, making WNV a major issue for public and veterinarian health.

1.2.1 Classification

WNV belongs to the Japanese encephalitis serocomplex, based on the cross-neutralization tests with polyclonal sera (Poidinger *et al.*, 1996). Phylogenetic studies classified WNV into nine different lineages (Pachler *et al.*, 2014). However, a recent study proposed a re-organization and harmonization of WNV lineages into seven (Rizolli *et al.*, 2015) (table 1-1). Lineage 1 has the mayor genetic variability and is divided in 3 clades. Lineage 1a is the most widely distributed and has been identified in Europe, America, Africa, Middle East and Israel (Bondre *et al.*, 2007; Calistri *et al.*, 2010). This clade includes strains causing severe diseases in humans, like WN-NY99 strain, responsible for the New York outbreak in 1999 (Lanciotti *et al.*, 1999) . Lineage 1b is formed by Kunjin virus (KUNV), a subtype of WNV circulating in Australia (Hall et al., 2001; Melinda et al., 2012). Lineage 1c is formed by strains isolated only in India (Bondre et al., 2007). Lineage 2 was traditionally limited to the sub-saharian Africa and

Madagascar (Bakonyi *et al.*, 2013). Strains of lineage 2 generally cause self-limiting illness that rarely progress to severe disease in humans. WNV strains of lineage 2 have been responsible for different outbreaks in Europe (Calistri *et al.*, 2010; Bakonyi *et al.*, 2013) since first identification of WNV lineage 2 in a wild goshawk in Hungary (Bakonyi *et al.*, 2006). This makes this lineage the second most widespread. Phylogenetic analyses revealed that all European WNV lineage 1 and 2 strains are derived from a limited number of independent introductions, most likely from Africa, followed by local spread and evolution (Rizolli *et al.*, 2015).

Table 1-1. Overview of West Nile virus lineages and suggested lineage numbering. Adapted from (Rizolli *et al.*, 2015).

Suggested lineage numbering	Other lineage labelling in the literature	Representative strain	GenBank accession number	Note
Lineage 1a	Lineage 1	NY99-flamingo382-99, New York 1999	AF196835	Most widespread WNV lineage
Lineage 1b	Lineage 1	Kunijin MRM61C, Australia, 1960	D00246	Kunijin virus strain, Australia
Lineage 1c	Lineage 5	804994, India, 1960	DQ256376	Only found in India
Lineage 2	No	B956, Uganda, 1973	AY532665	Second most widespread WNV lineage
Lineage 3	No	Rabensburg virus 97-103, Czech Republic 1997	AY765264	Only found in central Europe
Lineage 4a	Lineage 4	LEIV-Krnd88-190, Russia, 1998	AY277251	Originally isolated from Dermacentor ticks
Lineage 4b	Lineage 6 - 7	HU2925/06, Spain	GU047875	Only partial sequence available
Lineage 4c	Lineage 9	WNV-uu-LN-AT-2013, Austria, 2013	KJ831223	Identified in Uranotaenia mosquitoes
Lineage 5	Lineage 6	Kunijin virus KUN MP502-66, Malaysia, 1966	GU047874, HQ840762	Only partial sequences available
Lineage 6	Lineage 7	Dak Ar D 5443, Senegal	EU082200	Koutango virus
Lineage 7	Lineage 8	ArD94343, Senegal 1992	KJ131502	Only partial sequence available

1.2.2 Morphology and genome

WNV is a small spherical icosahedral virus with 50 nm of diameter surrounded by a lipid envelope. The nucleocapsid is formed by capsid (C) proteins (Rossi *et al.*, 2010) while the envelope is formed by a lipid bilayer derived from the host cell. The envelope is constituted by 180 copies of membrane (M) proteins and an equal number of E glycoprotein (Martin-Acebes *et al.*, 2012). The WNV genome is a single stranded, positive sense RNA of ~ 11 kilobase encoding for a single open reading frame (ORF), flanked by 5′ and 3′ untranslated regions (UTR) (Rossi *et al.*, 2010) (Figure 1-5).

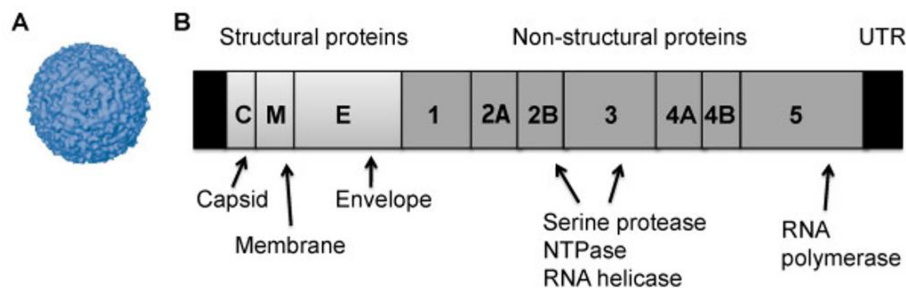

Figure 1-5: The structure (a) and RNA genome (b) of West Nile virus. From (De Filette *et al.*, 2012).

1.2.3 WNV transmission cycle

WNV is maintained in nature in an enzootic transmission cycle between avian host and mosquito vectors (Figure 1-6). Mosquito species involved in the primary transmission cycle are referred to as amplification vectors and generally they are strongly ornitophilic. Mosquito with more general feeding behavior can transmit WNV to non-avian vertebrate, after feeding from an infected avian host. They are referred to as bridging vectors (Blitvich, 2008).

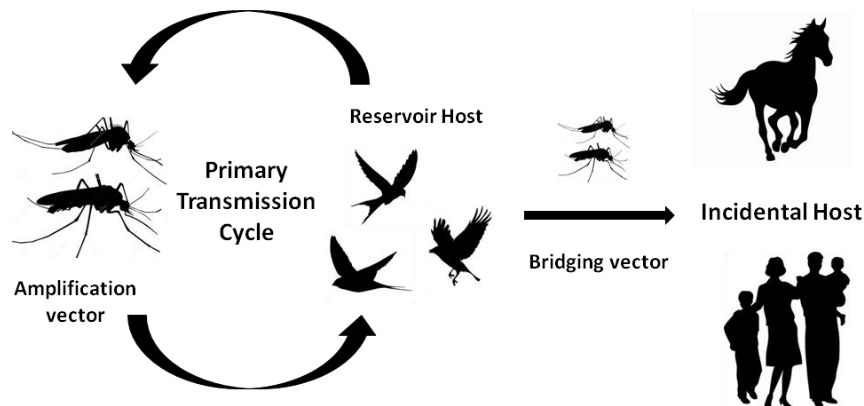

Figure 1-6: WNV transmission cycle. WNV is transmitted primarily by the bite of infected mosquitoes. Mosquitoes acquire the virus by feeding on infected birds.

Birds are the natural reservoir of WNV. The virus has been detected in more than 150 species of wild and domestic birds (Komar, 2003; van der Meulen *et al.*, 2005). *Passeriformes* (corvids, sparrow, finches etc.), *Charadriiformes* (woodcocks, gulls, etc.) *Stigiformes* (owls, eagle, owls. etc.) are the most affected birds by WNV. They develope high viremia and mortality rate (Komar *et al.*, 2003; Marka *et al.*, 2013). By contrast, birds belonging to the order of *Paciformes*, *Columbiformes* and *Aseriformes* develop a low viremia and they would not participate in the transmission cycle (Marka *et al.*, 2013). Many avian species shed large quantities of virus in their feces or oral secretion (Busquets *et al.*, 2012) and direct transmission from bird-to-bird has been experimentally proved (McLean *et al.*, 2001). Moreover, pray-to-predators transmission has been suggested (Garmendia *et al.*, 2000). Antibodies against WNV have been detected in more than 30 non avian-vertebrates, including mammals, reptiles and amphibians (van der Meulen *et al.*, 2005; Blitvich, 2008). Non-avian hosts are defined as "incidental hosts" or "dead end hosts" such as humans and horses. The incidental hosts develop a low viremia, insufficient to allow transmission to feeding mosquitoes (Hayes *et al.*, 2005a). Generally, a host should have a viremia > 10^5 PFU/ml to serve as reservoir hosts, as showed in

experimental infection done in birds (Turell *et al.*, 2000; Komar *et al.*, 2003). The virus can also be spread among human beings thought blood transfusion and organ transplantation (Charatan, 2002). Vertical transmission from mother to newborn has been rarely described via the intrauterine route (CDC, 2002b) or via breast-feeding (CDC, 2002a).

1.2.3.1 Disease in humans

The human incubation period of WNV ranges from 2 to 14 days and the symptoms usually last from 2 to 5 days (Campbell et al., 2002). About 80% of human infections are apparently asymptomatic (Hayes et al., 2005b). The remain 20% of patients develop symptoms of West Nile fever (WNF) characterized by severe non-specific flu-like symptom: acute onset of fever, headache, fatigue, malaise, muscle pain and weakness; in some cases, macular rush and gastrointestinal problems have been reported (Watson et al., 2004). Less than 1% of the people infected by WNV has developed a severe neurological disease (Hayes et al., 2005b). Three different neuroinvasive forms have been reported: West Nile meningitis (WNM), West Nile encephalitis (WNE), and West Nile poliomyelitis (WNP) with an estimated case-fatality rate respectively of <1% (WNM), 10% (WNE) and 20-50% (WNP) (Hughes et al., 2007).

1.2.3.2 Disease in horses

WNV infection in horse generally passes without evident symptoms. Approximately 10% of infected animals develop clinical signs (Bunning *et al.*, 2002; Gardner *et al.*, 2007). Except for fever, clinical signs of WNV in horses are almost exclusively of neurological nature: ataxia, depression or

anxiety, stupor, behavioral changes, paresis or paralysis of one or several limbs, clinical signs of cranial nerve paralysis, teeth grinding, muscle twitching, fasciculation and tremors, convulsions, colic, and intermittent lameness, or death with mortality rates of 50-60% of infected animal (Castillo-Olivares *et al.*, 2004; Saegerman *et al.*, 2016).

1.2.3.3 Disease in birds

The disease in birds is typically associated with neurological signs such as ataxia, paralysis, inability to hold head upright and unusual posture as well non-neurological signs as ruffled feathers and in some American crow external hemorrhage, either from the mouth or from the cloaca (Komar *et al.*, 2003). As mentioned before, the disease affects the *Passeriformes*, the *Charadriiformes* and the *Stigiformes* more severely, and high viremia, associated with high mortality, is reported among these orders of bird. In contrast chicken, duck and pigeon show no symptoms and developed low viremia (Hurlbut *et al.*, 1956; Komar *et al.*, 2003).

1.2.4 WNF epidemiology

The first WNV infection in human in the area of the Mediterranean basin was reported in the 1950s in Israel (Bernkopf *et al.*, 1953; Murgue *et al.*, 2001). Since then, several sporadically outbreaks were reported in central Europe (Martin-Acebes *et al.*, 2012). Three dates mark the evolution in the perspective of WNV: Romania 1996, New York 1999 and Hungary 2004. The human outbreak of Romania points the re-emerge of WNV in Europe. An outbreak of human encephalitis in New York City in 1999 points the introduction of WNV in North America (Lanciotti *et al.*, 1999). The New

York strain (WN-NY99), isolated from a dead flamingo in the Bronx zoo and responsible of the outbreak, showed high similitude to a strain isolated in the Middle East. A possible introduction of WNV from this region have been suggested (Lanciotti *et al.*, 1999); however, the way of introduction is still unknown (Blitvich, 2008; Martin-Acebes *et al.*, 2012). The Center of Disease Control and Prevention (CDC) has reported 41,762 human infections (18,810 neuroinvasive cases) with a total of 1,765 death cases from 1999 to 2014, (CDC, 2015). The isolation of a lineage 2 WNV strain from a goshawks (*Accipiter gentilis*) in southeast of Hungary in 2004 (Bakonyi *et al.*, 2006) points the start of circulation of lineage 2 strain in Europe.

1.2.4.1 Europe and Mediterranean basin

Besides the first outbreak in 1950, the most notable outbreak in Europe before 1996 was reported in the Camargue region of France in 1962-1963 with encephalitic cases in humans and horses (Calistri *et al.*, 2010). After this outbreak, a period of 20 years of low WNV activity was registered in Europe, without outbreaks reported. However, the circulation of WNV in Europe is attested by serological studies done by the surveillance programs (Zeller *et al.*, 2004). In Spain, evidence of WNV circulation in the Ebro Delta in 1979 was attested by a retrospective serological study (Lozano *et al.*, 1998). Several outbreaks have been reported in humans from 1994 to 2004 in the Mediterranean basin and continental Europe. All of them were caused by WNV strains belonging to lineage 1: Algeria 1994 (50 cases), Morocco 1996 (1 case), Romania 1996 (393 cases), Tunisia 1997 (173 cases), Russia 1999 (318), Israel 1999 and 2000 (2 and 417 cases respectively), Russia 2000-2001 (120 cases), France 2003 (7 cases), and Spain 2004 (1 case) (Zeller *et al.*, 2004; Calistri *et al.*, 2010). The outbreak of Romania 1996 was considered the first recorded outbreak of WNV in an urban area (Tsai *et al.*,

1998) with 393 humans patients serologically positive. In the same period epizootics in horses were reported in Morocco 1996 (94 cases), Italy 1998 (14 cases), France (76 cases) and Israel 2000 (76 cases), (Zeller *et al.*, 2004; Calistri *et al.*, 2010).

In 2004 strain of both lineages (1, 2) started to circulate concurrently in Europe and the Mediterranean basin causing an increasing number of outbreaks, with reported cases in humans and animals. The human and horse outbreaks reported from 2004 to 2015 are shown in table 1-2. Horse outbreak and country with reported human cases from 2004 to 2015 are shown in figure 1-7.

Table 1-2: WNV outbreaks in Europe. ʎ = human outbreak; Ω= horse outbreak; in red= lineage 1 outbreak; in blue= lineage 2 outbreak; in green= lineage 1 and 2 outbreaks; in black: lineage data not available.

	2004	*2005*	*2006*	*2007*	*2008*	*2009*	*2010*	*2011*	*2012*	*2013*	*2014*	*2015*
Albania							ʎ	ʎ	Ω			
Austria							ʎ				ʎ	ʎ
Bosnia-Herzegovina										ʎ	ʎ	
Bulgaria							Ω		ʎ			ʎ Ω
Croatia									ʎ Ω	ʎ		ʎ
Czech Republic	ʎ		ʎ							ʎ		
France	Ω		Ω									ʎ Ω
Greece							ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	
Hungary					ʎ		ʎ	ʎ	ʎ	ʎ	ʎ Ω	ʎ Ω
Italy					ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω
Kosovo									ʎ			
Macedonia								ʎ	ʎ	ʎ		
Montenegro									ʎ	ʎ		
Portugal							Ω					ʎ Ω
Romania					ʎ	ʎ	ʎ Ω	ʎ Ω	ʎ Ω	ʎ Ω	ʎ	ʎ
Russia		ʎ	ʎ	ʎ	ʎ	ʎ	ʎ	ʎ	ʎ	ʎ	ʎ	
Serbia						Ω	Ω		ʎ	ʎ	ʎ	
Slovenia										ʎ		
Spain	ʎ						ʎ Ω	Ω	Ω	Ω	Ω	Ω
Turkey							ʎ	ʎ Ω				
Ukraine								ʎ	ʎ	ʎ		

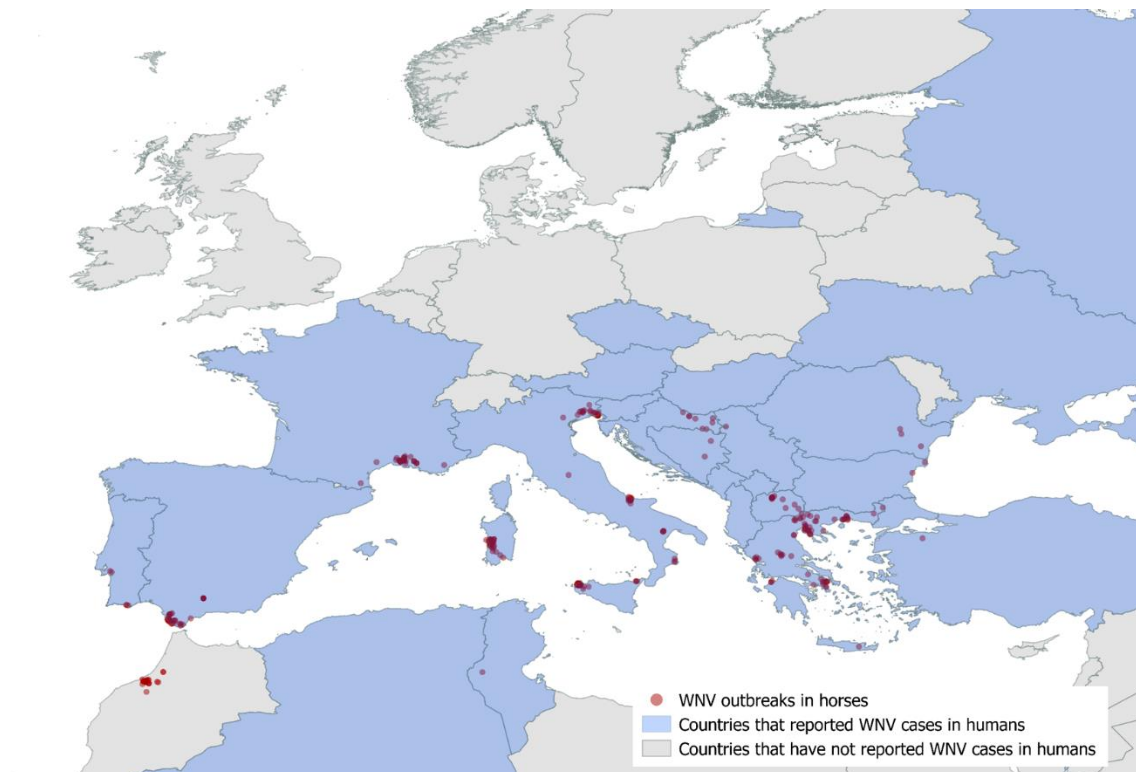

Figure 1-7: Distribution of WNF cases by affected areas in the European countries and Mediterranean basin (2004 – 2015).

1.2.4.2 Arthropod vector

WNV has been isolated from mosquitoes belonging to 12 genera: *Aedes*, *Aedeomyia*, *Anopheles*, *Coquilletidia*, *Culex*, *Culiseta*, *Deinocerites*, *Mansonia*, *Mimomyia*, *Orthopodomyia*, *Psorophora* and *Uranotaenia* (Hribar *et al.*, 2003; Zeller *et al.*, 2004). Ornitophilic mosquitoes from *Culex* genus are considered the major amplification vectors of WNV (Hayes *et al.*, 2005a). In Europe the virus was isolated mostly from *Cx. pipiens, Cx. antennatus*, *Cx. univittatus* and *Cx. modestus* (Hubalek *et al.*, 1999; Zeller *et al.*, 2004). However, mosquitoes of *Cx. pipiens* complex and their hybrids are considered the principal vectors (Rizolli *et al.*, 2015). In North America, WNV has been isolated in more than 59 mosquito species, but less than 10

of these are considered principal vector (Hayes *et al*., 2005a). Among them the most importants are *Cx. pipiens, Cx. quinquefasciatus*, *Cx. tarsalis, Cx. restuans, Cx. salinarius,* and *Cx. nigripalpus.* Several other species are reported as competent vectors in different regions of the world: in India and Pakistan *Cx. vishnui* and *Cx. tritaeniorhynchus* are considered principal vectors (Brault, 2009), and in Australia *Cx. annulirostris* is the principal one. In Africa and the Middle East, *Cx. univittatus* is the major vector although *Cx. poicilipes*, *Cx. neavei* and *Cx. decens* are important in certain areas (Hubalek *et al.*, 1999). Despite the predominant role of *Cx.* species as vectors, many other species, such as *Ae. albopictus* and *Ae. vexans,* have been described as competent vectors (Turell *et al*., 2000; Turell *et al*., 2001) and they can play an important role as bridging vectors.

