WEST NILE VIRUS IN PORTUGAL

VECTOR POPULATION, HOST INTERACTION AND DETECTION OF NEW FLAVIVIRUSES

Hugo Costa Osório

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Biologia

ÉVORA, OUTUBRO 2013

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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À Ana Paula

This dissertation includes results that have been or are being under consideration for publication in co-authorship. According to the decree law no. 388/70, Article 8, paragraph 2, the author of the dissertation, Hugo Costa Osório, states that has intervened and contributed the most in the execution of experimental work, data analysis, interpretation of results and drafting of the manuscripts published or submitted.

Na presente dissertação incluem-se resultados que foram ou estão a ser alvo de publicação em co-autoria. De acordo com o decreto de lei n.º 388/70, artigo 8, nº 2 o autor da dissertação, Hugo Costa Osório, declara que interveio e mais contribuiu na execução do trabalho experimental, análise de dados, interpretação de resultados e na redação dos manuscritos publicados ou submetidos.

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ABSTRACT

This thesis describes the studies undertaken in the epidemiology of West Nile virus (WNV) in Portugal exploring several key components of its natural cycle: identification of mosquito vector-populations – geographical and seasonal distribution; analysis of genetic and environmental determinants on feeding patterns of mosquito vectors; experimental transmission of WNV; identification of flaviviruses detected in wild mosquitoes; serological survey of wild birds potentially involved in the epizootic cycle of WNV; finally, a description of a clinical human case of WNV infection reported in 2010.

Culex pipiens was one of the most abundant mosquito species demonstrating ecological and intrinsic competence to transmit WNV. No positive mosquito pools for WNV were detected, but new mosquito-only flaviviruses were identified. The presence of antibodies anti-WNV in a juvenile turtle-dove suggests local virus circulation.

In conclusion, Portugal meets suitable conditions for epizootic circulation of WNV and for the occurrence of accidental human infections.

RESUMO

O vírus West Nile em Portugal
Vetores, interação com hospedeiros e deteção de novos flavivírus

Esta tese descreve os estudos desenvolvidos na epidemiologia do vírus West Nile (VWN) em Portugal explorando várias componentes do seu ciclo natural: identificação das populações de mosquitos vetores – distribuição geográfica/ sazonal; análise de determinantes genéticos/ ambientais na preferência de hospedeiro de mosquitos vetores; transmissão experimental do VWN; identificação de flavivírus detectados em mosquitos selvagens; estudo serológico em aves potencialmente reservatórios do vírus; finalmente é descrito um caso clínico humano de infecção por VWN detetado em 2010.

Culex pipiens foi uma das espécies de mosquito mais abundantes e demonstrou competência ecológica e intrínseca para transmitir o VWN. Não foram detectados *pools* de mosquitos positivos para o VWN, mas foram identificados novos flavivírus específicos de insetos. A presença de anticorpos anti-WNV numa rola juvenil sugere circulação local do vírus.

Em conclusão, Portugal reúne condições adequadas para a circulação epizoótica do VWN e para a ocorrência de infecções humanas acidentais.

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ABBREVIATION LIST

ABTS – 2,2'azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid)

*Ae*FV – *Aedes* flavivirus

*Aeve*FV – *Aedes vexans* flavivirus

BLAST – Basic local alignment searches

BSL – Biosafety level

C6/36 – *Aedes albopictus* salivary glands cell line

$CCID_{50s}$ – 50% cell culture infective dose

CDC – Centers for Disease Control and Prevention

cDNA – Complementary DNA

CEVDI – Centro de Estudos de Vetores e Doenças Infecciosas Dr. Francisco Cambournac

CFAV – Cell fusing agent virus

CNS – Central nervous system

CO_2 – Carbon dioxide

CPE – Cytopathic effect

CRP – C-reactive protein

CSF – Cerebrospinal fluid

*Cx*FV – *Culex* flavivirus

*Cxth*FV – *Culex theileri* flavivirus

DNA – Deoxyribonucleic acid

Eb-ELISA – Epitope blocking ELISA

EBV – Epstein-Barr virus

ECDC – European Centre for Disease control

EIP – Extrinsic incubation period

ELISA – Enzyme-linked immunosorbent assay

ENIVD – European Network for Diagnostics of "Imported" Viral Diseases

ER – Endoplasmic reticulum

ESR – Erythrocyte sedimentation rate

ftpn – females per trap night

HSB – Hospital São Bernardo

HHV6 – Human herpes virus 6

HI – Haemagglutination inhibition

HSB – Hospital de São Bernardo

IFA – Immunofluorescence assay

IgG, IgM – Immunoglobulin G, immunoglobulin M

INSA – Instituto Nacional de Saúde Dr. Ricardo Jorge

JE – Japanese encephalitis

KDa – Kilodalton

KRV – Kamiti River virus

MAb – Monoclonal antibody

MIR – Minimum Infection Rate

MOFs – Mosquito-only flaviviruses

MVE – Murray Valley encephalitis

*Oc*FV – *Ochlerotatus* flavivirus

PCR – Polymerase chain reaction

PRNT – Plaque reduction neutralization test

RES – Reticuloendothelial system

REVIVE – Rede de Vigilância de Vectores

RH – Relative humidity

RNA – Ribonucleic acid

RT-PCR – Reverse transcription polymerase chain reaction

SLE – Saint Louis encephalitis

TBE – Tick-borne encephalitis

UK – United Kingdom

USA – United States of America

USUV – Usutu virus

VBORNET – Vector-borne network

VNT – Virus neutralization test

WHO – World Health Organization

WNV – West Nile virus

YF – Yellow fever

INTRODUCTION

OUTLINE AND OBJECTIVES

The West Nile virus (WNV) is a *Flavivirus*, family Flaviviridae, and was first isolated in 1937 from a febrile woman in the West Nile region of Uganda (Smithburn *et al.*, 1940). This virus is maintained in nature in a transmission cycle of mosquito-bird-mosquito, in which birds are the natural reservoirs and mosquitoes the arthropod vectors. It is a neuropathogenic arbovirus (arthropod-borne virus) for humans and horses, accidental and dead-end hosts who develop disease but do not contribute to the maintenance of virus in nature (Hayes, *et al.* 2005[a]). About 80% of WNV human infections are typically mild or subclinical; 20% may result in sudden fever, nausea, vomiting, malaise, headache, myalgias, rash and lymphadenopathy. Severe disease is usually found in about 1% of cases, in which serious neurological and often fatal symptoms occur, such as encephalitis and meningitis (Hollidge, *et al.* 2010).

West Nile virus circulates in Africa, Asia, southern Europe, Australia and North American Continent. It was responsible for some outbreaks of minor severity during the 1950s in Israel, during 1962 in France, and during 1974 in South Africa. From 1996 more severe outbreaks have occurred, first in Romania, then in Israel during 1998 and in Russia during 1999. In this year, WNV was for the first time detected on the American continent, and within three years spread to all United States (Hubálek and Halouzka, 1999; Komar, 2003).