It is interesting to note that WNV has been sporadically isolated in other blood-sucking arthropods: soft ticks *Argas hermanni* and *Ornithodoros capensis*; hard ticks *Hyalomma marginatum, Rhipicephalus turanicus, Amblyomma variegatum* and *Dermacentor marginatus (Hubalek et al., 1999).* Soft ticks have been demonstrated to be able to transmit the virus under laboratory conditions while hard ticks (*Ixodidae*) allow the virus to pass transstadially but they are incompetent vector (Mumcuoglu *et al*., 2005). Other arthropod like mites and hippoboscid flies have been described as possible vectors, however, their role in the transmission cycle is unclear (Martin-Acebes *et al*., 2012).

1.3 RIFT VALLEY FEVER VIRUS

Rift Valley Fever virus (RVFV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Phlebovirus* of the *Bunyaviridae* family. RVFV was first described in 1931, in the Rift Valley province of Kenya (Daubney *et al.*, 1931). The virus is maintained in nature in an enzootic cycle involving mosquitoes, especially of *Culex* and *Aedes* species, and domestic and wild ruminants. RVFV can also infect humans causing, in a minority of cases, a severe hemorrhagic disease. Outbreaks of Rift Valley Fever (RVF) are traditionally reported in several countries of Africa. However, since 2000 the virus was first detected outside Africa, in the Middle East (Ahmad, 2000).

1.3.1 Morphology and genome

Virions of RVFV are spherical (80-120 nm diameter), consisting of an envelope and a ribonucleocapsid (Ikegami, 2012). Four different structural proteins form the virion: the glycoproteins Gn and Gc, the nucleoprotein (N) and the RNA-dependent RNA polymerase (RdRp). The RNA segments of RVFV genome are associated with the N protein and the RdRp to form pseudohelicoidal ribonucleoproteins (Raymond *et al.*, 2010). A total of 122 capsomers project as spike from the lipid bilayer on an icosahedral lattice T=12 quasisymmetry (Freiberg *et al.*, 2008) (Figure 1-8a). The capsomers are formed by hexamers and pentamers of Gn-Gc heterodimers.
The genome of RVFV is about 12 kilobases. It is composed of 3 segments of single strand RNA called S (small), M (medium) and L (large), reflecting the relative nucleotide length (Walter *et al.*, 2011). The S segment is encoded

in both sense and antisense, while M and L segments are negative sense (Figure 1-8b).

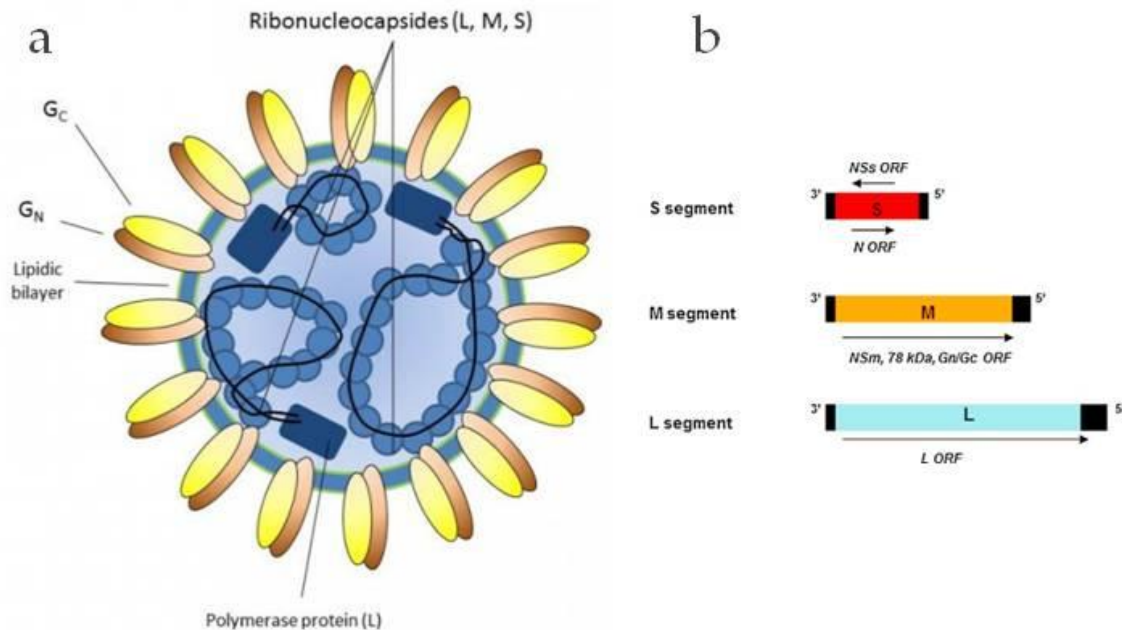

Figure 1-8: RVFV virion structure and genome organization. a) virion structure Modified from *www.bunyavirus.org.* b) Schematic representation of small (S), medium (M) and large (L) segments. Direction of open reading frames and protein are evidenced for every segment. From: (Mansfield *et al.*, 2015).

1.3.2 RVFV transmission cycle

RVFV transmission cycle involves mosquitoes and domestic/wild ruminants (Figure 1-9). Two different but overlapping cycles are involved in the ecology of RVFV: a low-level enzootic cycle and an epizootic/epidemic cycle (Bird *et al.*, 2009).

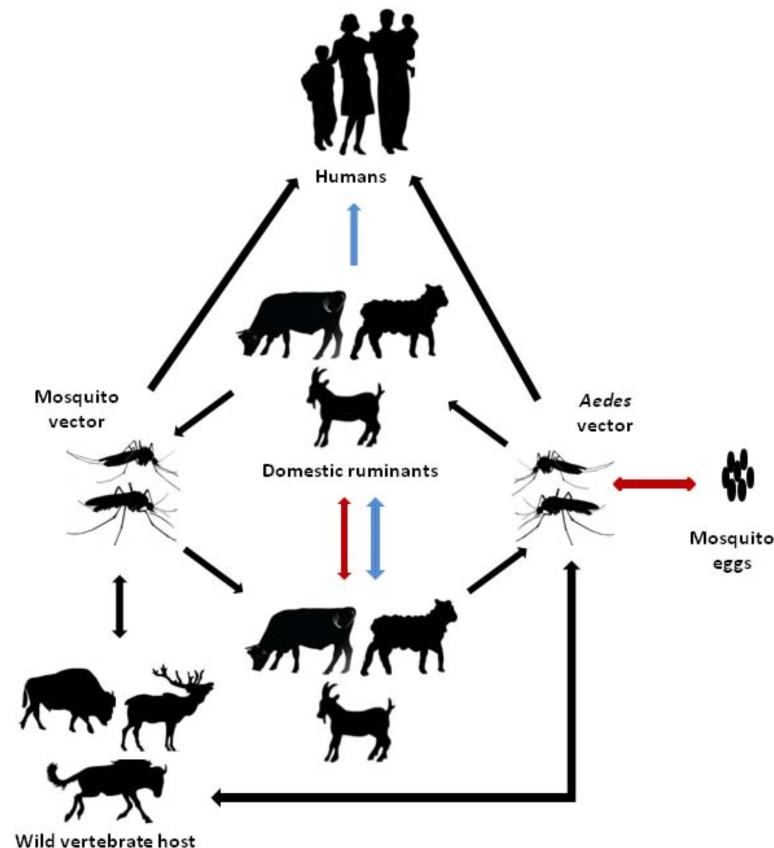

Figure 1-9: RVFV transmission cycle. Black Arrow: vectorial transmission; red arrow: vertical transmission; blue arrow: direct transmission. RVFV is maintained in nature in a mosquito-vertebrate cycle. During inter-epizootic period the capacity of some *Aedes spp.* to vertically transmit the virus to the offspring maintain the virus in the cycle. Despite the possibility to be infected by mosquito bite the principal source of infection for humans is the direct contact with body fluids of infected animal.

RVFV has an epizootic cycle that affects livestock, causing long outbreaks of 1-3 years of duration with a huge number of animals deaths and big economical losses (Davies *et al.*, 1985). The outbreak usually follows a period of intensive rainfalls and generally occurs every 5-15 years (Davies *et al.*, 1985). Large-scale weather phenomenon *El Niño* can influence these two cycles causing heavy rainfall in eastern and southern Africa (Linthicum *et al.*, 1999). The virus is maintained during large inter-epizootic period by transovarian transmission of floodwater mosquito, as showed for *Ae. mcintoshi* (Linthicum *et al.*, 1985). The eggs of *Aedes* mosquitoes of *Neomelaniconion* subgenus are drought-resistant and remain viable for months/years in field's depression (*dambos*) (Bird *et al.*, 2009). After a rainfall the flooding of *dambos* induces the hatching of the eggs (Davies *et al.*, 1985; Zeller *et al.*, 1997) and the rise of infected mosquito. Flooding mosquitoes generally start the epizootic cycle, biting a sensible host, typically cows and sheep, and transmitting the pathogens. In a few days, the host develops a high-titer viremia (10^6-10^8 PFU/mL) and a naïve mosquito can be infected once it is fed. Mosquitoes of other genus, especially *Culex*, serve as secondary amplification vectors. Aborted fetal materials and placenta membranes contain large number of virus particles that can infect animals in close contact making possible direct infection and sustaining the amplification (Pepin *et al.*, 2010). Moreover, lactating animals can infect their young via milk. Serological evidence of RVFV in wild animals have been reviewed and a large number of species seems to have been affected, especially among wild artiodactyls (Olive *et al.*, 2012), like the African buffalo (*Syncerus caffer caffer*) (LaBeaud *et al.*, 2011b). Evidence of infection in small rodents has been described but their role in the transmission cycle is not clear (Pretorius *et al.*, 1997; Youssef *et al.*, 2001). Humans can be infected but do not develop sufficient viremia to sustain the transmission cycle and they are considered a dead-end host (Chevalier *et al.*, 2010). Despite the possibility of being infected by mosquito (McIntosh

et al., 1973), the principal source of infection for humans is the contact with body fluids of infected animal; for this reason, veterinarians, farmers and slaughterhouse workers are within the most affected job categories (Archer *et al.*, 2011). Drinking raw milk has also been reported to cause infection in humans (Nicholas *et al.*, 2014).

1.3.2.1 Disease in humans

The first clinical signs in human appear after a period of 2-6 days of incubation. Human infections generally pass asymptomatically or associated with a mild flu-like syndrome with no long-term sequelae (Ikegami *et al.*, 2011). A minority of infected people can develop a severe RVF disease characterized by acute hepatitis with associated jaundice, renal failure and hemorrhagic complications (Madani *et al.*, 2003). Ocular form of the disease associated with maculopathy or retinopathy is reported in less than 2% of the cases and can cause the permanent loss of vision (Ikegami *et al.*, 2011). Approximately 1% of patients develop a neurological form of the disease characterized by severe encephalitis (Alrajhi *et al.*, 2004). Less than 1% of affected people develop a hemorrhagic form of the disease. In these cases, the percentage of mortality can reach the 50% of infected people (Madani *et al.*, 2003).

1.3.2.2 Disease in animals

Many vertebrate species are susceptible to RVF infection (Olive *et al.*, 2012), however, RVFV is primary a pathogens of livestock. The typical hallmark of RVF epizootics is the sudden development of extensive "abortion

storm" (Bird *et al.*, 2009). The general symptoms of RVF in livestock include fever, peracute to acute onset of inappetence, nasal discharge and fetid/hemorrhagic diarrhea (Bird *et al.*, 2009); high levels of viremia are reached in infected animals (Busquets *et al.*, 2010).
Young lambs are highly susceptible to RVF, the mortality rate reaches 90-100% and animals usually die in 24-72 hours. Adult sheep are less susceptible to infection, with a mortality rate of 10-30%, but the abortion rate in this species can reach 90-100% (abortion storm). Young calves are less susceptible to lethal RVF infection and the mortality rate ranges from 10 to 70%. Among adult cattle the case fatality is lower (5-10%). RVF in cattle is often unapparent and unusual abortion rate may be the only signal of infection. Goats are highly susceptible to infection and mortality rate of kids can reach 48%. Clinical signs and abortions have also been reported occasionally in camels, water buffalo, and some wild ungulate species (Bird *et al.*, 2009).

1.3.3 RVF epidemiology

The first big outbreak of RVF was reported between 1950 and 1951 in South Africa (Alexander, 1951) with 100,000 death sheep estimated and 500,000 abortions among ewes (Pienaar *et al.*, 2013). RVFV was identified as the cause of the epidemic, after the infection of a veterinarian that performed a post-mortem inspection of an infected bull (Mundel *et al.*, 1951). In the following years, evidence of epidemic outbreaks were reported in Zambia, Zimbabwe and Namibia (Nanyingi *et al.*, 2015). In 1977, RVFV was reported for the first time in Egypt (Meegan, 1979). The outbreak caused 200,000 human infections and at least 594 death cases among hospitalized patients (Bird *et al.*, 2009). The extensive deaths of livestock cause losses for 115 million US dollar. After the epidemic of 1977, other minor outbreaks

were reported in Egypt in 1993, 1994, 1997 and 2003 (Ahmed Kamal, 2011). In 1978 a large outbreak occurred in Zimbabwe with 70,000 estimated cases among animals (10,000 death cases) (Nanyingi *et al.*, 2015). First cases of RVF were detected in the Western African countries of Senegal and Mauritania in 1987 (Nanyingi *et al.*, 2015). However, the presence of virus in Senegal has been reported since 1974 (Fontenille *et al.*, 1998). During 1997-1998 large outbreaks of RVF were reported in Kenya and Tanzania and coincide with an anomalous activity of *El Niño* (Nanyingi *et al.*, 2015). Only in the Garissa district of Kenya 27,500 human infections were estimated (Woods *et al.*, 2002). In 2000, RVFV was first detected outside Africa in Saudi Arabia and Yemen (Ahmad, 2000). In Saudi Arabia 883 people ended up infected and 124 died; in addition in Yemen 1,328 people were infected and 166 death cases were reported. However, it might be reasonable to estimate that more than 40,000 people have contracted the disease in a mild form during the entire outbreak (Al-Afaleq *et al.*, 2011). Between November 2006 and May 2007 a large epizootic/epidemic outbreak occurred in Somalia, Tanzania and Kenya with a total of 1,062 human cases (315 fatalities), 32,000 animals infected and 4,200 death cases reported only in Kenya (CDC, 2007; Pepin *et al.*, 2010; Nanyingi *et al.*, 2015). In 2009 RVFV was detected outside continental Africa in Mayotte (Sissoko *et al.*, 2009), Madagascar (2010) (Andriamandimby *et al.*, 2010) and Republic of Comoros (2009) (Roger *et al.*, 2011). Numerous others outbreaks have been reported in central and southern African countries: in the Republic of Sudan (2007-2008, 2010) (Hassan *et al.*, 2011; Aradaib *et al.*, 2013); in the Republic of South Africa (2008-2011) with 14,342 animal reported cases (8,877 death cases) and 242 human cases (26 death cases) (Metras *et al.*, 2015; Nanyingi *et al.*, 2015); in Uganda (2008, 2016) and in the Republic of Namibia (2011 - 2012) (Monaco *et al.*, 2013; Capobianco Dondona *et al.*, 2016). In western Africa numerous outbreaks have been reported in Mauritania (2010 - 2011, 2013 – 2015) (El Mamy *et al.*, 2011; Jackel *et al.*, 2013;

Sow *et al.*, 2014a) and in Senegal (2011-2012) (Sow *et al.*, 2014b). The geographical distribution of RVF outbreaks from January 2004 to April 2016 is shown in figure 1-10.

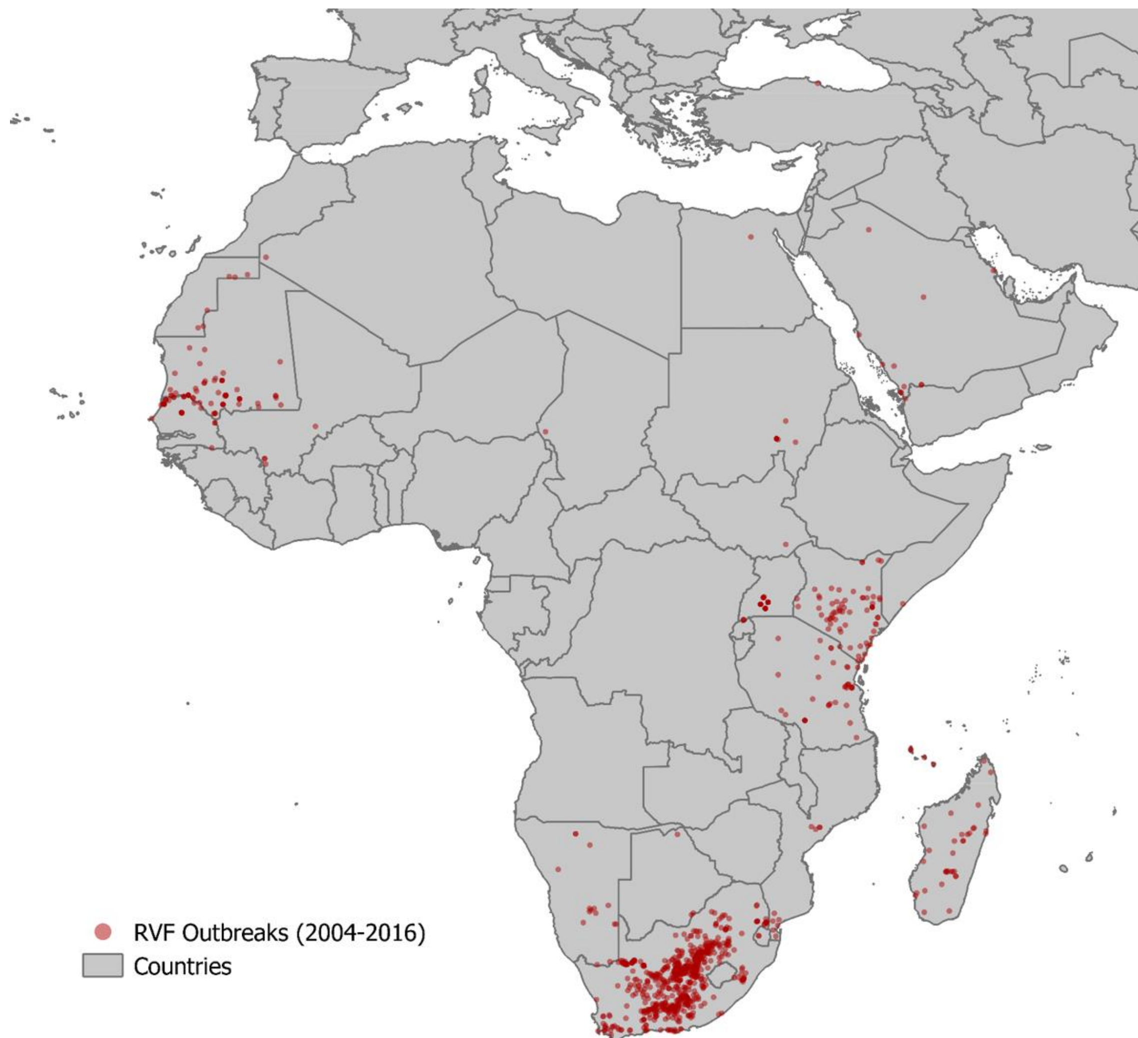

Figure 1-10: Geographical distribution of Rift Valley fever outbreak from January 2004 to April 2016.

1.3.3.1 Arthropods vectors

RVFV has been isolated from 53 species in 7 mosquito genera (Linthicum *et al.*, 2016). Mosquito species of the genus *Aedes* and *Culex* are the most important vectors of RVFV. Other mosquito genera involved belong to *Anopheles*, *Coquillettidia*, *Eretmapodites*, *Mansonia* and *Ochlerotatus* (Chevalier *et al.*, 2010). In case of being introduced to Europe, the principal species that could be competent vectors are: *Cx. pipiens*, *Cx. perexiguus*, *Ae. albopictus* and *Oc. caspius* (Moutailler *et al.*, 2008; Chevalier *et al.*, 2010).
Among Sandfly, *Phlebotomus duboscqi* is able to transmit RVFV (Turell *et al.*, 1990). Additionally, *P. papatasi, P. sergenti,* and *Sergentomyia schwetzi* have been demonstrated to be laboratory competent vector (Dohm *et al.*, 2000). Mechanical transmission of RVFV has been described in *Stomoxys calcitrans* (Turell *et al.*, 2010), and *Lutzomyia longipalpis* (Hoch *et al.*, 1985). Transtadial (infection from a development stage to the next one) and horizontal transmission in laboratory infected *Hyalomma truncatum* has been described, however, it is not considered a competent vector (Linthicum *et al.*, 1989).

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Chapter 2

Objectives

Mosquito borne diseases are increasing their burden in the entire world. The relevance of these diseases for the public and the veterinary health, require a comprehensive study of the vectors of these diseases. To know the vector competence and the distribution of mosquito populations present in the country provides useful information to Public and Animal Health authorities in order to establish appropriate surveillance programs and entomological control strategies.

With the aim to provide information to the abovementioned issues, this thesis presents two general purposes: i) to understand the population dynamics of the invasive mosquito *Aedes albopictus* in Spain; and ii) to improve the knowledge of vector competence of autochthonous *Culex pipiens* and invasive *Aedes albopictus* for two zoonotic arboviruses: West Nile virus and Rift Valley Fever virus.

To accomplish the general purposes the following three objectives were formulated:

I. To study the genetic structure of established populations of *Aedes albopictus* in Spain *(Chapter 3)*.

II. To determinate the vector competence of *Culex pipiens* and *Aedes albopictus* for West Nile virus *(Chapter 4).*

III. To evaluate the vector competence of *Culex pipiens* and *Aedes albopictus* for Rift Valley fever virus *(Chapter 5).*

PART II

Studies

"You make observations, write theories to fit them, try experiments to disprove the theories and, if you can't, you've got something"

Kary Mullis

Chapter 3

Study I: Phylogeography of *Stegomyia albopicta* (*Aedes albopictus*): deciphering the introduction event in Spain and insights of global patterns

3.1 ABSTRACT

Stegomyia albopicta is one of the most efficient invasive species in the world and its presence has been reported globally. *S. albopicta* was detected in 2004 in NE Spain. The mosquito successfully established itself there and spread southwards along the Mediterranean coast of the Iberian Peninsula and into the Balearic Islands. The present study analyzes the genetic structure of Spanish *S. albopicta* populations based on the subunit I of the *cytochrome oxidase I* (COI) gene. Landscape genetic analysis tools were used to infer introduction and dispersion patterns of the vector. The same analyses were performed with a worldwide *S. albopicta* dataset. Though the most likely hypothesis for the *S. albopicta* introduction in Spain was through a single event followed by its successful spread towards neighboring regions, the genetic diversity and geographic distribution of Spanish populations does not apparently support such a hypothesis. Eight haplotypes are described, five of them previously unpublished. On a global scale, 43 COI haplotypes have been recorded and grouped into four clusters. The results obtained depict a common scenario for Spain and worldwide which supports the hypothesis that globalization is the main driver responsible for the global spread of the Asian tiger mosquito.

3.2 INTRODUCTION

Stegomyia albopicta (Aedes albopictus) (Skuse), also referred to as *Stegomyia albopicta*, is one of the most efficient invasive species in the world (Baldacchino *et al.*, 2015). This mosquito is native to tropical forests of South-East Asia, from where it has spread worldwide (Paupy *et al.*, 2009). *S. albopicta* is a major public health concern due to its capacity to transmit pathogens. *S. albopicta* is expected to be a potential vector for at least 26 different arboviruses, some of them producing major diseases as Dengue, Chikungunya or Zika (Moore *et al.*, 1997; Gratz, 2004). *S. albopicta*, known as the Asian tiger mosquito, is an aggressive species that prefers human blood (Paupy *et al.*, 2009), but is also an efficient opportunistic feeder. As a result, *S. albopicta* is now considered a major public health concern in addition to its relevance as an invasive species.

The global spread of the Asian tiger mosquito has dramatically increased in the past three decades (Gratz, 2004; Paupy *et al.*, 2009; Medlock *et al.*, 2012). This has been related to increased commercial trade with Asian countries, especially in used tires and decorative plants, mainly *Draecana* spp (Gratz, 2004; Paupy *et al.*, 2009). The successful spread of *S. albopicta* is related to its physiological and ecological plasticity. A high physiological plasticity confers to *S. albopicta* mosquitoes the ability to adapt to the colder temperatures typical of temperate areas (Nawrocki *et al.*, 1987; Paupy *et al.*, 2009; Medlock *et al.*, 2015). In addition, a wide ecological plasticity confers to *S. albopicta* a rapid adaptation to different habitats including urban and peri-urban areas (Paupy *et al.*, 2009).

Historically, *S. albopicta* was distributed across SE Asia and neighboring regions (Gubler, 2003). *S. albopicta* was probably introduced into the islands of Madagascar and Reunion in the late 19th century and around the 1980s into the Pacific islands of Hawaii and Marians via ships (Gubler, 2003; Usmani-Brown *et al.*, 2009). Its first introduction into the USA was reported

in 1985 through trading in used tires from Asia (Zhong *et al.*, 2013). Later introductions were linked to "Lucky Bamboo" plant importation from China (Linthicum *et al.*, 2003). In South America, *S. albopicta* was first reported in Brazil in 1986 (Gratz, 2004), and since then its presence has been reported in the Caribbean and in Central and South America (Savage *et al.*, 1992). In Africa, since the year 2000, the presence of *S. albopicta* has been reported in Cameroon, Gabon, Equatorial Guinea and Central African Republic where it shares an ecological niche with *Ae. aegypti* and other endemic species (Kamgang *et al.*, 2013). The first detection of *S. albopicta* in Europe was reported in Albania (1979) and was linked to the importation of used tires from China (Adhami *et al.*, 1998; Gratz, 2004). Since then, the Asian tiger mosquito has been reported in several EU member countries (ECDC, 2015; Medlock *et al.*, 2015). In Spain, while active entomological surveillance provided no evidence of *S. albopicta* in imported used-tire stores (Roiz *et al.*, 2007), its presence was first reported in a residential area of Sant Cugat del Vallès in August of 2004 (Catalonia, NE Spain) (Aranda *et al.*, 2006). Since then, and within 10 years, the Asian tiger mosquito has spread along the Mediterranean coastline. Mosquito populations moved northwards to Girona province (Catalonia Autonomous Community) and southwards as far as the Region of Murcia. Subsequently, *S. albopicta* reached the overseas territory of the Balearic Islands (Alarcón-Elbal *et al.*, 2014). The first detection of *S. albopicta* in Alicante province (Valencian Community) was in 2005 (Lucientes-Curdi *et al.*, 2014). However, populations could apparently not become established there until 2009. In 2010, the Asian tiger mosquito was identified in Castellón province (Valencian Community), located between Catalonia and Alicante. Since then, *S. albopicta* has been reported in the Region of Murcia in 2011, in the Balearic Islands in 2012 (Lucientes-Curdi *et al.*, 2014) and in 2014 in Andalusia and the Basque Country (Delacour-Estrella *et al.*, 2014; Delacour Estrella *et al.*, 2015). The rapid colonization of the Mediterranean coast of

Spain and the evidence of successful outdoor winter reproductive activity (Collantes *et al.*, 2014) confirms the high ecological plasticity of the species. The specific drivers responsible for the rapid spread of the mosquito in the country are unknown, as are the dynamics of the metapopulation.

The growing interest in understanding the rapid spread of *S. albopicta* has resulted in several phylogeographic studies aimed at characterizing populations of interest (Benedict *et al.*, 2007; Raharimalala *et al.*, 2012; Shaikevich *et al.*, 2013). Mitochondrial regions are the usual targets for such studies. Two mitochondrial genes have been routinely used: nicotinamide adenine dinucleotide dehydrogenase subunit 5 (ND5) and cytochrome oxidase subunit I (COI). The COI gene has been proposed as the most informative gene for the analysis of *S. albopicta* (Shaikevich *et al.*, 2013).

In the present study, we investigate the genetic clustering, haplotype diversity and introduction dynamics of the Asian tiger mosquito in Spain, using a fragment of the COI gene. In addition, we analyze on a worldwide scale the genetic clustering and global distribution of *S. albopicta*.

3.3 MATERIAL AND METHODS

3.3.1 Mosquito strain

Specimens from the Autonomous Community of Catalonia (NE Spain) were collected as eggs or larvae and were reared to adult stage at CReSA facilities. Specimens from other Mediterranean Autonomous Communities of Spain were kindly provided by Dr. Javier Lucientes Curdi (Universidad of Zaragoza). *S. albopicta* populations were sampled in every Spanish NUTS3 region where it had become established before and up to October 2013. The collection sites are shown in Figure 1, and the geographical coordinates and number of specimens analyzed are shown in Table S-1 (APPENDIX).

Figure 1. Geolocalization of sampling locations. 1) Figueres; 2) Sant Julià de Ramis; 3) Lloret de Mar; 4) Sant Cugat del Vallès; 5) El Prat de Llobregat; 6)

Vilanova del Camí; 7) Llorenç del Penedès; 8) Constantí; 9) Mont-Roig del Camp; 10) Vinarós; 11) Peníscola; 12) Benicarló 13) Castelló de la plana; 14) Palma 15) Cullera; 16) Benijòfar; 17) Torrevella; 18) Murcia.

3.3.2 DNA isolation, PCR amplification and sequencing

Total DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's recommendations. A fragment of 658-bp of the COI gene was amplified using this set of primer: 5′-TCW ACA AAT CAT AAR GAT ATT GG-3′ (LCO1490_Modified) and 5′-TAA ACT TCA GGR TGW CCA AAR AAT CA-3′ (HCO2198_ Modified) (Folmer *et al.*, 1994). PCR was carried out with GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with a reaction mix of a final volume of 50µL containing 5µL of 10X NH4 Reaction Buffer (Bioline, London, UK), 3µL of MgCl2 [50 mM] (Bioline, London, UK), 0.5µL of dNTPs [10 mM] (Applied Biosystems, Foster City, CA, USA), 0.5µL of each primer, 0.2µL of BIOTAQ DNA Polymerase [5 U/µl] (Bioline, London, UK) and 2-5µL of DNA depending on its quantification. The thermal cycling condition consisted of 5 min at 94ºC, 40 cycles of 1 min at 94º C, 1 min at 40º C and 1.5 min at 72º C, followed by 7 min at 72ºC. PCR products were detected by agarose gel electrophoresis in Tris-borate-EDTA buffer (TBE), stained with ethidium bromide, and visualized over UV light. PCR products were commercially purified and both strands sequenced (Macrogen Inc., Seoul, Korea).

3.3.3 Genetic diversity

The DNA sequences generated were manually trimmed using BioEdit v7.0.5.3 software (Hall, 1999). Analyses were performed on two sequence data sets: Spanish data set (SDS, n=199), including all newly obtained COI sequences, and Worldwide data set (WDS, n=1578). WDS included sequences derived from the NCBI database and the 199 SDS sequences (Table S-2; APPENDIX).

Both data sets were analyzed with BioEdit and aligned using Clustal W (Thompson *et al.*, 1994). The SDS had a length of 658 bp. The WDS length was delimited at 374-bp and overlapped the sequenced SDS fragment from position 228 to 601. Alignment was submitted to the online FaBox (Villesen, 2007). Basic sequence statistics of number of segregating sites (S), haplotype (Hd) and nucleotide (π) diversity, number of haplotypes (H) and average number of nucleotide differences (K), as well as neutrality test (Tajima's *D* (Tajima, 1989), Fu's *Fs* (Fu, 1997)) were performed in DnaSP v5.10.01 (Librado *et al.*, 2009) only for SDS.

3.3.4 Population structure

Departures from values expected under panmixia (i.e. F_{ST} = 0) between Spanish locations and their correct *P* values for population differentiation among pairs of populations were tested with ARLEQUIN with 1000 permutations of the data.

Haplotype data files were analyzed using Arlequin v3.5 (Excoffier *et al.*, 2010). A minimum spanning network (MSN), based on the number of nucleotide differences between haplotypes, was constructed from the distance matrix generated by Arlequin, using Hapstar v0.7 (Teacher *et al.*, 2011). To better visualize the interrelationships between the haplotypes

and the weight of each haplotype, the network was modified using the Inkscape™ software.

An additional clustering method was used to infer the spatial genetic structure of *S. albopicta* for both sequence datasets. First, the number and the composition of panmitic groups, as well as the spatial boundaries among them, were estimated using a Bayesian model computed with the Geneland package, version 2.0.0 (Guillot *et al.*, 2005) in the R environment (R, version 2.4.1; (Ihaka *et al.*, 1996)). This software implements a Markov chain Monte Carlo (MCMC) procedure to determine the best clustering of samples with regard to genetic and geographical information. Geographical information was taken into account at the Bayesian prior level, so that clusters corresponding to spatially structured groups are considered to be more likely than clusters that are randomly distributed in space. Five million MCMC iterations sampled each 1000 steps with a 50000 burn-in period, and a maximum number of clusters K = 10 were run to estimate the model parameters and posterior probabilities of group membership.

3.4 RESULTS

3.4.1 Genetic diversity of Spanish populations

DNA sequences (COI) were obtained for 199 specimens. No insertions or deletions were detected. Eight single nucleotide polymorphisms (SNP) were detected (overall nucleotide diversity, π= 0.00065) and defined eight haplotypes (Genbank accession numbers: KU319443 - KU319450). All haplotypes were defined by a single nucleotide mutation, except H7 which had two mutations (Table 1). The statistical data are summarized in Table 2.

Table 1. COI haplotypes for *Aedes albopictus* using the Spanish data set. Only polymorphic positions are shown. N: the number of times the haplotype was found; [ ] = Genbank accession numbers.

Haplotypes	N	Polymorphic position							
		10	38	56	85	88	284	508	554
H1 [KU319443]	160	C	G	G	T	T	C	G	G
H2 [KU319444]	13	-	-	-	-	-	T	-	-
H3 [KU319445]	2	-	-	-	-	C	-	-	-
H4 [KU319446]	4	-	-	A	-	-	-	-	-
H5 [KU319447]	2	-	-	-	C	-	-	-	-
H6 [KU319448]	6	T	-	-	-	-	-	-	-
H7 [KU319449]	5	-	A	-	-	-	-	A	-
H8 [KU319450]	7	-	-	-	-	-	-	-	A

Tajima's D and Fu's Fs statistics showed concordant positive or negatives values for municipalities, but values lacked statistical significance. When all sequences were analyzed together, D and Fs statistics were negative but not statistically significant. Haplotype diversity for each province (NUTS 3) was also calculated. Valencian community showed the highest Hd value (Hd= 0.505), followed by Balearic Islands (Hd=0.389) and Catalonia

(Hd=0.257). The region of Murcia had an Hd value of 0 due to the absence of different haplotypes in the territory.

Table 2. Gene polymorphism data for *Aedes albopictus* using the Spanish data set. N, number of analyzed sequences; H, haplotypes; S, number of segregating sites; Hd, haplotype diversity; π, nucleotide diversity; K, average number of nucleotide differences; D, Tajima's statistics; Fs statistics; NC, not computed.

Locality	N	H	S	Hd	π	K	D	Fs
Costantí	11	H1, H2, H3	2	0.618	0.001	0.690	0.036	-0.113
El Prat de Llobregat	10	H1	0	0	0	NC	NC	NC
Figueres	10	H1,H4	1	0.467	0	0.466	0.819	0.818
Lloret de Mar	10	H1	0	0	0	NC	NC	NC
Llorenç del Penedès	14	H1	0	0	0	NC	NC	NC
Mont-Roig del Camp	17	H1,H3	1	0.118	0	0.117	-1.163	-0.748
Sant Cugat del Vallès	20	H1	0	0	0	NC	NC	NC
Sant Julià de Ramis	10	H1	0	0	0	NC	NC	NC
Vilanova del Camí	10	H1,H2	1	0.467	0	0.466	0.819	0.818
Palma	9	H1,H5	1	0.389	0	0.388	0.156	0.477
Benicarló	9	H1,H6	1	0.389	0	0.388	0.156	0.477
Benijòfar	10	H1,H7	1	0.2	0	0.4	-1.4	0.586
Castelló de la plana	9	H1,H4	1	0.222	0	0.222	-1.088	-0.263
Cullera	10	H1,H2,H8	2	0.689	0.001	0.911	0.931	0.745
Murcia	10	H1	0	0	0	NC	NC	NC
Peníscola	11	H1,H6,H7,H8	4	0.745	0.001	1.127	-0.639	-0.486
Torrevella	9	H1,H7	2	0.5	0.001	1	1.234	2.079
Vinarós	10	H1,H6	1	0.2	0	0.2	-1.111	-0.339

3.4.2 Genetic structure of Spanish populations

The population differentiation between each pair of Spanish population combinations is shown in Table S-3 (APPENDIX). The populations of Cullera and Vilanova del Camí were the most differentiated populations when compared to the remaining populations.
The MSN generated by Hapstar illustrates the relationship between the 8 haplotypes (Figure 2).

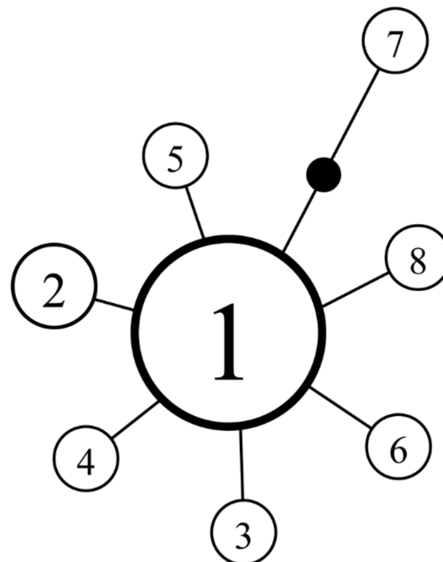

Figure 2. Minimum spanning networks showing COI haplotypes found for *Aedes albopictus* in Spain. Circle size is proportional to haplotype abundance, straight lines and black dots reflect mutations and unsampled or extinct haplotype

The network displays a star-like shape characteristic of a recent colonization or rapid expansion events. The most frequent haplotype, H1 (80.4%), was present in all the municipalities. Haplotype H5 is the only one detected in a single municipality (from the Balearic Islands). Haplotypes H6, H7 and H8 were exclusive to southern NUTS2 (Valencian Community and the Region of Murcia) municipalities. Haplotype H3 was exclusive to

Catalan municipalities (Catalonia NUTS2). A precise geographical distribution of haplotypes in the SDS is given in Table 2.

Some of the haplotypes have been previously reported outside Spain. Haplotype H1 is the most extensively distributed haplotype worldwide. H4 has been reported in Italy (JX679377) and Costa Rica (AB907796, AB907798-AB907800), while, the Balearic Islands haplotype H5 has been identified in samples collected in Japan (KC690920). The remaining haplotypes reported in Spanish populations of *S. albopicta* are new (H2, H3, H6, H7, H8).

The model-based Bayesian clustering algorithm implemented in Geneland detected two main clusters (K = 2), the first one including Vilanova del Camí, Constantí and Cullera (Figure S-1a; APPENDIX), and the second one for the remaining municipalities (Figure S-1b; APPENDIX). Values of cluster membership were high for all municipalities (P>0.9).

3.4.3 Worldwide scenario of *S. albopicta* distribution

The MSN generated by Hapstar illustrates the relationship between the 43 mtDNA haplotypes found (Figure 3). The network displays a star-like shape characteristic of a recent colonization or rapid expansion events. The network is composed of five major groups. WH1 is a haplotype present in all countries included in the study. WH3 and its related haplotypes (WH2, WH5, WH7 and WH22) form a specific cluster where all the sequences were from the Oriental and Australasian region (Australia, Indonesia and Papua New Guinea). WH4 together with its related haplotypes (WH6, WH11, WH13, WH21, WH30, WH33 and WH36) form a group of sequences distributed worldwide. Haplotype WH8 and related haplotypes (WH31, WH32 and WH34) form a cluster belonging to the Australasian (Australia and Papua New Guinea) and, interestingly, the Ethiopian

regions (Cameroon). WH14 is the root of the last group, with sequences from the Palearctic (Pakistan, China), Ethiopian (Madagascar, Mayotte, Reunion Island) and Nearctic (United States) regions. All WH14-related haplotypes (WH16, WH40, WH41, WH42 and WH43) were from Pakistan.