In Portugal, the presence of WNV was first reported in 1966 in a serological survey carried out to the people of Aljustrel. In 1969, the virus was isolated from the mosquito *Anopheles maculipennis* in the same region of Alentejo. In 1970, 24 horses who survived an outbreak of equine encephalomyelitis were studied and 30% were seropositive (Filipe and Pinto, 1972). A new isolation came in 1996, also from *Anopheles maculipennis* in Tagus estuary (Fernandes *et al.*, 1998). In 2004, two human cases occurred in Algarve (Connell *et al.*, 2004) and the virus was isolated from *Culex pipiens* and *Cx. univittatus* mosquitoes (Esteves *et al.*, 2005). In 2010, virus activity was detected in horses and a probable human case was notified (Barros *et al.*, 2011; Alves *et al.*, 2012).

West Nile virus was initially considered a minor arbovirus in public health, however it has a high impact in countries where it is or has become endemic, as in the North American Continent (Komar, 2003). In Europe, the distribution is discontinuous, multifocal and unpredictable. The epidemic features of WNV are complex, and climate change as well as globalization processes will certainly influence the distribution patterns of this and other vector-borne pathogens (Reiter, 2008).

The general objectives of this thesis were:

1. The identification of mosquito species captured in different geographic regions of Portugal, proceeding to an update on the composition and distribution of the culicidae fauna;
2. The molecular identification of host blood meals in engorged females captured in nature, in order to analyze the host-feeding patterns of potential mosquito vectors;
3. The genetic analysis of the primary vector of WNV - *Culex pipiens* - towards the identification of biotypes with different vector capacities and its habitat determinants;
4. The analysis of vector competence of *Cx. pipiens* for WNV;
5. The detection and identification of flaviviruses in the collected mosquitoes;
6. The detection of anti-WNV antibodies in resident and migratory wild birds, and identification of the species involved in the natural cycle of WNV.

Studies in vectors

Mosquitoes were collected from April to November 2008-2012. The mosquito collection was held in collaboration with the Regional Health Administrations, with which was concluded a protocol of collaboration and also within the project "Understanding the factors that promote the prevalence of infectious diseases in migratory shorebirds (PTDC/BIA-BDE/64063/2006)." Fieldwork took place for three to four consecutive days per month in each area. Adult mosquitoes were trapped with CDC light-traps considering: 1) height, ground level or tree canopy; 2) proximity to potential natural breeding sites such as lakes, marshes, channels, holes in trees and abandoned containers with rainwater; 3) presence of potential hosts such as birds, horses or humans; 4) and vegetation. Larvae and pupae were collected in natural breeding sites. Samples were kept alive until arrival at the laboratory, identified and sorted by species. Females were separated and organized in pools from 25 to 50 specimens according to species, date and location. Mosquito pools were then used for flaviviruses detection after RNA extraction and RT-PCR. The engorged female mosquitoes were processed by PCR after DNA isolation in order to identify the blood meal host. *Culex pipiens* mosquitoes were used to analyse the biotype after DNA isolation and PCR. Larvae of *Cx. pipiens* and *Anopheles atroparvus* mosquitoes were set in insectary conditions for further studies regarding vector competence.

Studies in reservoirs

Birds were sampled in bird ringing sessions undertaken by the Institute for Nature Conservation and the project mentioned above. Blood samples were collected from birds and

sera was analyzed by enzyme-linked immunosorbent assay (ELISA) and virus micro-neutralization tests (VNT).

Studies in flaviviruses

In all positive mosquito pools screened for flaviviruses, direct sequencing and phylogenetic analyses of the virus were performed.

DISSERTATION PLAN

This thesis is organized in eight chapters.

The first chapter is the introduction to the theme providing general information about arthropod-borne viruses, an updated review of WNV and a broad perspective on mosquito biology and importance as vector of disease agents.

Chapter two presents the studies on mosquito composition and distribution that provided the basis for developing the research work plan.

Chapter three addresses vector-host interaction by analyzing together the feeding patterns of potential vector species and the biotypes of the *Culex pipiens* mosquitoes relating to host preference.

Chapter four is devoted to determine the vector competence for WNV of two important mosquito species in WNV epidemiology: *Anopheles atroparvus* and *Cx. pipiens*

Chapter five presents the results on mosquito screening for flaviviruses and discusses the importance of these viruses in a European context.

Chapter six is devoted to a serological survey of WNV in several wild bird species.

Chapter seven describes an autochthonous human case of WNV fever identified in CEVDI and notified to Health Authorities during 2010.

Finally chapter eight summarizes the results previously discussed emphasizing their implications in Public Health and proposing new lines for future research.

CHAPTER 1

LITERATURE REVIEW

1.1 ARBOVIRUSES

The arboviruses (arthropod-borne viruses) are currently the most important etiologic agents of emerging infectious diseases with public health importance (Hollidge *et al.*, 2010). Out of 534 described arboviruses 134 can cause human disease (Karabatsos, 1985). This amazing number of viruses is distributed in eight families, of which Bunyaviridae, Flaviviridae, and Togaviridae are the most important concerning medical and veterinary health. Arboviruses are maintained in natural cycles involving vertebrate hosts and arthropod vectors (Table 1).

Table 1. Most representative arboviruses with public health importance.

Vector	Family	Virus	Hosts [a]	Pathology [b]	Distribution [c]
Mosquito	Flaviviridae	Dengue 1-4	HUM, PRI	F, HF	TROPREG
		Yellow fever	HUM, PRI	F, HF	AFR, SAME
		Japanese encephalitis	AVI, PIG	F, ME	AS, PAC
		Murray Valley encephalitis	AVI	SF, ME	AUS
		Rocio	AVI	F, ME	SAME
		Saint Louis encephalitis	AVI	F, ME	AM
		West Nile	AVI	F, ME	AFR, AS, EUR,
	Bunyaviridae	Rift Valley fever	?	F, HF, ME	AFR, MEAS
		La Crosse encephalitis	ROD	F, ME	NAME
		California encephalitis	ROD	F, ME	NAME
		Oropouche	?	F	NAME, CAME
	Togaviridae	Chikungunya	HUM, PRI	F	AFR, AS, EUR
		Ross River	HUM, MARS	F	AUS, SPAC
		Mayaro	AVI	F	SAME
		O'nyong-nyong	?	F	AFR
		Sindbis	AVI	F	AM, AFR, AS, AUS, EUR
		Barmah Forest	?	F	AUS
		Easter equine encephalitis	AVI	F, ME	AM
		Western equine encephalitis	AVI, RAB	F, ME	AM
		Venezuelan equine encephalitis	ROD	F, ME	AM
Sandfly	Bunyaviridae	Sandfly fever	?	SF	AFR, AS, EUR
Tick	Flaviviridae	Kyasanar forest disease	PRI, ROD, CAM	F, FH, ME	SARA, IND
		Omsk hemorrhagic fever	ROD	F, HF	AS
		Tick-borne encephalitis	AVE, ROD	F, ME	NAME, AS, EUR
	Bunyaviridae	Crimea-Congo hemorrhagic fever	ROD	F, HF	AFR, AS, EUR

[a] CAM, camels; HUM, humans; MARS, marsupials; PIG, pigs; PRI, primates; RAB, rabbits; ROD, rodents. [b] HF, hemorrhagic fever; ME, meningo-encephalitis; F, fever. [c] AFR, Africa; AM, America; CAME, Central America; NAME, North America; SAME, South America; AS, Asia; SARA, Saudi Arabia; AUS, Australia; EUR, Europe; IND, India; MEAS, Middle East; TROPREG, tropical region; PACISL, Pacific Islands; SPAC, South Pacific (Adapted from Gubler, 2002).