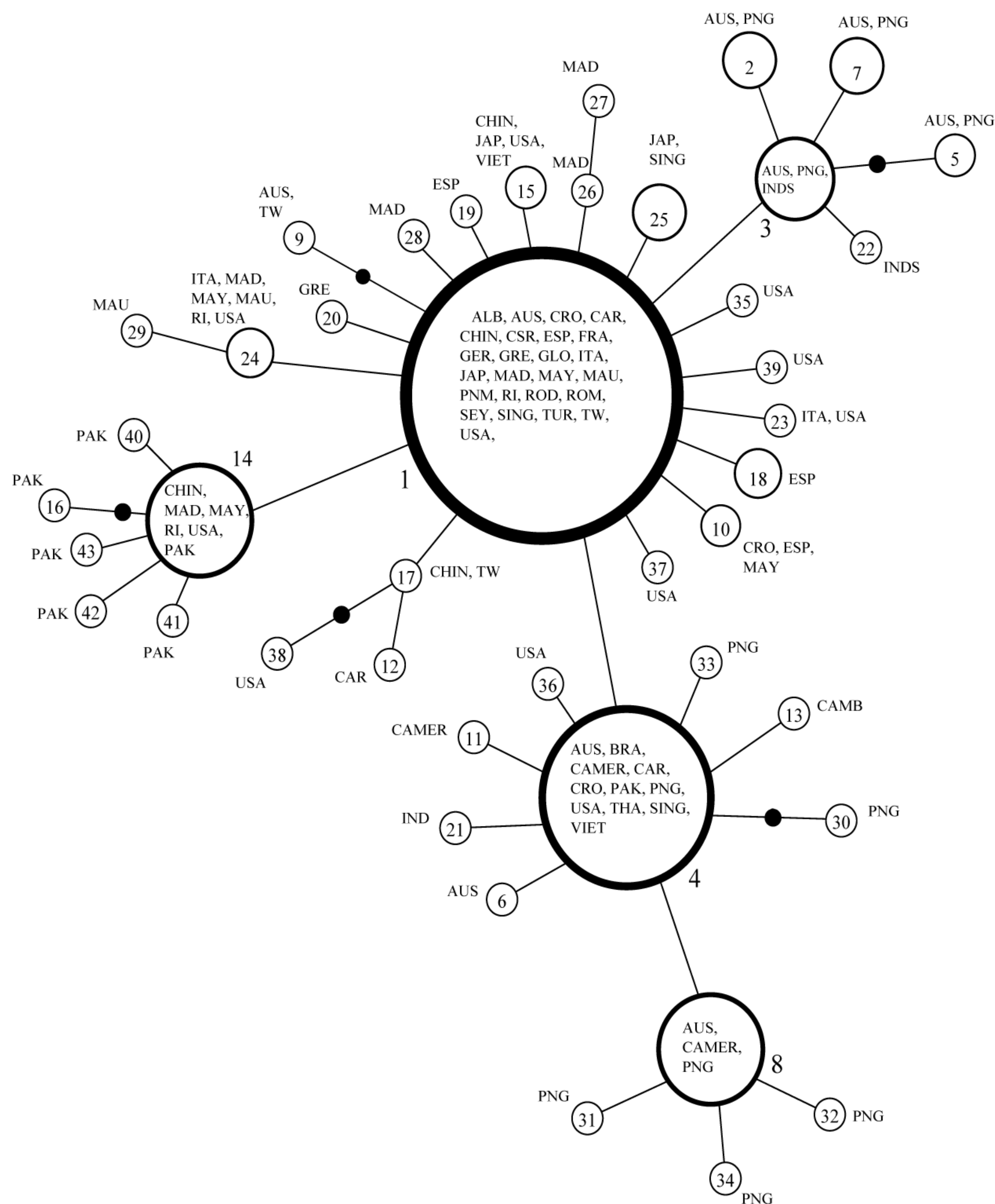

Figure 3. Minimum spanning networks showing COI haplotypes found for *Aedes albopictus* worldwide. Circle size is proportional to haplotype abundance, straight lines and black dots reflect mutations and unsampled or extinct

haplotype. For each haplotype the name of the countries in which it was described is reported. ALB= Albania, AUS= Australia, BRA= Brazil, CAMB= Cambodia, CAM= Cameroon, CAR= Central African Republic, CHIN= China, CR= Costa Rica, CRO= Croatia, FRA= France, GER= Germany, GLO= Glorieuses Island, GRE= Greece, IND= India, INDS= Indonesia, ITA= Italy, JAP= Japan, MAD= Madagascar, MAU= Mauritius, MAY= Mayotte, PAK= Pakistan, PNG= Papua New Guinea, RE= Reunion Island, ROD= Rodrigues Island, ROM= Romania, SEY= Seychelles, SING= Singapore, ESP= Spain, TW= Taiwan, THA= Thailand, TUR= Turkey, USA= United State of America, VIET= Vietnam.

The most likely number of genetic clusters (K) inferred by Geneland using the WDS was $K = 4$. Pakistan localities clustered together with the one in Los Angeles (USA) and two Chinese locations, Xiamen and Guangzou (Figure S-2a; APPENDIX). An additional cluster was formed by Australian and Southeast Asian localities, with the exception of Papua New Guinea (Figure S-2b; APPENDIX). The third cluster was composed of the localities of Brazil, India and Central Africa (Figure S-2c; APPENDIX). The final wide cluster was comprised of the remaining USA localities, Madagascar and its geographically adjacent islands (Reunion, Seychelles, Glorieuse and Mayotte islands), Europe, Japan and the remaining Chinese localities (Figure S-2d; APPENDIX). Values of cluster membership were circa 0.5 for most localities.

3.5 DISCUSSION

3.5.1 The Asian tiger mosquito in Spain

More than a decade after its introduction, the lack of knowledge about the genetic structure of the established *S. albopicta* populations make this study crucial. Our results show that the genetic diversity (π, Hd) of Spanish populations is higher than that found in populations from newly invaded areas (Kamgang *et al.*, 2011; Kamgang *et al.*, 2013), especially in southern locations. Conversely, genetic diversity resembles that obtained in areas where *S. albopicta* populations have been established for some time (Beebe *et al.*, 2013; Ashfaq *et al.*, 2014). However, the negative values of D and Fu's Fs statistics in some locations suggest a recent demographic expansion event in Spanish populations of *S. albopicta*. Such event is supported by the star-like shape of the haplotype network which is characteristic of a recent colonization event or of a rapid expansion event. Haplotype diversity was heterogeneous between provinces.

Taken together, the results support two hypotheses. The first is the spread of *S. albopicta* specimens from Catalonia by terrestrial vehicles, as suggested by *Lucientes et al* (Lucientes-Curdi *et al.*, 2014). Local dispersion of *S. albopicta* by road is known (Medlock *et al.*, 2012), and the introduction of *S. albopicta* by car has previously been described in Germany (Werner *et al.*, 2015). The dispersion from north to south (and vice-versa) along the Spanish Mediterranean region is made possible by the presence of the Mediterranean highway, a high traffic highway used by a large number of heavy commercial trucks. The second hypothesis suggests that more than one *S. albopicta* introduction event might have occurred in Spain since 2004. The high genetic diversity found in southern locations reinforces such a hypothesis. In addition, the Geneland analysis shows the presence of two different clusters with heterogeneous spatial distribution (Figure S-1;

APPENDIX). The first cluster includes northern and southern locations sharing haplotype H2. The second cluster grouped together the remaining localities.

Ae. albopicus was detected in the Balearic Islands in 2012. To our knowledge, haplotype H5 is exclusive to the Balearic Islands. However, we cannot rule out that the haplotype remains undetected in mainland Spain in an untested population. A new introduction event could have occurred more recently in the NW of Spain where *S. albopicta* was detected in 2014 in the Cantabrian region (Delacour Estrella *et al.*, 2015), with the species already present in the nearby French region.

3.5.2 Worldwide scenario of *S. albopicta* distribution

Human activity has greatly influenced the distribution of *S. albopicta* populations around the globe (Reiter, 1998; Medlock *et al.*, 2012; Medlock *et al.*, 2015). This makes the association of haplotypes with specific geographic areas difficult. This was the case with all the clusters of the haplotype network with one exception. The cluster comprised of WH2-WH3-WH5-WH7-WH22 is restricted to countries in the North Australian region (Australia, Papua New Guinea) and the South Oriental region (Indo-Malayan). The remaining clusters included populations from different regions of the world. The haplotype network displays the typical genealogy (Figure 3) of a species under geographic expansion. The Geneland analysis output was in accordance with the major hypothesis behind the *S. albopicta* origin and global expansion event (Gratz, 2004). The cluster aggregation inferred apparently correlates with some of the major commercial axes on a global scale, namely: a) West Indian Ocean route between West Africa and SE Asia (Pakistan/East India), b) East Indian and South Pacific Ocean routes through south Oriental and North Australian

regions, c) South Atlantic Ocean route between (sub-Saharan) West Africa and South America (Brazil mainly), d) Atlantic Ocean route between North/Central America and Europe, the route between Europe and the Indian Ocean Islands (including EU outermost regions: Reunion, Mayotte), and the route between Japan and North America. Interestingly, the four Geneland clusters share a common geographic area in SE Asia. This wide area represents the native geographic distribution of *S. albopicta*. These results highlight the unexpected usefulness of even a short region (<400 bp) in the COI gene of the mitochondrial DNA to infer global trends for *S. albopicta* populations.

The molecular characterization of populations throughout the world is very scant. The regions from where information is available are heterogeneous, with large areas of interest with no available data. An additional constraint is the absence of a gold-standard technique or marker for population genetics. Most studies have targeted COI/ND5 for sequencing (Kamgang *et al.*, 2011; Žitko T, 2011; Kamgang *et al.*, 2013; Shaikevich *et al.*, 2013; Zhong *et al.*, 2013). This is the most commonly used approach as it is highly informative and affordable in terms of budget and time required for analysis. However, comparison of the data available in public databases is not feasible due to a heterogeneous overlapping rate of the sequences.

The Asian tiger mosquito is a major public health concern everywhere it becomes established due to its high capacity to transmit a wide range of pathogens. It is crucial to understand the complex and dynamic phylogeographic patterns of *S. albopicta* throughout the different regions of the world. Preparation and control interventions could be valuably improved if we were able to understand the mechanisms behind the introduction, establishment and expansion events into naïve areas of *S. albopicta*.

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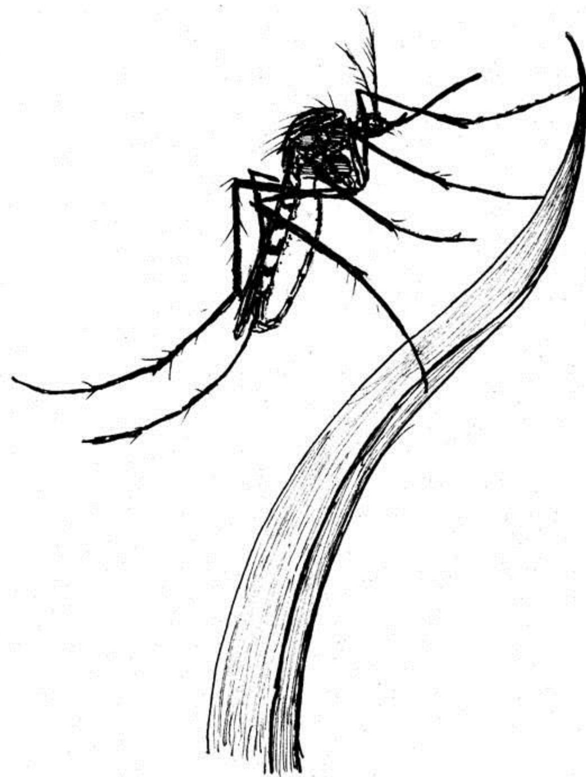
Chapter 4

Study II: *Culex pipiens* and *Stegomyia albopicta* (*Aedes albopictus*) populations as vectors for lineage 1 and 2 West Nile virus in Europe



4.1 ABSTRACT

The emerging disease West Nile fever (WNF) is caused by West Nile virus (WNV), one of the most widespread arboviruses. This is the first vector competence test involving European *Culex pipiens* Linnaeus 1758 and *Stegomyia albopicta* (*Aedes albopictus*) (Diptera: Culicidae) populations for linage 1 and 2 WNV isolated in Europe. *Cx. pipiens* and *S. albopicta* populations were susceptible to WNV infection, had disseminated infection, and were capable of transmitting both WNV lineages. This was the first WNV competence assay to maintain mosquito specimens under environmental conditions mimicking the field (day/night) conditions associated with the period of maximum expected WN activity. The importance of environmental conditions is discussed and how fixed, high temperatures could overestimate previous WNV vector competence results with respect to natural environmental conditions is analyzed. The information presented should be useful for policy makers and public health authorities to establish effective WNV surveillance and vector control programs. This would improve preparedness to prevent future outbreaks.

4.2 INTRODUCTION

The recent West Nile virus (WNV) outbreaks in the USA and Europe have caused global concern for human and animal health due to its demonstrated capacity to cause severe disease and death. WNV is a positive-sense RNA virus belonging to the genus *Flavivirus* of the *Flaviviridae* family. The primary enzootic cycle of WNV affects avian hosts and mosquitoes. Humans and horses are accidental hosts and do not develop sufficient viremia to support the transmission cycle (Martin-Acebes *et al.*, 2012). WNV is the most widespread member of the Japanese encephalitis virus complex (Bakonyi *et al.*, 2006) and can be phylogenetically divided into nine distinct genetic lineages (Pachler *et al.*, 2014). The most frequently recognized of these lineages are: lineage 1, which is present in Europe, America, Africa and Australia; and lineage 2, which has traditionally been found in Madagascar and sub-Saharan Africa, but which has more recently also been reported in Europe (Calistri *et al.*, 2010; Di Sabatino *et al.*, 2014). There have been several major outbreaks of WNV in recent years, affecting both humans and horses: Romania 1996, Russia 1999, France 2000, Israel 2000, Italy 2008, Spain 2010, Italy 2010-2013 and Croatia 2013 (Calistri *et al.*, 2010; García-Bocanegra *et al.*, 2011; Di Sabatino *et al.*, 2014). Until the last decade there had been no evidence of circulation of WNV lineage 2 strain in Europe. However, WNV lineage 2 has been reported responsible for several human and animal outbreaks of disease across Europe: Russia 2004, Hungary 2004–2008, Austria 2008, Greece 2010, Romania 2010, Russia 2011, Italy 2011, Italy (Sardinia) 2012, Serbia 2012 and Italy 2013 (Hernández-Triana *et al.*, 2014).

In temperate climates, viral infections affecting human and equine species are generally reported in the warmest months, when they are associated with increases in vector density and influenced by bird population dynamics (Rappole *et al.*, 2003). The ability of a vector to acquire, maintain and transmit microbial agents (vector competence) involves a dynamic

balance between vector genetics, hosts, virus strains, viremia and environmental conditions (Hardy *et al.*, 1983). The vector competence for WNV lineage 1 has been well studied in the USA, particularly since the initial New York outbreak of 1999. *Cx. pipiens, Cx. nigripalpus* Theobald and *Cx. quinquefasciatus* Say are the species that have been most studied on the American continent (Turell *et al.*, 2001; Goddard *et al.*, 2002; Richards *et al.*, 2007; Anderson *et al.*, 2008; Richards *et al.*, 2011; Richards *et al.*, 2012). However, very little information about WNV vector competence has been reported outside the USA. In the Maghreb region of Africa, *Amraoui et al.* demonstrated that *Cx. pipiens* were competent for a WNV lineage 1 strain isolated in France (Amraoui *et al.*, 2012), while *Cornel et al.* studied the vector competence of *Cx. univittatus* Theobald with lineage 2 in South Africa (Cornel *et al.*, 1993). To our knowledge, only one study had previously compared the vector competence of two different African vectors for different African WNV lineages (Fall *et al.*, 2014). In Europe, *Cx. pipiens*, *Cx. perexiguus* Theobald, and *Cx. modestus* Ficalbi are potential vector species (Hubalek *et al.*, 1999; Engler *et al.*, 2013). However, to our knowledge, only two studies have so far evaluated the vector competence of European culicid species. *Balenghien et al.* tested the vector competence of *Cx. pipiens*, *Cx. modestus* and *Ochlerotatus caspius* Pallas (*Aedes caspius*) from the Camargue region (France) (Balenghien *et al.*, 2008) while *Fortuna et al.* tested four different populations of Italian *Cx. pipiens (Fortuna et al., 2015)*. These studies were performed using two different strains of WNV lineage 1, isolated in France in 2000 and Italy in 2011 respectively.

The main objective of the current study was to explore WNV vector competence with respect to two mosquito species that are widely established in Europe: the *Cx. pipiens* (two forms were assayed) and the *S. albopicta* species. These assays were performed using strains from two European isolates representing lineages 1 and 2; this was the first time that a European linage 2 isolate had been tested for vector competence. Two

probable host viremias were assayed and, for the first time in this type of WNV studies, circadian temperature fluctuations were applied in the experiments to simulate natural conditions in Europe.

4.3 MATERIAL AND METHODS

4.3.1 Mosquito populations

Two mosquito species found in Catalonia (NE of Spain), *Cx. pipiens* and *S. albopicta*, were used for the experimental infections. Two different *Cx. pipiens* populations were used: *Cx. pipiens* form *molestus* from Empuriabrava and a hybrid between *pipiens* and *molestus* form, from Gavà. A molecular characterization of the *pipiens* forms was performed as previous described (Bahnck *et al.*, 2006). One population of *S. albopicta* from Sant Cugat del Vallès was used (Table 1).

Table 1. **Mosquito species used for experimental infection**. Place, year of collection and number of filial generations obtained in laboratory.

Mosquitos Species	Source	Filial generations
Cx. pipiens* form *molestus	Empuriabrava, 2011	> 30
Cx. pipiens hybrid	Gavà, 2012	> 30
S. albopicta	Sant Cugat del Valles, 2005	> 30

Empuriabrava is located near the *Aiguamolls de l'Èmpordà*, a wetland area with a high concentration of wild birds. Gavà and Sant Cugat del Vallès are towns belonging to the metropolitan area of Barcelona. Gavà is a touristic coastal town, while Sant Cugat del Vallès is a residential settlement. *S. albopicta* was identified for the first time in Spain at Sant Cugat del Vallès in 2004 (Aranda *et al.*, 2006). All mosquito populations have been reared in the laboratory to obtain stable colonies. These colonies were tested for the presence of *Flavivirus* and *Alphavirus* by RT-nested PCR (Sanchez-Seco *et al.*, 2001; Sánchez-Seco *et al.*, 2005) to confirm mosquitoes had no previous infections.

4.3.2 Virus strains

Two different WNV strains were used in the present study: PaAn001, a lineage 1 strain (Wn_lin1) isolated from a horse in the French region of Camargue in 2001 (EVA project, Ref-2651), and 178907/2013, a lineage 2 strain (Wn_lin2), isolated from a mosquito in Italy in 2013. The Wn_lin1 strain was propagated in *S. albopicta* larvae (C6/36) cells (ATCC, CRL-1660). The virus was titrated by 50% tissue culture infective dose ($TCID_{50}$) in African green monkey kidney (Vero) cells. The Wn_lin2 strain was propagated and titrated in Vero cells.

4.3.3 Vector competence assay design

Mosquitoes were reared under environmental conditions simulating those found around their natural breeding sites from the 3rd week of July until the 3rd week of August, corresponding with the period of maximum expected WNV activity. The photoperiod was 14.25h:9.75h (light:dark)

with two crepuscular cycles of 30min between to simulate dawn and dusk. The mean day temperature was 25.7ºC and the mean night temperature was 21.3ºC; relative humidity (HR) was maintained constant at 70%. Mosquitoes were housed in 0.5L volume plastic cages with mesh screening and fed on a 10% sucrose solution *ad libitum*. The sucrose solution was retired 30h before feeding the mosquitoes with infectious blood meals. Ten-to-twelve day-old female mosquitoes that had not previously been bloodfed were used in the experimental assays. The mosquitoes were fed using a Hemotek feeding system (Discovery Workshop, Accrington, UK) and with a specific pathogen-free chicken skin as a membrane. Bovine blood (38ºC±0.5) with heparine and ATP ($5x10^{-3}$ M) (Sigma-Aldrich, St. Louis, MO) were fed to the mosquitoes. Each population was exposed to infected blood containing 5 and 7 $log_{10}TCID_{50}$/mL of virus. The total number of mosquitoes, for each population, exposed to infectious bloodmeals is showed in table 2. After infectious bloodfeeding, the mosquitoes were anesthetized with CO_2 and fully-engorged females (FEF) were selected. To test the rates of individual infection, dissemination and transmission, some FEF were individually transferred to cardboard cages (Watkins & Doncaster, Leominster, UK) sealed with mesh screening on top. The remaining FEF were pooled and transferred, as a group, to a single plastic cage to evaluate group transmission. Cardboard and plastic cages were stored inside a climatic cabinet. Sucrose solution was administered with soaked cotton pledgets on the mesh screen, which were changed every day. Following a previous study (Dohm *et al., 2002)*, an extrinsic incubation period (EIP) of 12 days was established; at twelve days post infection (DPI) all the surviving mosquitoes were then sacrificed at -80ºC. All the assays were performed in Biosafety Level 3 facilities at the *Centre de Recerca en Sanitat Animal* (CReSA).

4.3.4 Sample collection

Each mosquito was dissected, its legs were detached from its body and both parts were separately homogenized in 0.5mL of Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland). The samples were homogenized at 30Hz for 1min using TissueLyser II (QIAGEN, Hilden, Germany) and stored at -80ºC until they were tested for WNV. The presence of virus in the body but not in the legs was considered non-disseminated infection, while the presence of virus in both the body and the legs was considered disseminated infection (Richards *et al.*, 2007). Screening was performed in two steps: first, mosquito bodies were analyzed and when they tested positive, their legs were analyzed to confirm the presence of disseminated infection. For transmission studies, two strategies were followed: i) the sucrose cotton pledgets were collected from the plastic cage (from the mosquitoes housed in group) at 10, 11 and 12 DPI, homogenized in 2mL of DMEM medium and then stored at -80ºC until further testing; and ii) the cotton was collected at 10 DPI from the mosquitoes individually housed in cardboard cages and a FTA™ card (GE Healthcare, Little Chalfont, UK) soaked with Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zeeland) was placed in the cages until 12 DPI (Hall-Mendelin *et al.*, 2010; Van den Hurk AF, 2012). The FTA cards were then collected, re-suspended in 0.3mL of PBS and stored at -80ºC until further testing. All the saliva samples collected from the groups and from individual females with disseminated infection were analyzed to know the transmission rate.

4.3.5 Virus detection and isolation

Viral RNA was extracted using NucleoSpin® RNA Virus (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations. Quantitative real time RT-PCR (RT-qPCR) was carried out as previous described (Jiménez-Clavero *et al.*, 2006), with minor modifications. The amplification was performed using primers at concentrations of 900nmol/L and using 200nmol/L for probes. The samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) programmed as follows: 48ºC for 10min, 95ºC for 10min, 45 cycles at 97ºC for 3s and at 61ºC for 30s. Standard curves were based on data acquired from 10-fold serial dilutions of recombinant plasmid as previous described (Busquets *et al.*, 2012). The limit of detection for the Wn_lin1 strain was 1.18 $\log_{10}$ genome equivalent copies (GEC) of plasmid per reaction and it was 0.22 $\log_{10}$ GEC/reaction for the Wn_lin2 strain. We tried to isolate each positive sample in Vero cells.

4.4 RESULTS

4.4.1 Mosquito feeding and mortality

Mosquito bloodfeeding varied according to the mosquito species and population. For *Cx. pipiens* (both population) 437 females were fed with infected blood out of 2,236 exposed females. In the case of *S. albopicta,* 438 females were fed with infected blood out of 781 exposed females (Table 2).

Table 2. Rates of FEF after feeding with bloodmeal containing Wn_lin1 or Wn_lin2 at final titer of 5.00 log_{10} $TCID_{50}$/mL and 7.00 log_{10} $TCID_{50}$/mL for the two populations of *Cx. pipiens* and for the population of *S. albopicta* at day 0 DPI. The mortality 12 DPI is expressed as ratio of dead FEF over total number of FEF.

Mosquito population	**WNV Strain**	**5.00 log_{10} $TCID_{50}$/mL**		**7.00 log_{10} $TCID_{50}$/mL**	
		FEF 0 DPI (%)	**Dead FEF 12 DPI (%)**	**FEF 0 DPI (%)**	**Dead FEF 12 DPI (%)**
Cx.pipiens* form *molestus	Wn_lin1	89/351 (25.3)	20/89 (22.4)	40/205 (19.51)	3/40 (7.5)
	Wn_lin2	85/329 (25.8)	15/85 (17.6)	24/193 (12.4)	0
Cx. pipiens hybrid	Wn_lin1	63/349 (18.0)	5/63 (7.94)	54/258 (20.9)	2/54 (3.70)
	Wn_lin2	58/371 (15.6)	1/58 (1.72)	24/180 (13.3)	3/24 (12.5)
S. albopicta	Wn_lin1	138/205 (67.3)	44/138 (31.8)	98/192 (51.0)	14/98 (14.2)
	Wn_lin2	100/151 (66.2)	26/100 (26)	102/233 (43.78)	14/102 (13.7)

As pointed out above, the percentage of FEF *Cx. pipiens* was lower than that of *S. albopicta*. The mean FEF values per population were: 20.75% (Standard Deviation, SD= 6.25) for the *molestus* form of *Cx. pipiens*, 16.95% (SD= 3.25) for hybrid population of *Cx. pipiens*, and 57.07% (SD= 11.56) for *S. albopicta*.

The mortality at 12 DPI was similar for the *molestus* form of *Cx.pipiens* and for *S. albopicta,* with average values (total for both WNV strains) of 11.87% (SD= 10.06) and 21.42% (SD= 8.95), respectively. The mortality rate for the *Cx. pipiens* hybrid was lower, with an average value of 6.46 % (SD= 4.76) (Table 2).

4.4.2 Mosquito infection

One female specimen of *Cx. pipiens* form *molestus* out of sixty-three (1.6%) exposed to 5.00 log_{10} $TCID_{50}$/mL of the Wn_lin1 strain was infected but this infection was not disseminated. For the same mosquito population, no infected females were detected at 12 DPI after they had been exposed to 5.00 log_{10} $TCID_{50}$/mL of Wn_lin2. In contrast, *Cx. pipiens* hybrid and *S. albopicta* exposed to the same viral load were neither infected nor disseminated for both viral strains tested (Table 3).

Table 3. Rates of infection and dissemination for *Cx. pipiens* and *S. albopicta* at 12 DPI after feeding with bloodmeal containing Wn_lin1 or Wn_lin2 at final titer of 5.00 log_{10} $TCID_{50}$/mL and 7.00 log_{10} $TCID_{50}$/ml. IF= Infected females; FDI= females with disseminated infection

		5.00 log_{10} $TCID_{50}$/mL		**7.00 log_{10} $TCID_{50}$/mL**	
Mosquito population	**WNV Strain**	**IF, *n* (%)**	**FDI, *n* (%)**	**IF, *n* (%)**	**FDI, *n* (%)**
Cx. pipiens form molestus	Wn_lin1	1/63 (1.6)	0 (0)	4/36 (11.1)	0 (0)
	Wn_lin2	0/68 (0)	0 (0)	6/22 (27.2)	0 (0)
Cx. pipiens hybrid	Wn_lin1	0/54 (0)	0 (0)	1/23 (4.3)	0 (0)
	Wn_lin2	0/54 (0)	0 (0)	11/21 (52.4)	2/11 (18.2)
S. albopicta	Wn_lin1	0/94 (0)	0 (0)	15/60 (25)	7/15 (46.6)
	Wn_lin2	0/72 (0)	0 (0)	21/85 (24.7)	9/20 (45)

At the higher concentration, of 7.00 log_{10} $TCID_{50}$/mL, both viral strains were able to produce infection in *Cx. pipiens* and *S. albopicta* (Table 3). The infection rate for the Wn_lin2 strain was higher than for the Wn_lin1 strain for both *Cx. pipiens* forms. The *molestus* form of *Cx. pipiens* presented 27.2% and 11.1% of infection, using Wn_lin2 and 1, respectively. Infection rates for *Cx pipiens* hybrid population were 52.4% for Wn_lin2 strain, and 4.3%

for Wn_lin1 strain. On the other hand, for the *S. albopicta* population, no significant difference in the infection rate was observed between the Wn_lin2 and 1 strains (24.7% vs. 25%). Disseminated infection was detected in *S. albopicta* mosquitoes exposed to both of the viral strains, with similar ratios (46.6% Wn_lin1 strain vs. 45% Wn_lin2 strain). The disseminated infection was also detected in *Cx. pipiens* hybrid exposed to Wn_lin2 strain (18.2%) (Table 3).

4.4.3 Viral quantification and isolation from mosquito samples

Two categories of WNV-infected females could be distinguished: i) females which replicated the virus at a low level and ii) females which replicated it at a high level. The GEC values of both WNV strains detected in infected female bodies at a low level ranged from 1.44 to 3.42 log_{10} GEC. On the other hand the GEC values detected in infected females at a high level ranged from 4.28 to 8.20 log_{10} GEC. *S. albopicta* presented both types of infections with similar rates of high level infection for both Wn_lin1 and Wn_lin2 (46.67% and 47.62%, respectively). Otherwise, the *Cx. pipiens* hybrid showed mainly high level infection for both the Wn_lin1 and Wn_lin2 strains (83.3% and 100%, respectively), while the *Cx. pipiens* form *molestus* presented a 100% rate of high infection for both of the WNV strains tested (Figure 1).

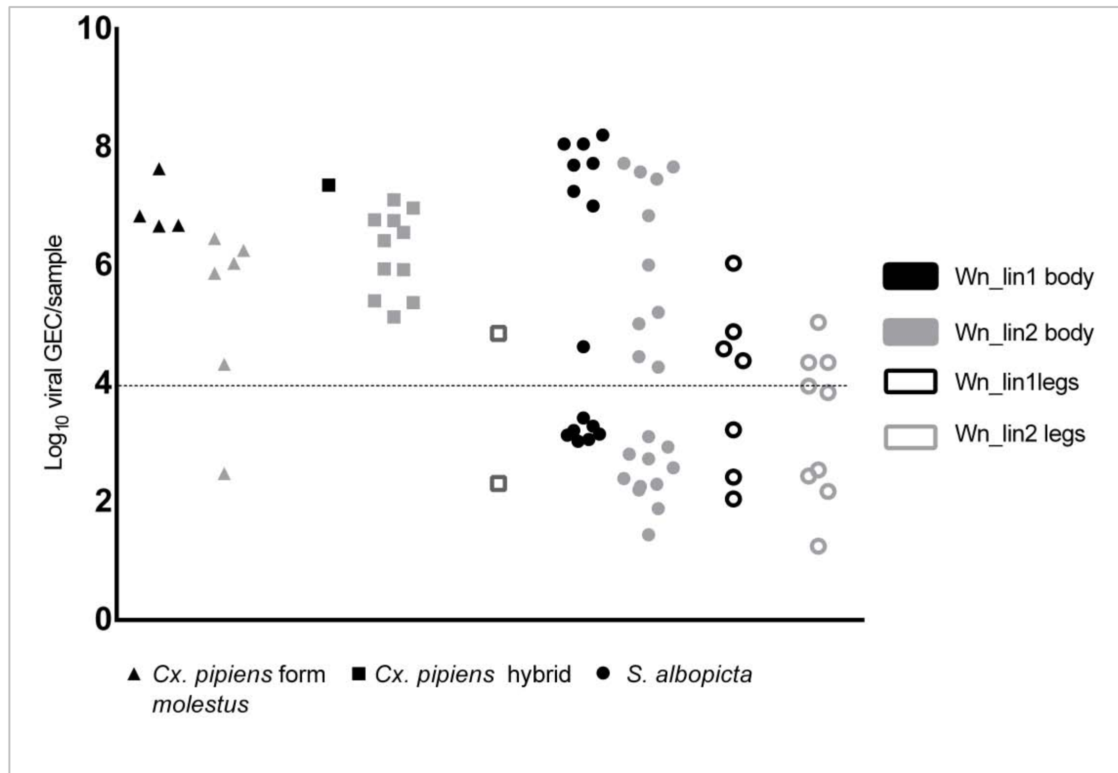


Figure 1. **Representation of GEC values for positive samples**. Solid symbol corresponds to infected bodies and empty symbol correspond to infected legs. Black is used for samples of Wn_lin1 and grey for samples of Wn_lin2. The separation between high infection and low infection is marked by discontinuous line. For each population, individuals are divided in different columns for better visualization.

In the mosquitoes with disseminated infection, the GEC values were always higher in the body than in the legs and a high viral load in the body correlated with a high viral load in the legs, and vice versa (Figure 2).

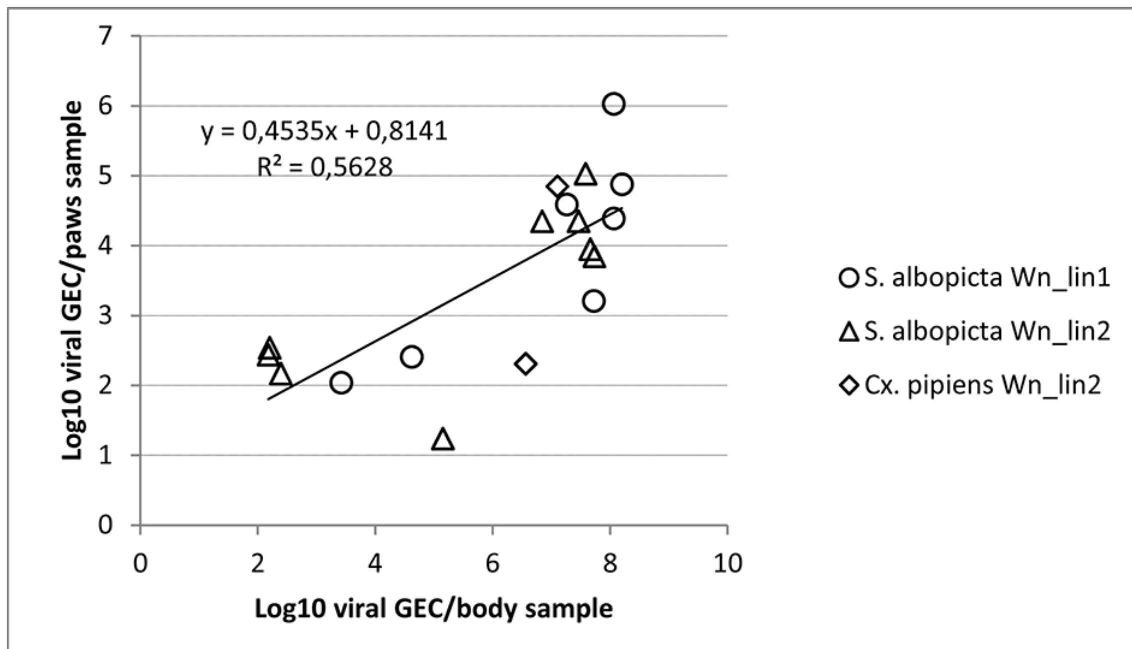


Figure 2. Figure showing the relationship between bodies titer and legs titer of *Cx. pipiens* and *S. albopicta* at 12 DPI with both WNV strain tested.

It was possible to isolate WNV from the bodies and legs in specimens of the three mosquito populations. This was mainly possible in the case of samples with high viral loads (more than 5.4 log_{10} viral GEC/sample). The percentage of isolation from bodies with high levels of infection was 38.46%, while that from legs was 50%.

4.4.4 Transmission

Cx. pipiens hybrid and *S. albopicta* populations proved able to transmit WNV (Table 4).

Table 4. Identification of WNV RNA in saliva of mosquitoes fed with infected blood at concentration of 7.00 log10 TCID50/mL. FDI= Females with disseminated infection

		Transmission study 1				**Transmission study 2**		
Mosquito population	**WNV Strain**	**FDI, *n* (%)**	**10 DPI**	**11 DPI**	**12 DPI**	**FDI, *n* (%)**	**10 DPI**	**Positive FTA / FDI**
Cx. pipiens form molestus	Wn_lin1	0	-	-	-	0	-	0/0
	Wn_lin2	0	-	-	-	0	-	0/0
Cx. pipiens hybrid	Wn_lin1	0	-	-	-	0	-	0/0
	Wn_lin2	2	-	-	+	0	-	0/0
S. albopicta	Wn_lin1	6	-	+	-	1	-	0/1
	Wn_lin2	8	-	-	-	1	-	1/1

In the first study of transmission, one cotton pledget from *S. albopicta*, which had been exposed to the Wn_lin1 strain, tested positive at 11 DPI (3.17 log_{10} viral GEC/sample). One cotton pledget from *Cx. pipiens* hybrid, which had been exposed to the Wn_lin2 strain tested positive at 12 DPI (3.47 log_{10} viral GEC/sample). The *S. albopicta* cotton pledget was collected from a group including females with disseminated infection (5 high level infected females and 1 low level infected female). The *Cx. pipiens* hybrid cotton pledget was collected from a group including 2 females with disseminated infection. In the second study of transmission, one FTA card from an *S. albopicta* female with disseminated infection (high level of

infection) that had been exposed to the Wn_lin2 strain tested positive. In this case, the viral load was 4.79 log_{10} viral GEC/sample.