Arboviruses life cycle requires a complex and dynamic interaction between the vertebrate hosts and the arthropod vectors both with the environment, in which the arboviruses must be competent to infect and replicate in vertebrates and invertebrates (Figure 1).

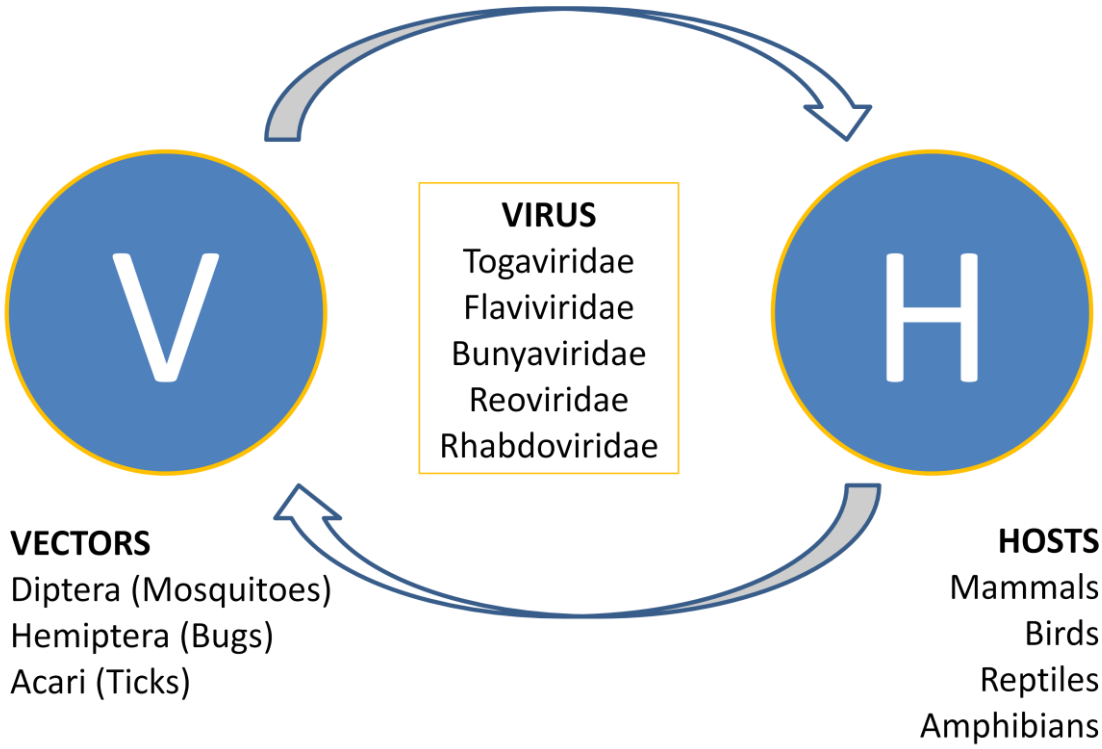


Figure 1. Natural cycle of arboviruses (Adapted from Higgs and Beaty, 2005).

Except African swine fever virus, family Asfarviridae (Tulman *et al.*, 2009), the arbovirus genome consists of ribonucleic acid, thus achieving greater adaptability to alternate replication in vertebrate and invertebrate hosts due to the high mutation rate of RNA genomes. The genome is usually small, encoding few structural and functional proteins. Arboviruses are obligate parasites and depend entirely on the host cell for replication and protein synthesis. The virion is composed of proteins and nucleic acids, which form the nucleocapsid, and some lipids and carbohydrates that form the outer envelope (Weaver, 1997).

Most arboviral infections in humans are characterized by fever or flu-like syndrome that can progress to neurological syndromes (Hollidge *et al.*, 2010), with invasion of the central nervous system (CNS) and development of meningitis and encephalitis, or hemorrhagic syndromes (Lupi, 2011). The treatment is symptomatic and prevention is based in reduction of mosquito bites. Yellow Fever (YF), Japanese Encephalitis (JE) and Tick-borne Encephalitis (TBE) vaccines are the only available for humans. A West Nile virus (WNV) vaccine has been approved for use in horses (Hall and Khromykh, 2004). The YF vaccine is recommended as a

preventative measure for travellers moving to endemic areas (Africa, Central America and South America) or for residents in these regions (Frierson, 2010). The vaccine of JE virus is recommended for residents and travellers from endemic areas of Asia (Wilder-Smith and Halstead, 2010). Strategies to develop tetravalent vaccines for dengue are ongoing in order to ensure immunization against all four serotypes and to prevent the dengue hemorrhagic fever, currently the most important viral disease transmitted by mosquitoes to humans (Thomas and Endy, 2011).

Arboviral transmission is always *biological*, which results in an *amplification* of the virus by replication within the cells of the vector (Chamberlain and Sudia, 1961). However, *mechanical* transmission in which no viral replication occurs is possible via contaminated mouthparts and may have high impact on the epidemiology of an agent (Figure 2). Vectors are usually infected by viremic blood ingestion, although transovaric and venereal infection may occur (Kramer and Ebel, 2003; Higgs and Beaty, 2005).

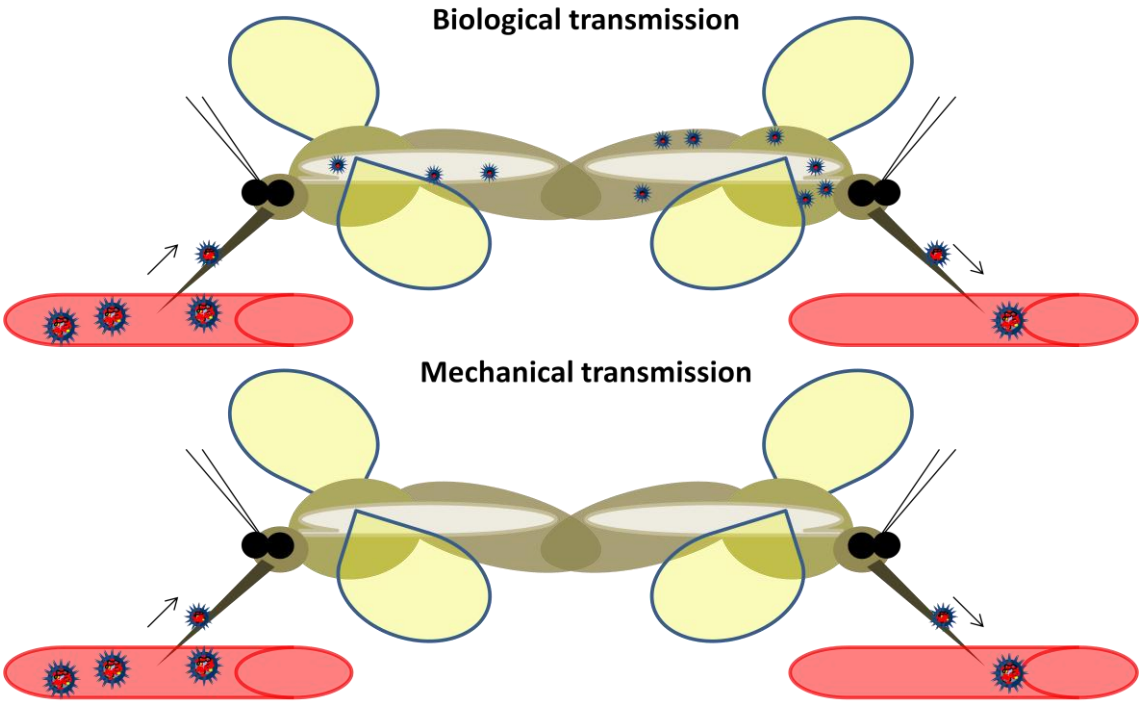


Figure 2. Two forms of transmission considering the development of the pathogen in the vector.