4.5 DISCUSSION

The results showed the presence of WNV in the saliva of Spanish *S. albopicta* (both lineages 1 and 2) and in the saliva of *Cx. pipiens hybrid* form (only lineage 2) that had been experimentally infected. Our data confirmed the results obtained in previous studies which had highlighted the importance of the viral load used to blood-feed mosquitoes (Turell *et al.*, 2001; Dohm *et al.*, 2002; Anderson *et al.*, 2010). Under laboratory conditions, a bloodmeal with a WNV load of five log_{10} $TCID_{50}$/mL allowed the development of infection in the *molestus* form of *Cx. pipiens*, but it was not sufficient to cause a disseminated infection. The same viral load failed to infect either the *Cx. pipiens* hybrid or the *S. albopicta* populations; consequently, no disseminated infection was tested on these populations. Viremia titers exceeding $10^{5.0}$ PFU/mL were considered sufficient to infect *Cx. pipiens* (Turell *et al.*, 2000). The hybrid form of the *Cx. pipiens* species is considered a superior bridge vector for WNV in nature (Farajollahi *et al.*, 2011) and our results confirmed this data. On the other hand, when we used a bloodmeal with a higher titer (7 log_{10} $TCID_{50}$/mL), both WNV strains tested produced infection in *Cx. pipiens* (both forms), as had been previously reported (Balenghien *et al.*, 2008). It is also relevant to highlight the fact that it was also possible for the invasive species *S. albopicta* to be infected by both WNV lineages. The European Wn_lin1 and Wn_lin2 strains exhibited differences in their respective vector competences for the two forms of *Cx. pipiens* tested. The Wn_lin2 strain produced a high rate of infection in both *Cx. pipiens* form; it also induced a disseminated infection and viral RNA was detected in the saliva of individuals from the *Cx. pipiens*

hybrid population. Otherwise, the Wn_lin1 did not produce disseminated infection in either of the *Cx. pipiens* forms; as a result viral RNA was not detected in the saliva of any of the samples analyzed. Our data suggested that the *Cx. pipiens* hybrid form would be an important WNV vector as it was more competent than the *molestus* form, as previous described (Farajollahi *et al.*, 2011); this was particularly true for the Wn_lin2. Interestingly, *S. albopicta* turned out to be the most competent population tested for Wn_lin1, while similar infection and dissemination rates were obtained for Wn_lin2. It has been described as a mainly mammalophilic mosquito in the test region, whereas *Cx. pipiens* has been reported to exhibit opportunistic feeding behavior (Muñoz *et al.*, 2011). *S. albopicta* would therefore not be expected to play an important epidemiological role for WN in urban areas. In relation to infection and dissemination, our results contrasted with those of *Fall et all* (Fall *et al.*, 2014) for *Cx. neavei* Theobald and *Cx. quinquefasciatus*. In that study, the authors obtained a better percentage of infection and dissemination with the WN lineage 1 strain than with the WN lineage 2 strain. This difference between our results and those of Fall *et al* (2014) could suggest the Wn_lin2 strain which has been circulating around Europe has adapted to the European population of *Cx. pipiens.* Differences can be appreciated between our results and those obtained in previous studies (Turell *et al.*, 2001; Dohm *et al.*, 2002). However, on the basis of our results, it is difficult to infer the reasons behind the differences in vector competence reported in these previous studies. This is mainly because we lack information on important biotic factors relating to the important binome mosquito-virus. The parameters for which we have precise information, and which could help to explain such differences, are bloodmeal and temperature. When mosquitoes are bloodfed using a living viraemic host (Turell *et al.*, 2001; Dohm *et al.*, 2002), the infection and transmission rates are expected to be more efficient (Lord *et al.*, 2006) than when they are bloodfed using an artificial system

involving spiked blood, as in our case. The temperature profile during the EIP was usually constant and high in previous studies. In contrast, this study mimic the local circadian cycle and the mean temperature profile of the period with high WN activity; this reduced the mean temperature of the EIP to 23.9ºC. In our experiments, both these factors would have contributed to lower rates of WNV infection and dissemination in mosquitoes as previously pointed out (Cornel *et al.*, 1993). The bloodmeal viral load and temperature profile proved to be important parameters that influenced the vector competence. This was evident when the data have been compared with previous studies infecting *Cx. pipiens* with the same strain we used (Wn_lin1). Populations from France infected with a higher titer and kept at constant temperature (26ºC) showed 38.5% of disseminated infection at 14 DPI (Balenghien *et al.*, 2008). The former rate was much higher than the one we observed. The studies performed with African (Maghreb region) and Italian populations were much more interesting (Amraoui *et al.*, 2012; Fortuna *et al.*, 2015) as the bloodmeal viral loads were similar to ours, while the temperature was higher and constant in both studies (28ºC). In the study of *Amraoui et al.*, all the mosquito strains had disseminated infection rates which ranged from 59.1% to 100% while in the second cited study the disseminated infection rates ranged from 37% to 47%. In all the studies cited, higher and constant temperatures seemed to be associated with better results with respect to infection, dissemination and transmission. Previous studies have reported that temperature can influence the WNV vector competences of *Cx. pipiens* (Dohm *et al.*, 2002; Kilpatrick *et al.*, 2008), *Cx. quinquefasciatus* (Richards *et al.*, 2007) and *Cx. tarsalis (Reisen et al., 2006).* In these studies, an increase in temperature during the EIP produced an increase in the infection, dissemination and transmission rates (Anderson *et al.*, 2010). Previous study showed that a bloodmeal of 1.2–1.4x10^8 PFU/ml could differentially infect *Cx. pipiens (Kilpatrick et al., 2008)* depending on the temperature. Major differences

were also found with regard to disseminated infection. Our fluctuating daily environmental conditions could have affected both the infection and dissemination rates of the mosquitoes tested, thereby reducing their vector competences. This theory is also supported by a recent study that measured the vector competence of *Ae. aegypti* for the transmission of Dengue virus (Lambrechts *et al.*, 2011) in which the authors assessed how fluctuations in daily temperature had an impact on the capacity of the vector to transmit the virus. The circadian temperature range in our experiments was narrower (only 4.4ºC) than that in Lambrechts *et al*. However, Killpatrick *et al*. demonstrated a nonlinear correlation between the extrinsic incubation temperature and vector competence in *Cx. pipiens (Kilpatrick et al., 2008)* and suggested that even a relatively small variation in temperature could have had a significant impact on vector competence for this species. The findings mentioned above would therefore support the argument that the low rates of infection, dissemination and transmission founded, could have been the result of the lack of a constant temperature during the assay. Nevertheless, the use of a cycling temperature profile to simulate real environmental conditions seems necessary in order to design a more realistic vector competence assay.

The results obtained showed the presence of two different levels of infection in mosquitoes infected with WNV. A low infection or high infection pattern was not linked to the particular WNV strain used, but depended on the mosquito species in question. *Cx. pipiens* almost exclusively presented a high infection pattern, while both high and low infection patterns were found in *S. albopicta*. This difference in the level of infection between *Cx. pipiens* and *S. albopicta* could probably be explained by the different adaptations of the virus to these two species. *Cx. pipiens* is considered a principal vector for WNV with the virus having adapted well to the vector, whereas *S. albopicta* is a secondary, or opportunistic, vector and the adaptation of the virus to the vector is probably not as complete.

The results obtained in the present study demonstrated how the European *Cx. pipiens* and *S. albopicta* populations are both laboratory competent vectors for two strains of WNV isolated in Europe. *Cx. pipiens* is considered a primary field vector for WNV in Europe. In contrast, *S. albopicta*, which is a non-primary vector, has demonstrated an unexpectedly good level of vector competence for both of the WNV strains tested. It is important to note that both forms of *Cx. pipiens* collected in the region were fed successfully in the laboratory and that the proportion of fed *S. albopicta* was more than twice that of *Cx. pipiens*. In central and northern Europe, *Cx. pipiens* is not considered a pest because it has a very low degree of anthropophilic behavior but this changes moving to the south, where it has a more aggressive behavior versus humans. (Gabinaud *et al.*, 1985; Ishii, 1991). The proportion of fed females in both species is a parameter of maximum importance as it can be deduced from classical formulas of vectorial capacity such as in the formula of Reisen (Reisen, 1989).

In Europe, we are currently witnessing the re-emerge of WNV. Human and equine outbreaks are continually reported in different countries of the European Community and the recent appearance of WNV lineage 2 strains could have critical implications for both human and animal health. The assessment of the vector competence of local mosquito populations can provide helpful information for establishing a targeted, and more effective, surveillance of WNV and for developing a vector control program at the regional level; this will help to improve our preparedness and to prevent future outbreaks of disease.

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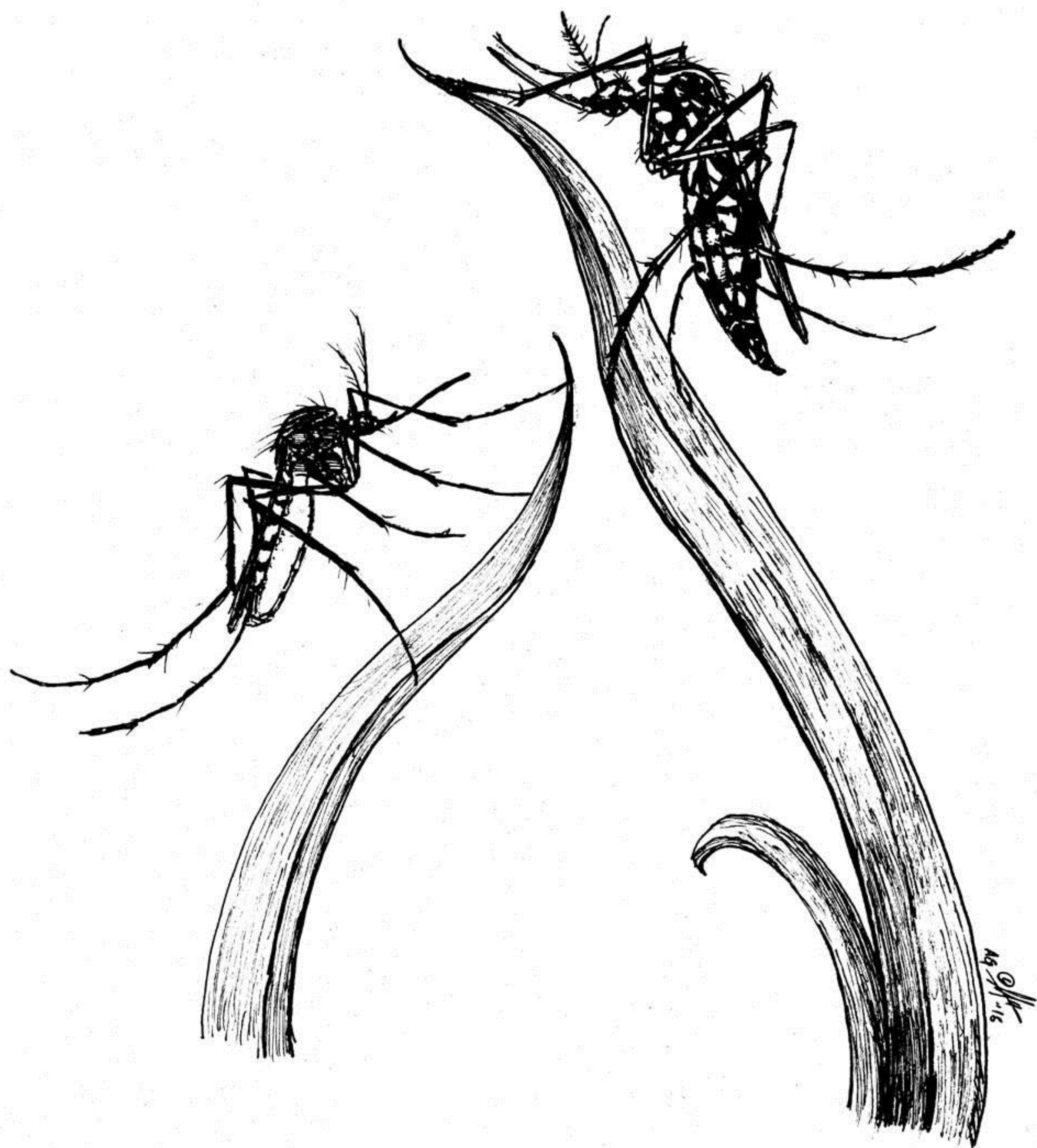
Chapter 5

Study III: Rift Valley fever virus and European mosquitoes: vector competence of *Culex pipiens* and *Aedes albopictus*



5.1 ABSTRACT

Rift Valley fever (RVF) is a mosquito-borne disease caused by the Rift Valley fever virus (RVFV). RVF affects a large number of species including humans. RVF has a severe impact on public health and economy, especially in African countries where numerous outbreaks have occurred since 1931. In the present study we evaluated the vector competence for RVFV of three different populations of European mosquitoes: *Culex pipiens* form *molestus*, *Culex pipiens* hybrid form and *Aedes albopictus*. Mosquitoes were artificially fed with blood containing virulent RVFV (strain 56/74). After a period of 14 days, infection, disseminated infection and transmission rates and transmission efficiency were evaluated. The virus was able to induce a disseminated infection in *Culex pipiens* hybrid form and in *Aedes albopictus*. Moreover, infectious viral particles were isolated from saliva samples of both species, showing their RVFV transmission capacity. On the other hand, *Culex pipiens* form *molestus* was not a performing experimental vector for competence studies of RVFV due to low propensity to the artificial feed. The presence of competent populations of *Cx. pipiens* and *Ae. albopictus* in Spain indicates that an autochthonous outbreak of RVF may occur in case of virus introduction. Our findings provide helpful information to health authorities in order to set up a future efficient entomological surveillance and vector control programs for RVFV.

5.2 INTRODUCTION

Rift Valley fever (RVF) is an arthropod borne zoonotic disease caused by Rift Valley fever virus (RVFV), an arbovirus of the *Phlebovirus* genus, belonging to the *Bunyaviridae* family. RVF is a zoonotic disease transmitted by infected mosquitoes to a large number of hosts, both domestic (especially sheep and goat) and wild (African buffalo, waterbuck, camel, rat) animals (Olive *et al.*, 2012). RVFV has been isolated in more than 50 mosquito species from seven different genus, the majority belonging to the *Culex* and *Aedes* genera (Linthicum *et al.*, 2016). RVFV was first described in 1931 in the Rift Valley province of Kenya (Daubney *et al.*, 1931). Since then, RVFV has caused numerous human and animal outbreaks, in several African countries. The presence of the virus has been reported in East African countries, from Egypt to South Africa, including Madagascar and, more recently, in the Mayotte island (Gerdes, 2004; Sissoko *et al.*, 2009; Nanyingi *et al.*, 2015). In the western part of Africa, RVFV was reported since the 1980s in the area between Mauritania and Senegal (Gerdes, 2004; Nanyingi *et al.*, 2015) and, in 2000, RVFV was reported for the first time outside the African continent, in Saudi Arabia and Yemen (Ahmad, 2000). The impact on public health and economy is important, as it was reported after the epidemic outbreak in Saudi Arabia in 2000, with 883 people infected, 124 human deaths and 40.000 animals dead or aborted (Al-Afaleq *et al.*, 2011); or the 1977 outbreak in Egypt with 200.000 human clinical cases, 600 deaths and economic losses of more than 115 million USD (Meegan *et al.*, 1980).

The presence of RVFV outside the African continent and especially in countries facing the Mediterranean Sea, such as Egypt, highlight the possibility of a RVF introduction event in Europe. The risk of RVFV introduction in Europe has been reviewed (Chevalier *et al.*, 2010; Rolin *et al.*, 2013; Mansfield *et al.*, 2015). Due to the restrictions imposed by the UE

on the importation of livestock and to the differences in climate and seasonal variations of vector and host density, compared to those presented in Africa, these authors classified as low the risk of introduction of RVFV in the EU. However, the illegal importation of infected livestock, specially between Africa and southern Europe and between Middle East and central Europe, has been indicated as the major source for virus introduction into Europe (Chevalier *et al.*, 2010). On the other hand, clime is a key factor to estimate the risk of RVFV outbreaks (Gerdes, 2004). The unusual strength of *El Niño* and the consequent rainfall anomalies reported, have enhanced the risk of further RVFV outbreaks in many African countries (2015; FAO *et al.*, 2015) due to the increase of vector density, an important parameter used to estimate the vectorial capacity (Garrett-Jones, 1964; Smith *et al.*, 2012). The climatic effects of *El Niño* are expected to affect also several European countries with rainfall anomalies (2015). Furthermore, several unpredictable factors like bioterrorism or intentional introduction of the virus could increase the risk of RFV introduction, as pointed out by *Rolin et al*. (Rolin *et al.*, 2013). This author suggests that entomological research and knowledge of vector ecology could help to the proper estimation of RVF risk assessment. Moreover, Northwest African countries (Mauritania and Senegal) with reported outbreaks (Nanyingi *et al.*, 2015), serological evidence of RVFV antibodies in camels in Morocco (El-Harrak *et al.*, 2011) and the presence of stable competent vector populations in Algeria, Morocco, and Tunisia (Moutailler *et al.*, 2008; Amraoui *et al.*, 2012), enhance our risk perception for a RVF introduction in Europe.

To date only one study of vector competence has been performed with European mosquitoes (Moutailler *et al.*, 2008). In that study the authors tested the capacity of two different strains of RVFV (the virulent strain ZH548 and the avirulent strain Clone 13) to produce a disseminated infection in several mosquito species from the Camargue region of France

(*Ae. caspius*, *Ae. detritus* and *Cx. pipiens*). In other regions of the world, RVFV vector competence studies have been more exhaustive: in Africa, 9 species have been tested as vectors of RVFV: *Ae. aegypti*, *Ae. calceatus*, *Ae. circumluteolus*, *Ae. mcintoshi*, *Ae. palpalis*, *Cx. antennatus*, *Cx. pipiens*, *Cx. quinquefasciatus, Cx. zombaensis* (Turell *et al.*, 1996; Turell *et al.*, 2007; Moutailler *et al.*, 2008; Turell *et al.*, 2008b; Amraoui *et al.*, 2012); 9 species were tested in Canada (Iranpour *et al.*, 2011); 4 species in Australia (Turell *et al.*, 1998) and 21 species in the USA (Turell *et al.*, 2008a; Turell *et al.*, 2010; Turell *et al.*, 2013a; Turell *et al.*, 2013b; Turell *et al.*, 2015).

Cx. pipiens complex is known as an efficient vector for RVFV (Turell *et al.*, 1996) and was proposed to be the principal vector during the 1977 outbreak in Egypt (Meegan *et al.*, 1980). In Europe, *Cx. pipiens* and *Ae. albopictus*, could be the main vector species due to their massive presence in the countries of the Mediterranean Basin. Other mosquito species of the *Culex* genus (*Cx. theileri*, *Cx. perexiguus* and *Cx. antennatus*) have been considered potential vectors due to their bio-ecology in terms of abundance, biting activity, feeding habits and longevity (EFSA, 2013). The important role of *Aedes* mosquitoes, will be not only linked to their ability to horizontally transmit the virus (Turell *et al.*, 1988; Turell *et al.*, 2010) but also to their capacity of maintaining virus viability during the coldest winter months. *Aedes* mosquitoes are able to transmit RVFV transovarially (Linthicum *et al.*, 1985). Moreover, the eggs of *Aedes* spp. could enter in diapause and survive at temperatures between 0 and -15ºC (Thomas *et al.*, 2012). The presence of potential vectors and areas with favorable environmental conditions (Sanchez-Vizcaino *et al.*, 2013) cannot exclude an RVF outbreak event in Spain. Considering the foregoing, the vector competence (infection, dissemination and transmission) of two different populations of *Cx. pipiens* and a population of *Ae. albopictus* were investigated using a virulent strain of RVFV. In this work, the experimental mosquito infections have been performed, for the first time, using cycling conditions that

simulated environmental conditions. This should allow a more realistic estimation of vector competence for mosquitoes present in Europe.

5.3 MATERIALS AND METHODS

5.3.1 Mosquito populations

Two different populations of *Cx. pipiens* were used: *Cx. pipiens* form *molestus* from Empuriabrava and a hybrid between *pipiens* form and *molestus* form, from Gavà. Empuriabrava is located near the *Aiguamolls de l'Èmpordà*, a wetland area in the north of Catalonia and Gavà is a touristic coastal town belonging to the metropolitan area of Barcelona. The population of *Ae. albopictus* came from Sant Cugat de Vallès, a town belonging to the metropolitan area of Barcelona and the place where the Asian tiger was first identified in Spain in 2004 (Aranda *et al.*, 2006). All mosquito populations have been reared in the laboratory to obtain stable colonies and the number of filial generations was more than 30. Mosquitoes were reared under the following environmental conditions: photoperiod was 14 h:10 h (light:dark) with two crepuscular cycles of 30 min between to simulate dawn and dusk; the mean temperature during day was 26ºC and 22ºC during night. Relative humidity (HR) was maintained constant at 80%. This environmental conditions corresponded to the mean temperature and photoperiod present at our latitude during the summer time, when the density and the activity of mosquitoes would be expected to be highest.

The mosquito colonies were tested for the presence of *Flavivirus* and *Alphavirus* by reverse transcription nested polymerase chain reaction (RT-

nPCR) (Sanchez-Seco *et al.*, 2001; Sánchez-Seco *et al.*, 2005) to confirm the absence of other viral infections. The colonies were also tested for the presence of *Wolbachia* spp. by PCR analysis of a fragment of *wsp* gene as previously described (Braig *et al.*, 1998). All colonies resulted *Wolbachia* spp. positives (data not shown).

5.3.2 Virus strains

Virulent strain RVF 56/74, originally isolated from cattle in 1977 (Barnard *et al.*, 1977), was used in the present study. RVF 56/74 was propagated in Baby Hamster Kidney fibroblasts 21 (BHK-21) cell (passages history (Busquets *et al.*, 2010) and titrated by 50% tissue culture infective dose for ml ($TCID_{50}$/ml) in African green monkey kidney (Vero) cells.

5.3.3 Vector competence assays design

Infection rate (IR), disseminated infection rate (DIR), transmission rate (TR) and transmission efficiency (TE) were evaluated. IR refers to the proportion of mosquitoes with infected body (abdomen, thorax and head) among tested mosquitoes. DIR corresponds to the proportion of mosquitoes with infected legs and wings among the infected mosquitoes. TR represents the proportion of mosquitoes with positive saliva samples among the total number of mosquitoes with disseminated infection. TE represents the proportion of mosquitoes with infectious saliva among the total number of mosquitoes tested. The efficiency of transmission in *Cx. pipiens hybrid* and in *Ae. albopictus* were compared using the Fisher's exact test.

Three assays were designed. In the first assay, the two forms of *Cx. pipiens* were tested with two different viral doses: 5.75 $log_{10}TCID_{50}$/mL and 7.01 $log_{10}TCID_{50}$/mL. This first trial was designed to elucidate the IR and the DIR of the two forms of *Cx. pipiens*. The TR was evaluated using FTA™ cards (GE Healthcare, Little Chalfont, UK). In the second and third assays two different approaches were used to test saliva samples and calculate TR and TE: the use of FTA cards and the use a capillary for direct extraction of saliva from mosquito. In the second assay, the population of *Cx. pipiens* hybrid was tested using a viral dose of 7.50 $log_{10}TCID_{50}$/mL. In the third assay the population of *Ae. albopictus* were tested using a viral dose of 6.23 $log_{10}TCID_{50}$/mL.