Maintenance and *amplification* are two major components of arboviruses transmission cycles. Maintenance is relative to mechanisms to increase virus survival time, as long term infections of hosts and vectors. Amplification is related to increased prevalence of infected hosts and vectors, increasing the amount of virus in nature, which usually occurs after periods

of reduced transmission of arboviruses, *e.g.* cold seasons that reduce vector activity. At this stage it is common the occurrence of outbreaks in vertebrates (Monath, 1988).

FAMILY FLAVIVIRIDAE

The family Flaviviridae comprises a large group of viruses that are distributed in three genera: *Flavivirus* (viruses transmitted to humans by arthropods – West Nile virus), *Pestivirus* (viruses that impact on the livestock industry – classical swine fever virus and bovine virus diarrhoea) and *Hepacivirus* (viruses that impact on blood transfusions – hepatitis C virus) that share morphogenetic characteristics and similar replication strategies. Flaviviruses and pestiviruses were initially considered Togaviridae family members given classical methods as a criterion for classifying viruses, but later evidence showed that these viruses represent a distinct evolutionary lineage and reclassified them in the family Flaviviridae.

The family Flaviviridae belongs to supergroup II positive-stranded RNA viruses and is the only member of this group that encode RNA helicases, thus showing a single strategy for genome replication (Lindenbach and Rice, 2001). The YF virus, prototype virus of the family, was the first to be isolated (1927) and cultivated *in vitro* (1932) and was the first virus which transmission was assigned to an arthropod vector (Eldridge, 1993).

CLASSIFICATION OF FLAVIVIRUSES

The genus *Flavivirus* consists of 73 viruses, of which 34 are transmitted by mosquitoes, 17 by ticks and 22 are zoonotic agents of unknown vector that circulate in nature in rodents and bats. A total of 40 viruses of the genus *Flavivirus* can cause disease in humans, 22 of 34 (65%) flaviviruses transmitted by mosquitoes, 13 of 17 (75%) flaviviruses transmitted by ticks and 5 of 22 (23%) no vector assigned. West Nile virus (WNV), dengue fever, YF, tick-borne encephalitis (TBE) and JE are currently the most important arboviral infections worldwide, with high rates of morbidity and mortality in all continents (Burke and Monath, 2001).

The flaviviruses are classified according to the classical serological criteria in antigenic complexes and sub-complexes or in groups (clusters), subgroups (clades) and species according to the molecular phylogeny. These studies have shown that mosquito-borne and tick-borne flaviviruses seem to have a no vector assigned *Flavivirus* as common ancestor.

All flaviviruses share common antigenic regions by haemagglutination inhibition (HI) with polyclonal serum, which was initially the basis for classification. Neutralization tests can distinguish viruses and genus subgroups. The membrane E protein is the viral hemagglutinin and the primary target of neutralizing antibodies. Combining data from neutralization assays that reflect a difference in the antigenic structure of the E protein, with HI test data, which

show the presence of serocomplex specific antigenic determinants, an analogue classification to the main biological and epidemiological characteristics of flaviviruses is obtained. A recent study that gathers data from molecular phylogeny with antigenic data sorts 68 flaviviruses in 14 phylogenetic subtypes and eight antigenic complexes (Kuno *et al.*, 1998). The use of specific monoclonal antibodies for the E protein has shown the presence of specific antigenic determinants enabling distinction of viruses to strain or sub-strain (Table 2).

Table 2. Classification of the genus *Flavivirus*.

Flaviviruses [a]	Clade	Antigenic complex
Non-vector cluster		
CFA Apoi **San Perlita; Jutiapa**	I	Modoc
Montana myotis leukoencephalitis; **Modoc; Cowbone Ridge; Sal Vieja**	II	Modoc
Bukalasa bat; Dakar bat; Rio Bravo; **Carey Island; Phnom Penh bat; Batu Cave**	III	Rio Bravo
Tick-borne cluster		
Gadgets Gully; **Royal Farm; Pow; Karshi; KFD; Langat; Omsk HF; TBE-far eastern subtype; RSSE; TBE-CE; Negishi**	IV	TBE
Kadam; Tyuleniy; Saumarez Reef; Meaban	V	Tyuleniy
Mosquito-borne cluster		
Edge Hill; Bouboui; Uganda S; Banzi; Jugra; Saboya; Potiskum	VI	Uganda S
Sepik; YF	VII	
Sokuluk; Entebbe bat; Yokose	VIII	
DEN-1 to -4 Kedougou	IX	DEN
Zika; Spondweni	X	
SLE; Rocio; Ilheus; **Tembusu**; THCAr; **Ntaya;** **Israel turkey meningoencephalitis; Bagaza Naranjal**	XI	Ntaya
Bussuquara; Aroa; Iguape	XII	
Kokobera; Stratford	XIII	JE
Cacipacore; Yaounde; **Koutango; Kunjin; WN;** **Alfuy; JE; Murray Valley encephalitis; Usutu**	XIV	JE

[a] Viruses in bold correspond to the antigen complex in the right column.
CFA, cell fusion agent; KFD, Kyasunur forest disease; Omsk HF, OmsK hemorrhagic fever; TBE, Tick-borne encephalitis, RSSE, Russian spring-summer encephalitis; CEE, Central European encephalitis; SLE, Saint Louis encephalitis; WN, West Nile. (Kuno *et al.*, 1998).

MOSQUITO-BORNE PATHOGENIC FLAVIVIRUSES

Out of the 73 flaviviruses described in the international catalogue of arboviruses (Karabatsos, 1985) 34 are transmitted by mosquitoes, 21 of which can cause human disease (Table 3).

Table 3. Pathogenic mosquito-borne flaviviruses.

Virus	Primary host [a]	Primary vector	Distribution [b]	Pathology [c]	Reference
Banzi	?	*Culex* spp.	AFR	F	(Fulop *et al.*, 1995)
Bussuquara	ROD	*Culex* spp.	SAME	F, ART	(Srihongse and Johnson, 1971)
Dengue 1 Dengue 2 Dengue 3 Dengue 4	HUM, PRI	*Aedes aegypti*	GLOBAL	F, HF	(Ross, 2010)
Edge Hill	MARS?	*Ae. vigilax*	AUS	F, ART, POLIART	(Aaskov and Phillips, 1993)
Enc Japonesa	AVI, PIG	*Cx. tritaeniorhyncus*	AS	ENC	(Wilder and Halstead, 2010)
Murray Valley Encephalitis	AVI	*Cx. annulirostris*	AUS	ENC	(Stich *et al.*, 2003)
Yellow fever	PRIM	*Ae. aegypti*	AFR, SAME	HF	(Gardner and Ryman, 2010)
Ilheus	AVI	*Psorophora* spp.	SAME	F, ENC	(Johnson *et al.*, 2007)
Kedougou	?		AFR		(Kuno and Chang, 2007)
Kokobera	AVI?	*Ae. vigilax,* *Cx. annulirostris*	AUS	F, RASH	(Boughton *et al.*, 1986)
Kujin	AVI	*Cx. annulirostris*	AUS	F, LFDP, RASH, ENC	(Scherret *et al.*, 2001)
Rocio	AVI	*Ae. scapularis,* *Ps. ferox*	SAME	ENC	(Medeiros *et al.*, 2007)
Sepik	?	*Armigeres* spp., *Ficalbia* spp., *Mansonia* spp.	AFR	F	(Kuno and Chang, 2006)
Spondweni	?	*Ma. uniformis*, *Ae. circuumluteolus*	AFR	F, NAU, EPIS	(Wolfe *et al.*, 1982)
Usutu	AVI	*Cx. perfuscus*	AFR	F, RASH	(Vazquez *et al.*, 2011)
Wesselsbron	OVI, ROD?	*Aedes* spp.	AFR, AS	F, MYAL, HPTM, RASH	(Justines and Shope, 1969)
West Nile	AVI	*Cx. pipiens* s.l.	AFR, NAME, EUR	F, MYAL, RASH, ENC	(Hayes *et al.*, 2005[b])
Zika	PRI	*Aedes* spp.	AFR, AS	F, RASH	(Hayes, 2009)

[a] AVI, birds; HUM, humans; MARS, marsupials; OVI, ovines; PIG, pigs; PRI, primates; ROD, rodents. [b] AFR, Africa; SAME; AS, Asia; AUS, Australia; NAME, North America; South America; [c] ART, arthralgia; ENC, encephalitis; EPIS, epistaxia; F, fever; HF, hemorrhagic fever; HPTM, hepatosplenomegaly; LFDP, lymphadenopathy ; MYAL, myalgia; NAU, nausea; POLIART, polyarthritis; RASH, cutaneous rash (Adapted from Burke and Monath, 2001).

WEST NILE VIRUS

STRUCTURE

The viral particle is spherical and has approximately 50nm in diameter (Petersen and Roehrig, 2001). It consists of a core of genomic RNA single-stranded positive sense of 11,000 to 12,000 nucleotides long enclosed by the capsid, which is involved by the virion envelope derived from the host cell membranes. The nucleocapsid is made of a 30-35nm icosahedral core, which contains multiple copies of 12KDa capsid proteins. Two integral membrane proteins E-glycoprotein (E) and prM protein characterize the virus tropism, the replication process, and the immune responses by stimulating B and T cells of vertebrate hosts. The E protein mediates virus host cell binding and is more immunologically important (Figure 3).

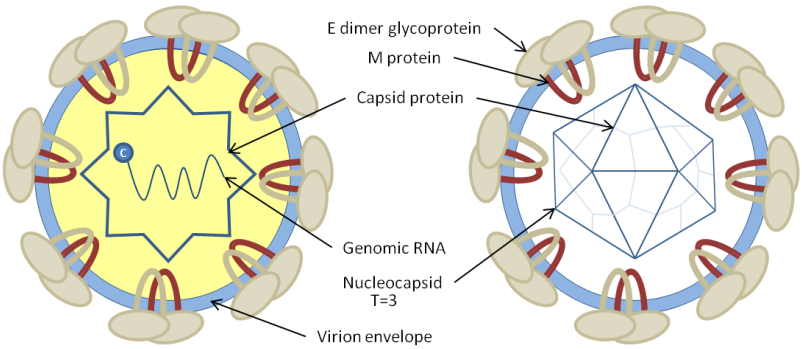


Figure 3. Structure of WNV virion.

The RNA genome consists of a 5' non-coding region with 100 nucleotides (NC), which is followed by an open reading frame that encodes three structural (C, M, and E) and seven non-structural (NS1, NS2a, NS2b, NS3, NS4A, NS4B, NS5) proteins ending with a non-coding region of 600 nucleotides. The M protein is synthesized as a precursor (prM) protein. The prM protein is processed to pr + M protein late in the virus maturation by a convertase enzyme (furin) (Figure 4).

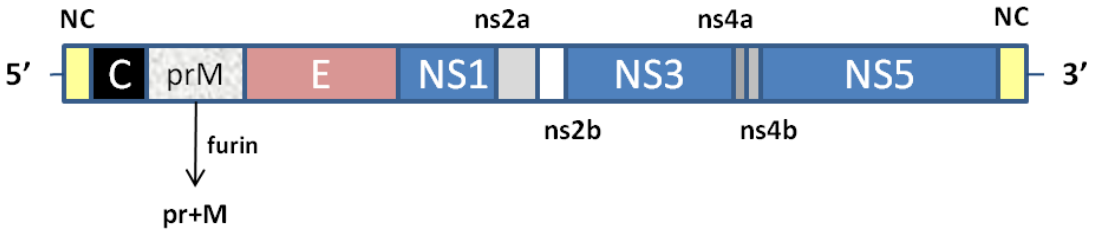


Figure 4. Genomic structure of WNV (Adapted from Peterson and Roehrig, 2001).

Viral replication occurs in the cytoplasm in close association with the endoplasmic reticulum (ER), from where new viral particles are then released via the cellular secretory apparatus (Campbell *et al.*, 2002).

PHYLOGENY

Two major phylogenetic lineages of WNV have been demonstrated: lineage 1 which has a worldwide distribution and includes strains of West Africa, Middle East, Eastern Europe, America and Australia (Kunjin virus); and lineage 2, which consists of enzootic strains of sub-Saharan Africa and Madagascar (Figure 5). These two lineages differ by approximately 30% nucleotide sequence based on a 255-bp region of the glycoprotein E gene (position 1402-1656 in the genome) (Lanciotti *et al.*, 2002). Certain strains of WNV remain in continuous genetic evolution (McMullen *et al.*, 2011) and the geographic distribution of the lineage 2 has been widening, as an example the case of Greece in 2010 (Valiakos *et al.*, 2011[a]).