In all the assays, seven-to-nine day-old female mosquitoes, that have never bloodfed before were used. Mosquitoes were housed in 0.5L volume plastic cages with mesh screening and fed on a 10% sucrose solution *ad libitum*. The sucrose solution was retired 30 h before feeding the mosquitoes with infectious blood meals. The mosquitoes were fed using a Hemotek feeding system (Discovery Workshop, Accrington, UK) with a specific pathogen-free chicken skin as a membrane. The mosquitoes were fed with infected bovine blood (38ºC±0.5) with heparin and ATP ($5x10^{-3}$ M) (Sigma-Aldrich, St. Louis, MO). After the infectious bloodfeeding, the mosquitoes were anesthetized with CO_2, fully-engorged females (FEF) were selected and individually transferred to cardboard cages (Watkins & Doncaster, Leominster, UK) sealed with mesh screening on top. Cardboard were stored inside a climatic cabinet with the same environmental condition mentioned above. Sucrose solution was administered with soaked cotton pledgets on the mesh screen, which were changed every day. All the assays were performed in Biosafety Level 3 facilities at the *Centre de Recerca en Sanitat Animal* (CReSA).

5.3.4 Sample collection

In all assays, FTA™ cards (GE Healthcare, Little Chalfont, UK) were used to take saliva samples at different time-points: 14 dpi in the first assay, 5 dpi and 14 dpi in the second and in the third ones. FTA™ cards were soaked with Manuka honey (Manuka Health New Zealand, Te Awamutu, New Zeeland) and a blue alimentary colorant. The FTA cards were left 24 h on the top of each mesh screen to allow the mosquito to feed from it. After FTA cards collection they were resuspended in 0.3 mL of PBS and stored at -80ºC until tested.

At 14-day post infection (dpi) each mosquito was anesthetized with CO_2 and dissected. The legs and wings were detached from the body and both parts were separately homogenized in 0.5 mL of Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland). The samples were homogenized at 30 Hz for 1 min using TissueLyser II (QIAGEN, Hilden, Germany) and stored at -80ºC until tested for RVFV detection.

In the second and in the third trials, the saliva was extracted from each mosquito at 14 dpi using a capillary technique, as previously described (Dubrulle *et al.*, 2009). Briefly, after the dissection of the legs and the wings, the proboscis was inserted into a P20 pipette tips filled with 7 μL of a solution 1:1 of Fetal Bovine Serum (FBS) and 50% of sucrose solution. To stimulate the salivation 1μL of 1% pilocarpine (Sigma-Aldrich, St. Louis, MO) prepared in PBS at 0.1% Tween 80, was applied on the thorax of each mosquito. After a period of 60 min, the solution containing the saliva was expelled into 1.5 mL tubes containing 190 μL of DMEM; 150 μL were used for viral RNA extraction and the remain were used for RVFV isolation in a monolayer of Vero cells. Cells were incubated for 7 days (37°C, 5% CO_2) and citophatic effect was evaluated.

5.3.5 Virus detection

Viral RNA was extracted from samples (bodies, legs and wings, FTA cards and saliva) using NucleoSpin® RNA Virus (Macherey-Nagel, Düren, Germany) following the manufacturer's recommendations. The RT-PCR was performed as previous described with minor modification (Drosten *et al.*, 2002). RT-qPCR was carried out using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems, Foster City, CA, USA) without adding supplementary $MgSO_4$. The samples were amplified using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) programmed as follows: 48ºC for 10min, 95ºC for 10min, 45 cycles at 95ºC for 15s and at 57ºC for 35s. The limit of detection was 0.09 $TCID_{50}$ per reaction.

5.4 RESULTS

5.4.1 Mosquito feeding and mortality

The two forms of *Cx. pipiens* presented different attitude to the artificial feeding; data are showed in the table 1. The mean feeding rate of *Cx. pipiens* hybrid was higher (25.69%, Standard Deviation (SD) = 6.62) than *Cx. pipiens* form *molestus* feeding rate (3.82%, SD= 1.96). The mean mortality rate at 14 dpi was 3.37% (SD= 1.10) and 6.25% (SD= 8.83) for *Cx. pipiens* hybrid and *Cx. pipiens* form *molestus* respectively. The feeding rate of *Ae. albopictus* was 24.50% and the mortality rate at 14 dpi was 7.31%.

Table 1. Feeding and mortality Rates. FEF = Full engorged female. NA= not applicable

	1st Assay				**2nd Assay**		**3rd Assay**	
	$10^{5,75}$ TCID$_{50}$/mL		**$10^{7,01}$ TCID$_{50}$/mL**		**$10^{7,5}$ TCID$_{50}$/mL**		**$10^{6,23}$ TCID$_{50}$/mL**	
	FEF	**Mortality**	**FEF**	**Mortality**	**FEF**	**Mortality**	**FEF**	**Mortality**
Cx. pipiens* form *molestus	11/211 (5.21%)	1/8 (12.50%)	8/329 (2.43%)	0/5 (0%)	NA	NA	NA	NA
Cx. pipiens hybrid	74/332 (22.28%)	3/66 (3%)	98/294 (33.33%)	1/40 (2.50%)	67/312 (21.47%)	3/65 (4.61%)	NA	NA
Ae. albopictus	NA	NA	NA	NA	NA	NA	49/200 (24.50%)	3/41 (7.31%)

5.4.2 Mosquito infection and dissemination

Infected mosquito bodies were detected in both *Cx. pipiens* forms tested with the lowest viral dose (5.75 log_{10} TCID$_{50}$/mL). However, disseminated infection was not detected in any mosquito tested with this viral titer. On the other hand, the viral dose of 7.01 log_{10} TCID$_{50}$/mL was able to induce both infection and disseminated infection in the *Cx. pipiens* hybrid form. At the same viral dose, C*x. pipiens* form *molestus* presented infection but not dissemination. Both infection and disseminated infection were detected in *Ae. albopictus*. The IR and DIR are summarized in the table 2. DIR from *Cx. pipiens* hybrid form and in *Ae. albopictus* populations showed the ratio of mosquitoes where the virus was able to cross the midgut barriers.

Table 2. Infection and disseminated infection rates. IR= Infection rate; DIR= disseminated infection rate. ND= No data.

	1st Assay				**2nd Assay**		**3rd Assay**	
	$10^{5,75}$ TCID$_{50}$/mL		**$10^{7,01}$ TCID$_{50}$/mL**		**$10^{7,5}$ TCID$_{50}$/mL**		**$10^{6,23}$ TCID$_{50}$/mL**	
	IR	**DIR**	**IR**	**DIR**	**IR**	**DIR**	**IR**	**DIR**
Cx. pipiens* form *molestus	1/7 (14.28%)	0/7	1/5 (20.00%)	0/5	ND	ND	ND	ND
Cx. pipiens hybrid	8/63 (12.69%)	0/63	3/39 (7.69%)	2/3 (66.66%)	18/62 (29.03%)	6/18 (33.33%)	ND	ND
Ae. albopictus	ND	ND	ND	ND	ND	ND	4/38 (10.52%)	1/4 (25%)

PART II

5.4.3 RVFV transmission

RVFV was detected in the saliva of *Cx. pipiens* hybrid form and *Ae. albopictus* using both FTA cards and capillary technique. The TR of *Cx. pipiens* hybrid form was 50% (3 mosquitoes with positive saliva up to 6 mosquitoes with disseminated infection); the TR of *Ae. albopictus* was 100% (1/1). These values referred to those mosquitos where the virus could have been able to cross the salivary glands barriers (legs and wings positives and saliva positive). However, other combinations had place. The relation among the different type samples of each mosquito with positive saliva samples is shown in table 3.

Table 3. Presence of virus in different samples of mosquitoes with positive saliva. R-*n*= *Cx. pipiens* hybrid, 1st assay; M-*n*= *Cx. pipiens hybrid*, 2nd assay; V-*n*= *Ae. Albopictus*, 3rd assay; - = negative; + = positives. ND= No data; Ct values of positives samples analysed by RT-qPCR are reported in brackets.

Mosquito	RT-qPCR				Citophatic Effect
	Legs and Wings	FTA 5 dpi	FTA 14 dpi	Saliva	Saliva
R-21	-	ND	+ (43,04)	ND	ND
M-5	-	-	-	+ (36,64)	+
M-14	+ (23,30)	-	-	+ (30,40)	+
M-35	+ (26,39)	-	-	+ (35,68)	-
M-46	-	+ (39,88)	-	+ (39,47)	-
M-49	-	-	+ (30,73)	-	-
M-59	+ (23,20)	-	-	+ (29,49)	+
V-3	+ (26,95)	+ (36,53)	-	+ (30,32)	+

In the first assay, one FTA belonged to a mosquito of *Cx. pipiens* hybrid form (named R-21 in table 3) resulted positive using the titer of 7.01 log_{10} $TCID_{50}$/mL but it did not present a disseminated infection.

In the second assay, two FTA cards resulted tested positives. These FTA cards belonged to two different specimens of *Cx. pipiens* hybrid form (M-46 and M-49) and were sampled at different time-point (5-dpi and14-dpi). Both mosquitoes also did not present a disseminated infection. Five saliva samples obtained by capillary technique from *Cx. pipiens* hybrid tested positives by RT-qPCR. Three of them produced citophatic effect once inoculated in Vero cells. Two specimens (M-14 and M-59) with infectious viral particles in saliva presented a disseminated infection. Conversely specimen M-5 did not present it. The FTA cards of these three specimens were negatives.

In the third assay, one FTA card belonged to *Ae. albopictus* specimen with disseminated infection (V-3) tested positive at 5dpi, although the FTA card resulted negative at 14dpi. However, the saliva sample extracted at 14dpi from the same mosquito tested positive by RT-PCR and was able to induce citophatic effect once inoculated in Vero cells.

In summary, the efficiency of transmission in *Cx. pipiens* hybrid was higher (3 mosquito with infectious saliva out of 62 mosquitoes fed, i.e. 4.83%) than in *Ae. albopictus* (1 out of 38, i.e. 2.63%), although differences were not statistically significant.

5.5 DISCUSSION

Mosquitoes belonging to *Culex* and *Aedes* spp. are considered main vectors for RVFV. For the first time, two populations of different *Cx pipiens* forms and a population of *Ae. albopictus* collected in Spain, have been demonstrated susceptible to RVFV infection. Moreover, *Cx. pipiens* hybrid and *Ae. albopictus* were able to transmit RVFV.

Cx. pipiens molestus exhibited lower propensity to feed from the artificial feeding system used, and resulted not to be a good laboratory species for competence studies.

In *Cx. pipiens* hybrid form, the rates of infection and dissemination tended to increase proportionally to the viral dose used during bloodfeeding, as previously observed in several species (Turell *et al.*, 2008a; Turell *et al.*, 2013a). This fact is probably due to the presence of dose-dependent midgut barriers that the virus have to overcome to successfully infect and disseminate in the whole mosquito body (Franz *et al.*, 2015). *Cx. pipiens* hybrid form presented higher infection and dissemination rates than *Cx. pipiens* form *molestus,* confirming what has been described in a previous

study using North American mosquito species (Turell *et al.*, 2014). The absence of disseminated infection in *Cx. pipiens* form *molestus* suggests the presence of a Midgut Escape Barrier (MEB) and the impossibility of RVFV 56/74 strain to overcome it. This hypothesis was already proposed by other authors using a different strain of RVFV (Turell *et al.*, 2014). However, the low number of *Cx. pipiens* form *molestus* successfully fed in this study does not provide sufficient data to strongly support this hypothesis and further studies are needed to clarify this point.

The rates of infection and dissemination of Spanish *Cx. pipiens* was lower than those of *Cx. pipiens* tested in France (Moutailler *et al.*, 2008) and Canada (Iranpour *et al.*, 2011). In both cited studies some differences in the experimental procedure could explain such differences: i) the titers used for challenge were higher (ranging from $10^{7.9}$ to $10^{9.4}$ PFU/ml); ii) the source of feeding was different (feeding directly from an infected hamster); iii) the temperature along the experimental procedures was constant: 28ºC for French specimens and 25ºC for Canadian specimens. As mentioned before, the viral dose influences directly both infection and dissemination rates. In the present study, the viral doses used correspond to the viral load found in blood from European lambs experimentally infected with the same virulent RVFV strain (Busquets *et al.*, 2010). The use of a living host for feeding also improves the competence ability of the mosquito specimens tested (Lord *et al.*, 2006). Moreover, is also known that a higher and constant Extrinsic Incubation Temperature (EIT), corresponds to high rates of infection, dissemination and transmission, as experimentally demonstrated for different arboviruses (Richards *et al.*, 2007; Kilpatrick *et al.*, 2008; Lambrechts *et al.*, 2011). In a recent study we showed how the use cycling environmental conditions can also affect to vector competence and we suggest to use them to mimic the environmental conditions in the field (Brustolin *et al.*, 2016). In another study, two forms of *Cx. pipiens* from USA were tested using a similar viral load ($10^{7.5}$ PFU/ml) to that used in the

present study. However, the authors used an infected hamster as blood source and the specimens were maintained at a constant EIT of 26ºC (Turell *et al.*, 2014). As result, higher infection and dissemination rates were obtained compared to those presented in the present study.

Regarding RVFV transmission, the results showed that the Spanish *Ae. albopictus* and the *Cx. pipiens* hybrid form populations were able to sustain the virus transmission cycle. In contrast, Spanish *Cx. pipiens* form *molestus* was not able to transmit RVFV under laboratory conditions and its role in the transmission cycle would remain unclear. One positive FTA card at 5 dpi provided evidence of early transmission capacity in *Cx. pipiens* hybrid form, as previously observed in *Cx pipiens* from Maghreb region (Amraoui *et al.*, 2012). In the populations of Maghreb, the presence of infectious viral particles was observed since 3 dpi.

The use of FTA cards was originally designed as a surveillance tool for the arboviruses detection in field studies to avoid the analysis of trapped vectors (Van den Hurk AF, 2012). The exposition period for FTA cards was 7 days for field studies. Probably a shorter period of exposition could limit the possibility of the mosquito to feed from it, resulting in a lower number of positive FTA cards compared to the number of positive saliva samples obtained by capillary extraction. The presence of blue colored belly would indicate if specimens had fed from FTA soaked with honey. The negative FTA cards from mosquitoes with positive saliva mainly corresponded to specimens without blue belly although the blue belly was not always evident to naked eye. On the other hand, some authors suggested that forcing salivation in a capillary for 30-45 minutes may be inaccurate and can produce an overestimation of the viral transmission (Smith *et al.*, 2006). Therefore, the different results obtained by FTA cards *versus* saliva directly extracted with the capillary technique were probably due to several factors: i) less sensibility of FTA cards technique, ii) insufficient exposition time for FTA card and iii) overestimation of viral shed in the capillary.

Three *Cx. pipiens* hybrid mosquitoes (M-5, M46 and M49) with positive saliva samples but without dissemination infection were observed. Previous study by *Romoser* et al. described the possibility of the RVFV to disseminate from midgut via trachea (Romoser *et al.*, 2005). This will provide a direct pathway to the salivary gland without the need of dissemination in hemocoel and other secondary organs.

The rates of infection, dissemination and transmission observed in the Spanish *Ae. albopictus* population are comparable to those obtained in previous vector competence study with *Ae. albopictus* mosquitoes from Texas (Turell *et al.*, 1988) fed with an infectious bloodmeal at a final titer of $10^{4.7}$ PFU. Presence of a positive FTA card at 5 dpi showed also an early transmission capacity in contrast to what was described previously for the Texan specimens, which were able to transmit RVFV only since 14 dpi.

The three populations of mosquitoes used in the present study were all naturally infected by *Wolbachia spp.*. This could have influenced the vector competence of infected mosquitoes, as showed in previous studies with several binomial vector/arbovirus (Moreira *et al.*, 2009; Walker *et al.*, 2011). However, further studies regarding this issue are needed to elucidate its possible role in arbovirus-vector interaction.

The risk of introduction of RVFV in regions of Spain with high density of livestock and favorable environmental conditions, has been analyzed in a previous study (Sanchez-Vizcaino *et al.*, 2013). Several Spanish regions, including Catalonia, were found suitable to an RVF outbreak and the results of vector competence presented in the present study would support this possibility since both Spanish *Ae. albopictus* and *Cx. pipiens* hybrid populations appear to be able to sustain the RVFV transmission cycle. These results would indicate that during a hypothetical outbreak in Europe, *Cx. pipiens* would probably act as a principal vector. *Aedes* mosquitoes can transmit RVFV to their offspring and the eggs of *Ae. albopictus* can remain viable at low temperature (Linthicum *et al.*, 1985;

Thomas *et al.*, 2012). The presence of competent *Ae. albopictus,* would suggest the possibility that the virus could persist during the winter months in egg of infected mosquitoes of this species. Further studies are needed to explore if the populations of *Ae. albopictus* present in Spain would be able to transmit RVFV vertically to their offspring and how long the binomial virus/egg could persist viable under cold environmental conditions.

In conclusion, the data presented in this work provide helpful information to establish decisions towards effective vector control programs and surveillance plans to prevent and control possible RVF outbreaks. Additional studies would be also required to evaluate the vector competence of other European autochthonous vectors and their possible role during an RVF outbreak.

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PART III

General discussion

and Conclusions

“Science is wonderfully equipped to answer the question ’How?’ but it gets terribly confused when you ask the question ’Why?”

Erwin Chargaff

Chapter 6

General discussion

Diseases transmitted by mosquito vectors represent a high percentage of global infectious diseases. The impact of these diseases on public health, on economy and on public opinion can be extremely high as demonstrated by the recent epidemics of ZIKV, which WHO declared to be a public health emergency of international concern (WHO, 2016).

The rapid spread of different invasive vector species -like the Asian tiger mosquito *(Ae. albopictus*)- and the global climatic situation are changing the risk of outbreaks for many vector-borne diseases.

The present thesis was formulated with the main objectives of elucidating the introduction and the diffusion of the invasive mosquito *Ae. albopictus* in Spain- a potential vector for several arboviruses- and with the intention to determine the vector competence of autochthonous and invasive mosquitoes in Catalonia for two different zoonotic arboviruses (WNV and RVFV).

In the first study, the results evidenced that, more than one introduction of *Ae. albopictus* have probably occurred in Spain. This invasive species has a great physiological plasticity which allows it to rapidly adapt to new environmental conditions and also to endure large journeys (Paupy *et al.*, 2009). These characteristics have allowed the global dispersion of the Asian tiger using human activities such as international trades or tourism. The global analysis performed in this thesis supports the hypothesis of the dispersion pattern linked to global trade. In public databases many datasets of *Ae. albopictus* are available and several phylogeografic studies have been performed. However, published datasets have heterogeneous overlapping rates or have been created using a different genetic marker. This limits the successful use of retrospective data from public databases to build a robust large scale study. A harmonization of the genetic marker used should be adopted by the scientific community in order to obtain more valuable and reliable data for phylogeografic studies concerving the invasive species *Ae. albopictus*.

Phylogenetic and phylogeografic studies offer powerful instruments to characterize populations and to correlate them with the territory. This information is useful to understand the dynamics of mosquito populations in the country. Surveillance of invasive mosquito species is more important than ever for a correct estimation of the risk of introduction of exotic diseases. Moreover, these invasive species could play an important role as a possible secondary vector for endemic diseases.

Among the invasive mosquito species present in Europe, *Ae. albopictus* is nowadays the most important. Its distribution area is huge compared to the others species and it has been reported as being responsible for different arboviral outbreaks. However, other invasive species such as *Ae. aegypti,* present in Madeira island and in the Black Sea region, require urgent attention due to their capacity to be vector of several arboviruses (Akiner *et al.*, 2016).

In the second study, the vector competence of Spanish *Cx. pipiens* form *molestus*, *Cx. pipiens* hybrid form and *Ae. albopictus* for WNV was investigated. For the first time in Europe we have studied the difference of the vector competence for WNV lineage 1 and lineage 2 strains. The results evidenced that the two forms of *Cx. pipiens* have different vector competence and highlighted the putative importance of *Ae. albopictus* as vector of WNV. Moreover, two different viral doses and no constant environmental conditions were employed.