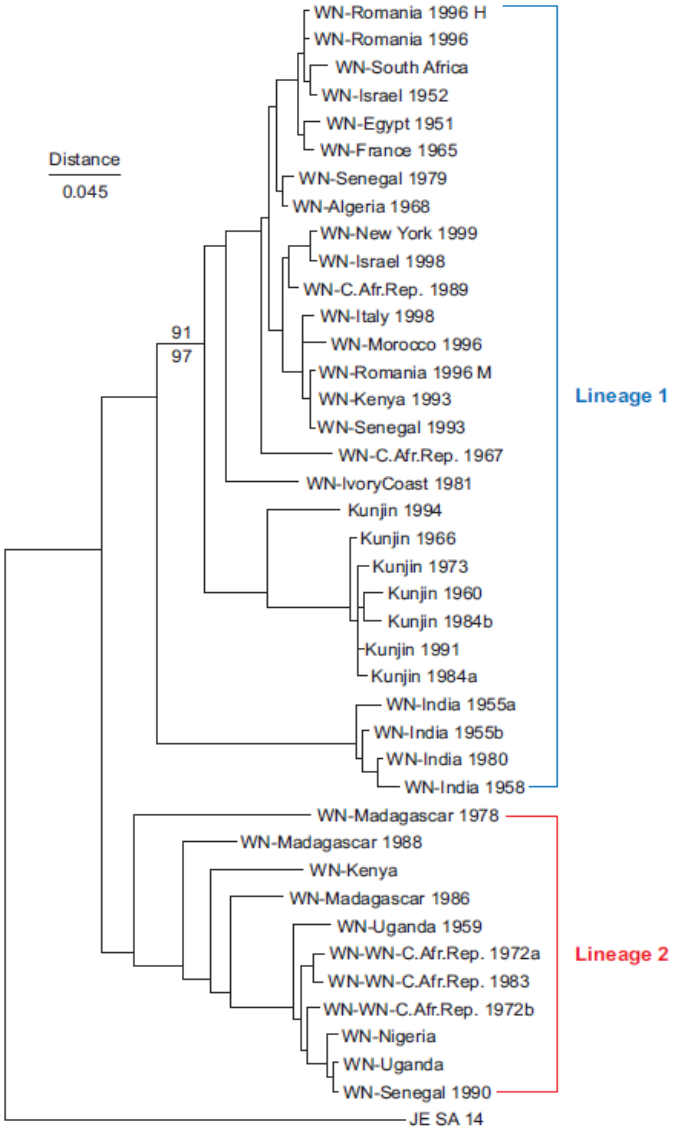


Figure 5. Phylogenetic tree based on E-glycoprotein nucleic acid sequence data (255 base pairs) constructed using MEGA by neighbor-joining with Kimura two-parameter distance (scale bar). Bootstrap confidence level (500 replicates) and a confidence probability value based on the standard error test were calculated using MEGA (Adapted from Kramer *et al.*, 2008).

INFECTIOUS AGENT

West Nile virus is serologically classified within antigenic subgroup of JE, which includes JE, Murray Valley Encephalitis (MVE) and Saint Louis Encephalitis (SLE) (Table 2). Kujin virus (Australia) of the same serocomplex presents the highest genetic and serologic similarity with WNV (Scherret *et al.*, 2001). Between JE virus or MVE and WN virus, there is a high degree of cross-immunization. Laboratory animals immunized with one of these viruses develop total or partial immunological protection for the other (Bosco-Lauth *et al.*, 2011). Studies show that genomic and antigenic strains of WNV may be organized into two distinct groups: Group I comprising strains from Africa, Europe, former Soviet Union and the Middle East; and Group II consisting of strains isolated in India and the Far East (Price and O'Leary, 1967). Some studies demonstrated a high heterogeneity in strains isolated from a limited geographic region (Morvan *et al.*, 1990). The analysis of antigenic and genomic variability agrees with the model of migrating birds, which are responsible for the maintenance and spread of the virus in the mosquito population at the local stopping of migratory routes (Burke and Monath, 2001).

The WNV develops and induce cytopathic effect and plaque formation in a variety of cell culture of avian origin (including chicken and turkey embryos), in human and primate, pigs, rodent, as well as amphibian cell lines. It also replicates in insect cells as *Drosophila*, *Aedes aegypti* and *Ae. albopictus*, producing cytopathic effect in the latter. Hamsters and rats of all ages are susceptible to lethal infection when the virus is inoculated intra-cerebrally. The rabbits and guinea pigs develop antibodies without symptoms or signs of serious infection. Rhesus monkeys and Bonnet developed fatal encephalitis after intracerebral or intranasal inoculation. All birds develop viraemia, including wildlife species, chickens and pigeons. Birds usually do not get sick except the Americans crows that are susceptible to fatal encephalitis. Horses are susceptible to infection and can develop fatal encephalitis, but as dead-end hosts develop low titer viraemia and produce few antibodies. Dogs are also susceptible to infection, but have a low titer viraemia. The cattle do not develop viraemia after experimental infection (Burke and Monath, 2001).

PATHOGENESIS AND PATHOLOGY

The pathogenesis of WNV is similar to that of other flaviviruses (Burke and Monath, 2001). The initial replication occurs in the skin and lymph nodes, at the periphery of the mosquito bite, and produces a primary viraemia in the reticuloendothelial system (RES). The replication of the virus in the RES results in a secondary viraemia, which can lead to infection

of CNS. The virus can be detected in the blood of a healthy person two days before and four days after the onset of symptoms, when the concentration of macrophages increases and IgM antibodies are formed. The viraemia caused by a natural infection is generally low and is not sufficient to infect new mosquitoes that feed on an infected human (Deubel *et al.*, 2001). The viraemia depends from factors specific to the virus and vertebrate host, and affects the clinical manifestations of disease. The virulence of the virus is related to the E protein, which is responsible for binding to the cell membrane and for processes that lead to invasion of the CNS (Chambers *et al.*, 1998). The invasion mechanism of CNS is not clear, but involves processes that promote replication of WNV in the endothelium of blood-brain barrier. Factors which cause the degeneration of the brain endothelium, such as age, hypertension or cerebrovascular disease, or other factors that increase the magnitude or the duration of viraemia, such as immunosuppression, increase the incidence of meningoencephalitis (Deubel *et al.*, 2001). The pathological changes in the CNS are direct result of: 1) proliferation of the virus in neuronal and glial cells; 2) cytotoxic immune response to infected cells; 3) diffuse perivascular inflammation; 4) microglial lumps (Shieh *et al.*, 2000; Armbrustmacher and Sampson, 2001). These lumps are composed of histiocytes and lymphocytes, and typically occur in areas where neuronal degeneration is high. In most patients when meningoencephalitis is detected, the immune response has been initiated and IgM antibodies are detected in serum and cerebrospinal fluid until the eighth day of disease. The increase of specific antibodies anti-WNV decreases viral replication by interfering with the binding of virus to the cell surface or by preventing fusion with the endosomes by promoting a structural alteration of intracellular E protein. Some persons who had survived the meningoencephalitis present permanent neurological sequelae, although there is no evidence of persistent WNV infections in humans (Tardei *et al.*, 2000).

CLINICAL DIAGNOSIS

Most human WNV infections are asymptomatic (Campbell *et al.*, 2002). The clinical syndrome is not specific and diagnosis may only be completed by serological analysis using ELISA assays, or indirect immunofluorescence, and/ or by nucleic acids detection using RT-PCR, only successful during viraemia period that typically last five days after the onset of clinical syndromes. In clinical cases of WNV fever, the disease resembles dengue fever with a typical incubation period of 2-6 days: sudden high fever (>39°C), headache and malaise accompanied by severe myalgias and sometimes by gastrointestinal symptoms. The symptoms tend to disappear in less than a week, but prolonged fatigue is common. In typical cases of fever about

half of patients develop maculopapular or roseolar skin rash, which tends to disappear without scaling and lymphadenopathy, followed by febrile syndrome.

The WNV encephalitis is clinically similar to arboviral encephalitis. A series of non-specific symptoms, as described above, followed by changes in mental status of patients, may occur, such as vomiting, loss of motor reflexes, muscle profound and diffuse weakness, flaccid paralysis, respiratory arrest, and in 15% of the cases coma and death. Mortality is directly related to the age, and patients over 60 years the most likely (Chowers *et al.* 2001; Nash *et al.*, 2001).

GEOGRAPHIC DISTRIBUTION AND EPIDEMIOLOGY

The WNV is widely distributed in Africa, Middle East, Southern Europe, former Soviet Union, India, Indonesia, and in 1999 was introduced in North America (Figure 6). Serological surveys conducted in the 1950s, after discovery and isolation of the virus in the Nile Valley region, demonstrated that human infections were extremely common in Egypt and Sudan, with a very high prevalence in the population (22% and 61% seropositive children and young adults) (Hurlbut *et al.*, 1956). Since the 1950s some outbreaks involving several hundred cases, although it was estimated that the incidence has been particularly higher, were reported in Israel as a result of WNV amplification and low immunity of human population (Goldblum *et al.*, 1956). The largest WNV outbreak took place in South Africa during 1974, in which 55% of the population in an area of 2500 km^2 in the province of Cape Town was infected. Thousands of clinical cases have been reported, but all without severe pathology or occurrence of encephalitis (Jupp, 2001). During the same decade, few cases were reported in Central Africa (Tomori *et al.*, 1978) and in Madagascar high annual prevalence of WNV infections in the population has been documented (Lonchampt *et al.*, 2003). In Egypt between 1966 and 1968 133 WNV infections with CNS invasion were reported (Abdel Wahab, 1970). In tropical and subtropical Asia infections are fairly frequent and the virus is endemic in many geographical areas (Thakare *et al.*, 2002). In Europe between 1962 and 1964 an outbreak of the WNV with 13 clinical cases with CNS invasion was reported (Murgue *et al.*, 2001[a]).

More recently, in the eastern hemisphere human cases of WNV infection were reported during 1994 in Algeria, during 1996 in Morocco, from 1996 to 1997 in Romania, during 1997 in the Czech Republic, between 1997 and 2003 in Tunisia, during 1999-2000 in Israel, 1999-2000 in Russia, during 2003 in France, during 2004 and 2010 in Portugal, during 2008 in Italy and from 2010 to 2011 in Greece. Epizootics in horses have been reported in

Morocco during 1996 and 2003, in Italy during 1998, in Israel during 2000, in south of France during 2000, 2003 and 2004 and in Portugal during 2010 (Table 4).

Table 4. Outbreaks of WNV in Europe and Mediterranean basin over the past two decades.

Year	Country	Humans		Horses	Birds	Months	Reference
		Cases	Fatalities				
1994	Algeria	50	8	-	-	Aug-Sep	(Murgue *et al.*, 2001[a])
1996	Morocco	1	1	+	-	Aug-Oct	(Murgue *et al.*, 2001[a])
	Romania	393	17	-	-	Jul-Oct	(Campbell *et al.*, 2001)
1997	Tunisia	173	8	-	-	Sep-Nov	(Murgue *et al.*, 2001[a])
1998	Italy	-	-	+	-	Aug-Oct	(Autorino *et al.*, 2002)
1999	Russia	826	40	-	-	Jul-Sep	(Platonov *et al.*, 2001)
	Israel	2	2	-	-	Aug	(Giladi *et al.*, 2001)
2000	Israel	439	29	+	-	Aug-Oct	(Bin *et al.*, 2001)
	France	-	-	+	-	Aug-Nov	(Murgue *et al.*, 2001[b])
	Russia	56	-	-	-	-	(Platonov, 2001)
2003	Morocco	-	-	+	-	Sep-Oct	(Schuffenecker *et al.*, 2005)
2003	France	6	-	-	+	-	(Durand *et al.*, 2004)
2004	France	-	-	-	+	-	(Jourdain *et al.*, 2007[a,b])
2004	Portugal	2	-	-	-	Aug	(Connell *et al.*, 2004)
2008	Italy	33	-	-	+	Aug	(Monaco *et al.*, 2010)
2010	Portugal	1	-	+	-	Jul	(Alves *et al.*, 2011)
2010	Greece	197	33	-	+	Aug	(Danis *et al.*, 2011[a])
2011	Greece	31	-	-	-	Jul-Aug	(Danis *et al.*, 2011[b])
2011	Italy	1	-	-	-	Sep	(Bagnarelli *et al.*, 2011)

In 1999 WNV was first detected in the Western Hemisphere, in the metropolitan New York, United States (USA). In the initial WNV outbreak 60 human cases and 7 fatalities, about 100 birds and 10 infected horses were reported (Hayes and Gubler, 2006). The virus soon established in the northeastern USA and from year to year the affected geographic area increased considerably. In 2003 it was first detected on the west coast in the states of California, Arizona and Colorado (Hayes *et al.*, 2005). The strain introduced in the USA was phylogenetically similar to the strain isolated in the Middle East, Israel, during the epidemic of 1999-2000 (Jia *et al.*, 1999).

Most human cases reported in the Western Hemisphere come from the USA, although there are also evidences of WNV in South America (Pauvolid-Correa *et al.*, 2011).

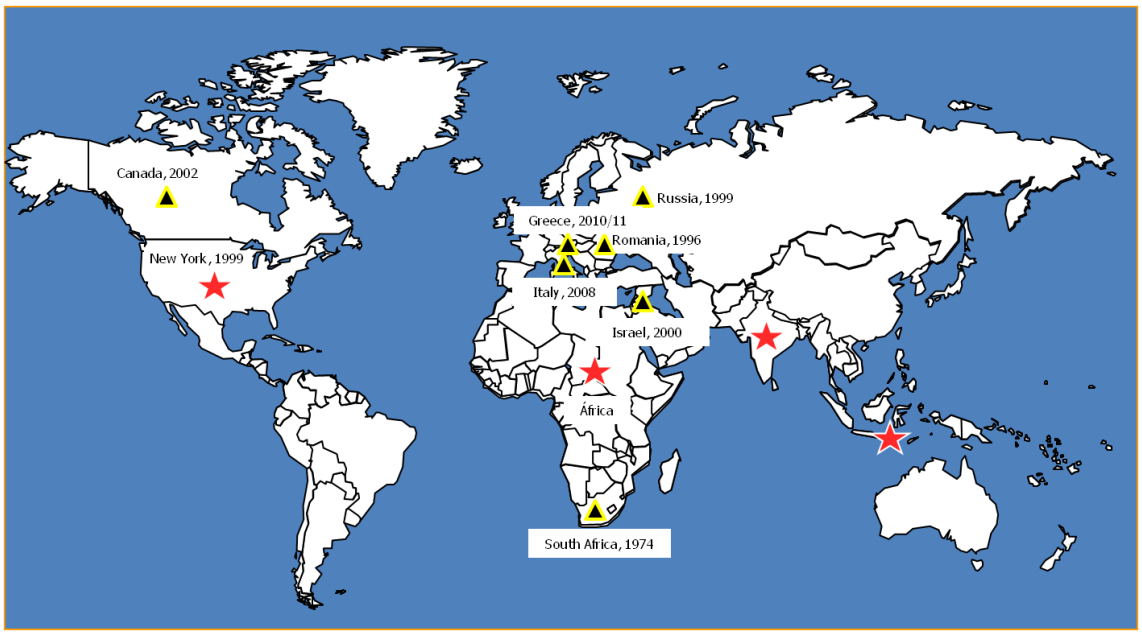


Figure 6. Major WNV outbreaks worldwide. Countries with human cases occurrences (▲) and endemic regions (★). Israel, 1957: 12 cases of human encephalitis; South Africa, 1974: 10,000 WN fever cases; Romania, 1996: 393 human cases of encephalitis, 17 fatalities; Russia, 1999: 826 cases, 84 human cases of encephalitis, 40 fatalities; New York, 1999: introduction of WNV in North America; Israel, 2000: 439 cases, 29 fatalities; Canada, 2002: 416 cases, 1 fatality; Italy, 2008: 33 cases; Greece, 2010: 197 cases, 33 fatalities; Greece, 2011: 31 cases.