The third study demonstrated that: i) mosquitoes of Catalonia are competent vector for RVFV, and ii) a RVF outbreak is possible in case of introduction. *Cx. pipiens* and *Ae. albopictus* were able to transmit RVFV under laboratory condition. In this study, two different approaches were used to test saliva samples. The results demonstrate that FTA cards were less sensible compared to the capillary technique.

The results obtained in both vector competence studies highlight the importance of using no constant temperature during the assays. The use of

variable circadian temperature -corresponding to the period of the maximum density of the vector in a particular region- allowed to estimate a more realistic vector competence of the populations present in that region. Meanwhile, constant temperature and same virus strain/titer should be only used in order to minimize the variability in the interlaboratory studies.

The susceptibility to infection of Spanish mosquito populations would seem low for both viruses. However, other factors such as the presence of naïve human/animal populations and a high rate of hosts density/biting mosquitoes in a determinate area, could contribute to the rapid spread of both viruses during a putative outbreak.

Different definitions for transmission rate (TR) can be found in the bibliography of vector competence studies. In our studies we refer to TR as the rate of mosquitoes with positive saliva with respect to mosquitoes with disseminated infection. This ratio is also used in recent studies for Zika virus (Chouin-Carneiro *et al.*, 2016). However, other studies– i.e. (Richards *et al.*, 2012; Di Luca *et al.*, 2016)- calculate the TR in a different way. Moreover, the transmission efficiency (TE) can be found with a different name (Di Luca *et al.*, 2016), making harder the comparison of the data. A harmonization of these ratios should be adopted by the scientific community to enable proprer comparison of results.

In the vector competence studies, the localization of the virus was performed analyzing different parts of mosquito: body, legs (or legs and wings) and saliva. This approach is the most used in this kind of studies. However, the use of an immuno-histochemical (IHC) technique would allow a better understanding of the pathogen's pathway through the mosquito's body and the eventual presence of infection and/or escape barriers. The low rates of feeding, infection and disseminated infection obtained during the experimental infections did not provide enough specimens to carry out the IHC studies.

Numerous strategies for the control of vector species involved in the transmission of zoonotic pathogens are available and can be divided into mechanical, environmental, biological, chemical and genetic methods (Baldacchino *et al.*, 2015). Among the biological methods, several microorganisms -and products derived from microorganisms- are currently employed for their larvicide and/or adulticide capacities: entomophatogenic fungi, copepods, *Bacillus spp.*, *Wolbachia spp.* and also *Spinosad* produced by *Saccharopolyspora spinose*. The use of *wMelPop Wolbachia* strain (originally identified in *Drosophila melanogaster*) is interesting because it can reduce: i) the length of the mosquito´s life (McMeniman *et al.*, 2009) and as consequence the mosquito density, and ii) the vector competence for several arboviruses (Moreira *et al.*, 2009; Walker *et al.*, 2011; van den Hurk *et al.*, 2012). The mosquito populations used in the vector competence studies of the present thesis were found naturally infected with *Wolbachia spp.*. The presence of *Cx. pipiens* and *Ae. albopictus* naturally infected with *Wolbachia spp.* in Catalonia represent a great opportunity to study if this symbiont can modulate the vector competence of Spanish mosquitoes. Further studies are needed to: i) analyze the presence and the geographical distribution of *Wolbachia* infected mosquitoes in Catalonia; ii) characterize the *Wolbachia* strain that infect the Spanish mosquitoes and iii) explore possible interactions between the bacteria and the vector.

The immune system response of the vector to arbovirus infection is a fascinating new field of research. In the last decade our knowledge of mosquito immune system has rapidly advanced, especially thanks to the new genetic and molecular biology technologies (Cheng *et al.*, 2016). Among the multiple possibilities offered by these techniques, the possibility to obtain the whole transcriptome results particularly

interesting. Comparing the transcriptome of tested mosquitoes (feed v*ersus* not feed, infected *versus* not infected, disseminated *versus* not disseminated, etc.) from a same population will allow to better understand the immune response mechanism involved in the control of arbovirus replication. The work presented in this thesis is the base for such kind of studies.

Vector competence is a complex balance between vector and pathogen and numerous factors affect it. Insect-Specific Viruses (ISVs) are specific viruses of insects which have historically generated limited interest due to their inability to infect vertebrates (Blitvich *et al.*, 2015). However, evidence of their impact on the vector competence in case of coinfection, has rapidly increased the interest of the scientific community. Possible application of ISVs as a strategy to reduce vector competence of co-infected vectors is linked to superinfection exclusion phenomenon. In the study of Kent *et al.*, Culex flavivirus (CxFV), an insect specific flavivirus, was able to enhance the transmission of WNV in *Cx. quinquefasciatus,* when both viruses were co-inoculated simultaneously (Kent *et al.*, 2010). Conversely, CxFV reduced WNV replication in *Cx. pipiens* during early infection but did not affect the transmission capacity at 14 dpi (Bolling *et al.*, 2012). Moreover, a recent study demonstrated how prior infection with Palm Creek virus -a flavivirus isolated from *Coquillettidia xanthogaster-* was able to suppress subsequent replication of WNV and Murray Valley encephalitis virus (MVEV) in mosquito cell cultures (Hobson-Peters *et al.*, 2013). Analyzing the results presented in these studies, it is evident that further studies are needed to investigate the effect of coinfection with ISVs and WNV/RVFV on vector competence of Spanish mosquitoes.

Evidence of co-circulation of different arboviruses in the same area is not a rare event (Terzian *et al.*, 2011; Caron *et al.*, 2012; Rezza *et al.*, 2014; Furuya-Kanamori *et al.*, 2016) and experimental evidence of vectors able to transmit

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two different arboviruses at the same time has been reported (Vazeille *et al.*, 2010). Similarly to coinfection with ISVs, coinfection with two mosquito-borne viruses can modify the replication capacity of both viruses as shown in a recent study (Bara *et al.*, 2014). In the cited study, vertebrate and invertebrate cells were co-infected with Sindbis virus (SINV) and La Crosse virus (LACV). The results evidenced how mixed infections suppress the replication of both viruses in vertebrate cells. Instead mixed infections had no effect on LACV replication and enhanced SINV replication in mosquito cells. In another study mosquito cells were co-infected with DENV-2 and YFV (Abrao *et al.*, 2016). In contrast to the results of *Bara et al.*, infection of C6/36 cells either with DENV-2 or YFV led to a significant impairment of the subsequent infection by the other virus, with a general reduction of replication level.
As in the case of ISVs, co-infection with two or more mosquito-borne viruses can produce alteration of the replication capacity in mosquito cells and, as consequence, an alteration of vector competence for one or both viruses involved. The results presented in this thesis show that mosquitoes of Catalonia are susceptible to WNV and RVFV; this represents a start point for future studies about the coinfection of these two viruses in Spanish mosquitoes although it would be an unlikely event.

To conclude, we can affirm that the results presented in this thesis not only have answered to the proposed objectives but also represent a start point for future studies, oriented to investigate the relationship between vectors and pathogens and to establish more efficient vector control programs.

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Chapter 7

Conclusions

I- High genetic diversity has been reported in Spanish populations of *Ae. albopictus*, suggesting a multiple introduction event.

II- Phylogeographic analyses indicated that *Ae. albopictus* distribution in Spain and worldwide is highly influenced by human activities and trade.

III- *Cx. pipiens* and *Ae. albopictus* from Catalonia are competent vectors for both WNV and RVFV. *Cx. pipiens* hybrid form and *Ae. albopictus* demonstrated to be able to transmit both viruses. Conversely, *Cx. pipiens* form *molestus* was not a competent vector for either viruses.

IV- Despite the historical and exclusive presence of WNV lineage 1 in Spain, the results evidenced that Spanish mosquitoes can transmit WNV lineage 2 strain. Moreover, WNV lineage 2 resulted more efficient in the infection and dissemination than lineage 1 in *Cx. pipiens*.

V- RVF infectious viral particles have been isolated from saliva of *Cx. pipiens* hybrid form and *Ae. albopictus* experimentally infected. This suggests that local mosquitoes can initiate a RVF outbreak in Catalonia in case of RVFV introduction.

VI- Early RVFV transmission reported for infected *Cx. pipiens* hybrid form and *Ae. albopictus* increases their vectorial capacity, thus enhancing outbreak probability.

APPENDIX

Table S1. **Sampling sites, geographical coordinates and number of specimens of *Aedes albopictus* used in the Spanish data set analysis.**

AUTONOMOUS COMMUNITY (NUTS2)	PROVINCE (NUTS3)	MUNICIPALITY	LATITUDE	LONGITUDE	Nº
Catalonia (ES512)	Girona	Figueres	42.2640	2.9700	10
Catalonia (ES512)	Girona	Sant Julià de Ramis	42.0315	2.8375	10
Catalonia (ES512)	Girona	Lloret de Mar	41.7062	2.8618	10
Catalonia (ES511)	Barcelona	Vilanova del Camí	41.5700	1.6331	10
Catalonia (ES511)	Barcelona	Sant Cugat del Vallès	41.4747	2.0866	20
Catalonia (ES511)	Barcelona	El Prat de Llobregat	41.3370	2.0768	10
Catalonia (ES514)	Tarragona	Llorenç del Penedès	41.2785	1.5522	14
Catalonia (ES514)	Tarragona	Constantí	41.1477	1.2162	11
Catalonia (ES514)	Tarragona	Mont-Roig del Camp	41.0882	0.9625	17
Valencian Community (ES522)	Castellón	Vinarós	40.4742	0.4778	10
Valencian Community (ES522)	Castellón	Benicarló	40.3598	0.4200	9
Valencian Community (ES522)	Castellón	Peníscola	40.3598	0.3933	11
Valencian Community (ES522)	Castellón	Castelló de la plana	39.9883	-0.0583	9
Balearic Island (ES701)	Balearic Islands	Palma	39.5462	2.6244	9
Valencian Community (ES523)	València	Cullera	39.1631	-0.2537	10
Valencian Community (ES521)	Alicante	Benijòfar	38.0774	-0.7373	10
Valencian Community (ES521)	Alicante	Torrevella	37.9712	-0.7022	9
Region of Murcia (ES621)	Murcia	Murcia	37.9661	-1.1701	10
Total					199

Table S-2. Detail of COI sequences included in *Aedes albopictus* for the Worldwide data set. For each country the GenBank accession Number and the number of sequences (N) used are specified; *= sequences with gaps or degenerated nucleotides have been excluded.

COUNTRY	GENBANK ACCESSION NUMBER	N
ALBANIA	HF912379	1
AUSTRALIA	KC572145-KC572266; GQ143719	123
BRAZIL	AJ971003;J971014	2
CAMBODIA	AJ971006	1
CAMEROON	JF309317-JF309320	154
CENTRAL AFRICAN REPUBLIC	KC979140 -KC979144	75
CHINA	KC690896-KC690911	91
COSTA RICA	KC690912; KC690932; AB907796-AB907800	57
CROATIA	HQ906848-HQ906851	39
FRANCE	AJ971008-AJ971009	2
GERMANY	JX675570; JQ388786	2
GLORIEUSE ISLAND	HQ623004-HQ623006	3
GREECE	AY748239; JF810659; AY748238	3
INDIA	AY729984	1
INDONESIA	KF042861-KF042885	25
ITALY	KC690898; KC690912; KC690931-KC690939; JX679373-JX679386	46
JAPAN	JQ004524; AB690835; KC690898; KC690919-KC690920	17
MADAGASCAR	HQ622959-HQ622985; NJ406654-NJ406732; NJ406795-NJ406797; NJ406804-NJ406809; NJ406822-NJ406826	94
MAURITIUS	HQ622980-HQ622988	9
MAYOTTE	HQ622929-HQ622958	29
PAKISTAN	KF406398-KF406579 *	101
PANAMA	KC690932; AB907801	16
PAPUA NEW GUINEA	KC572267-KC572496	230
REUNION ISLAND	HQ622905-HQ622928; AJ971012-AJ971013	25
RODRIGUES ISLAND	HQ622990-HQ62295	6
ROMANIA	HF536717	1
SEYCHELLES	HQ622996-HQ623003; HQ622904	9
SINGAPORE	KC690919; KC690921-KC690929	36
SPAIN	KU319443 - KU319450	199
TAIWAN	KC690898; KC690912-KC690918; AY072044	31
THAILAND	AJ971015	1
TURKEY	JQ412504	1
UNITED STATES OF AMERICA	KC690896; KC690898; KC690912; KC690914; KC690917; KC690920; KC690932; KC690934; KC690940-KC690961; AJ971011; AJ971005	145
VIETNAM	HQ398900-HQ398901; AJ971010	3
TOTAL		1578

Table S-3. Genetic differentiation among Spanish populations. Significant pairwise FST values ($P < 0.05$) are shown in bold. 1= Sant cugat del Valles; 2= Costantí; 3= El prat de Llobregat; 4= Figueras; 5= Llorenç del Penedès; 6= Lloret de Mar; 7= Mont-Roig del Camp; 8= Sant Julià de Ramis; 9= Vilanova del Camí; 10= Balearic Island; 11= Murcia; 12= Benicarló; 13= Benijofar; 14= Castellón de la plana; 15= Cullera; 16= Peñiscola; 17= Torrevella; 18= Vinarós.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	0.00000																	
2	**0.34178**	0.00000																
3	0.00000	0.22535	0.00000															
4	**0.34426**	**0.23004**	0.22222	0.00000														
5	0.00000	**0.27878**	0.00000	0.27835	0.00000													
6	0.00000	0.22535	0.00000	0.22222	0.00000	0.00000												
7	0.00983	**0.24199**	-0.03461	0.23356	-0.01196	-0.03461	0.00000											
8	0.00000	0.22535	0.00000	0.22222	0.00000	0.00000	-0.03461	0.00000										
9	**0.76143**	0.10087	**0.66667**	**0.53333**	**0.71272**	**0.66667**	**0.66088**	**0.66667**	0.00000									
10	0.25817	**0.19360**	0.14110	0.17935	0.19489	0.14110	0.14458	0.14110	**0.53459**	0.00000								
11	0.00000	0.22535	0.00000	0.22222	0.00000	0.00000	-0.03461	0.00000	**0.66667**	0.14110	0.00000							
12	0.25817	**0.19360**	0.14110	0.17935	0.19489	0.14110	0.14458	0.14110	**0.53459**	0.12500	0.14110	0.00000						
13	0.07455	0.16298	-0.00000	0.13333	0.03522	-0.00000	0.02683	-0.00000	**0.51852**	0.06559	-0.00000	0.06559	0.00000					
14	0.09661	0.17742	0.01235	-0.00411	0.05174	0.01235	0.01833	0.01235	**0.56953**	0.08333	0.01235	0.08333	-0.00332	0.00000				
15	**0.47787**	**0.20823**	**0.34921**	**0.31111**	**0.40910**	**0.34921**	**0.39329**	**0.34921**	**0.38492**	**0.28801**	**0.34921**	**0.28801**	**0.27160**	**0.29057**	0.00000			
16	**0.19901**	**0.16667**	0.10192	**0.14331**	**0.14659**	0.10192	**0.15037**	0.10192	**0.39704**	0.10388	0.10192	-0.03709	0.04032	0.08004	0.11587	0.00000		
17	**0.40100**	**0.25200**	0.26829	**0.24784**	**0.32979**	0.26829	**0.31653**	0.26829	**0.47003**	0.21875	0.26829	0.21875	0.05125	0.21429	**0.30166**	0.09838	0.00000	
18	0.07455	0.18889	-0.00000	0.16667	0.03522	-0.00000	0.01183	-0.00000	**0.58333**	0.09091	-0.00000	-0.05691	-0.00000	0.00062	**0.30556**	0.01959	0.22916	0.00000

Figure S-1. Spatial output form Geneland using *Aedes albopictus* Spanish populations. Points indicate the relative location of the sampled populations. Darker and lighter shading are proportional to posterior probabilities of membership clusters, with lighter (yellow) areas showing the highest probabilities of clusters. The number of clusters predicted based on the Bayesian clustering algorithm is shown (K = 2)

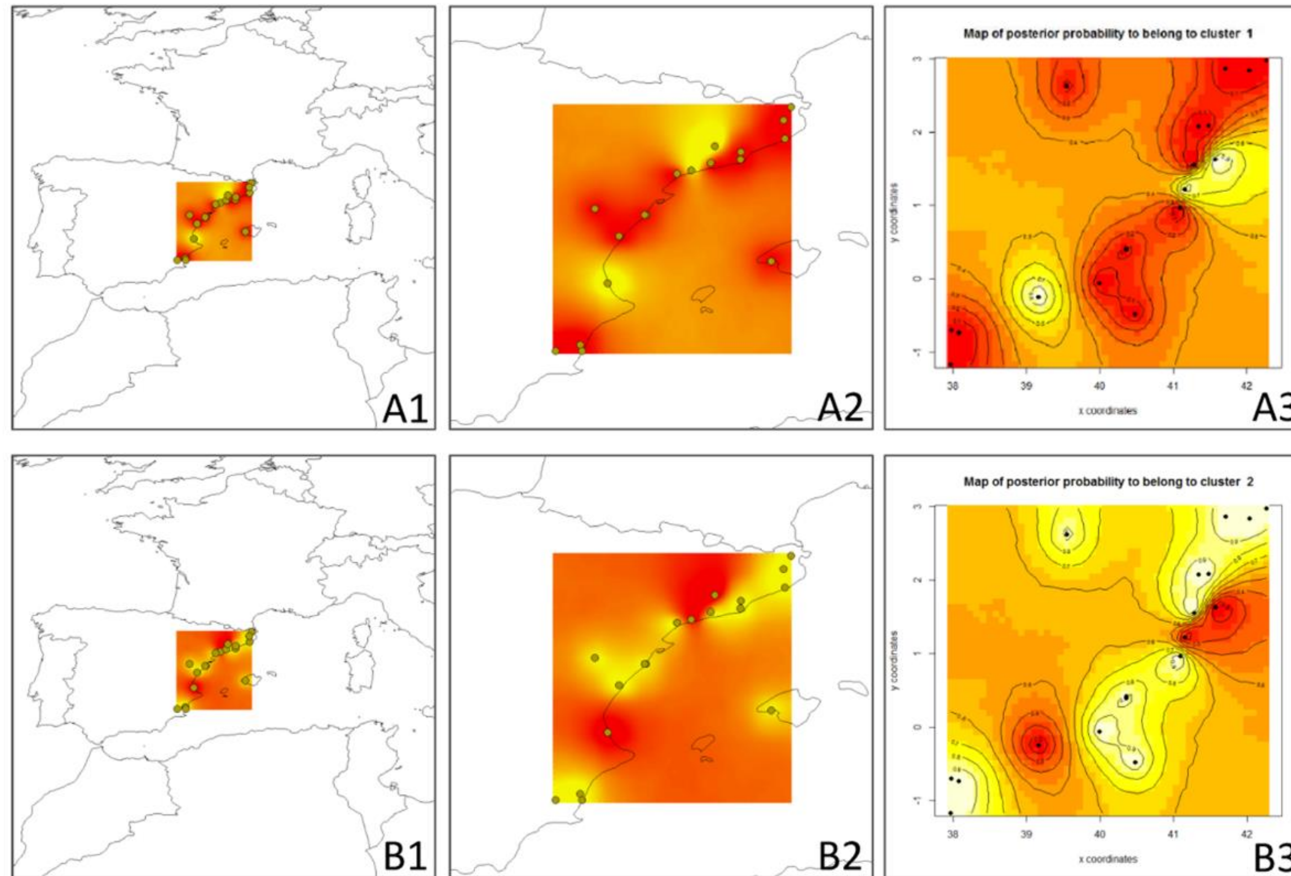

Figure S-2. Spatial output form Geneland using *Aedes albopictus* COI sequences from worldwide available populations. Points indicate the relative location of the sampled populations. Darker and lighter shading are proportional to posterior probabilities of membership clusters, with lighter (yellow) areas showing the highest probabilities of clusters. The number of clusters predicted based on the Bayesian clustering algorithm is shown (K = 4).