In relation to life cycle, WNV is maintained in nature in a transmission cycle of mosquito-bird-mosquito, in which birds are the natural reservoirs and mosquitoes the arthropod vectors (Figure 7). The WNV was isolated from several mosquito species, but appears to be transmitted primarily by *Culex* mosquitoes (Komar, 2003). In Europe and Africa the major WNV vectors are *Cx. pipiens* (*molestus*) and *Cx. univitattus*; in India the most important species are *Cx. vishnui*; in Australia Kunjin virus is transmitted by *Cx. annulirostris*; and in USA by *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. tarsalis*, *Cx. restuans*, *Cx. salinarius* and *Cx. nigripalpus* (Hayes *et al.*, 2005). Vertical transmission was demonstrated in *Cx. pipiens*, *Cx. quinquefasciatus* and *Cx. tarsalis* and the virus has been isolated from hibernating females of these species, which reflects an adaptation strategy in colder latitudes to withstand the winter (Goddard *et al.*, 2003; Reisen *et al.*, 2006).

Birds are the most important host amplifier of WNV. Passeriformes, Charadriiformes, Strigiformes and Falconiformes develop high and prolonged viraemia that is sufficient to infect vector mosquitoes. Columbiformes, Piciformes, and Anseriformes do not develop high levels of viraemia in laboratory studies (Komar *et al.*, 2003). In the USA, studies demonstrated that sparrows are often infected with WNV developing high and long viraemia, and that they are highly infectious for mosquitoes (Godsey *et al.*, 2005; Komar *et al.*, 2005). The migratory routes of birds contribute to the spread of WNV (Reisen *et al.*, 2010).

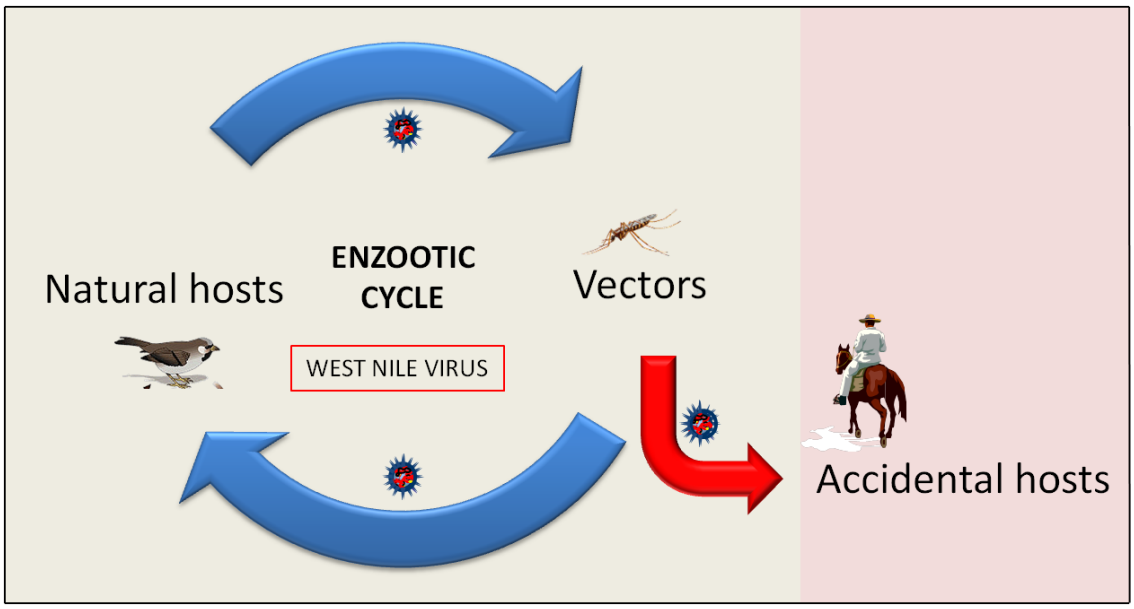


Figure 7. Transmission cycle of WNV.

Although WNV has been isolated from rodents in Nigeria and bats in India and Madagascar, most mammals do not generate sufficient levels of viraemia to contribute to transmission (Bunning *et al.*, 2002; Austgen *et al.*, 2004; Ratterree *et al.*, 2004). Some experimental studies on reptiles and mammals showed that these may be competent reservoirs of WNV, but they do not seem to contribute significantly to the epidemiological cycle (Klenk and Komar, 2003; Klenk *et al.*, 2004).

The magnitude of transmission is determined by mosquito vectors abundance and prevalence of infected mosquitoes, which can be translated as the minimum infection rate (MIR). This measure of the minimum number of infected mosquitoes by species and by geographic location and time evaluates the magnitude of epizootic transmission and relate it with epidemic risk (Hayes *et al.*, 2005). However, besides transmission by infected mosquitoes other risks of transmission between humans may occur, including: intrauterine (Hayes and O'Leary, 2004), blood transfusion (Busch and Petersen, 2010) and organ transplantation (CDC, 2009).

TREATMENT AND PREVENTION

Treatment is supportive and there is no human vaccine. For horses an approved inactivated virus vaccine is available (Hall and Khromykh, 2004). Prevention is based on reducing abundance of vector mosquitoes by control programs directed to larvae and adult and by personal protection measures. To prevent transmission by blood transfusion, blood donations from WNV endemic areas should be screened by PCR (Petersen and Busch, 2010).

WEST NILE VIRUS IN PORTUGAL

Evidence of WNV circulation in Portugal first emerged in 1967, when a preliminary serological survey in animals from the region of Alentejo showed high titers of hemagglutination-inhibiting antibodies and a high neutralization capacity to the WNV (Filipe, 1967). More serological information was obtained in 1969, when from 1,294 serum samples from cattle and sheep tested by HI 194 were positive to WNV with titers ranging from 1:20 to 1:5,120 (Filipe and Pinto, 1969). Thereafter a human serological survey in blood-donors was performed and from 1,649 analised sera by HI 38 (2.3%) were positive with titers ranging from 1:20 to 1:320 (Filipe, 1974; Filipe and Andrade, 1990). In 1971, the virus was isolated from the mosquito *Anopheles maculipennis* collected in Beja, in the region of Alentejo, during 1969 (Filipe, 1972) (Figure 8). A new isolation came in 1996, also from *Anopheles maculipennis* in Tagus estuary (Fernandes *et al.*, 1998). In 2004 two human cases were reported in the region of Algarve (Connell *et al.*, 2004) and a vector surveillance program was set in the district of Faro. The virus was isolated from *Culex pipiens* and *Cx. univittatus* mosquitoes. It belongs to lineage 1 and is phylogenetically close to the circulating strains in the Mediterranean basin, particularly in Italy, during 1998, in France, during 2000, and in Morocco, during 2003 (Esteves *et al.*, 2007; Parreira, *et al.*, 2007; Almeida *et al.*, 2008). In July 2010 the Health Authorities notified a human clinical case in the region of Lisbon and Tagus Valley, near the Sado estuary (Alves *et al.*, 2012). Moreover, the Veterinary Authorities detected the presence of two equine cases of WNV infection in that same region (Barros *et al.*, 2011). This topic is discussed in detail in Chapter 7.

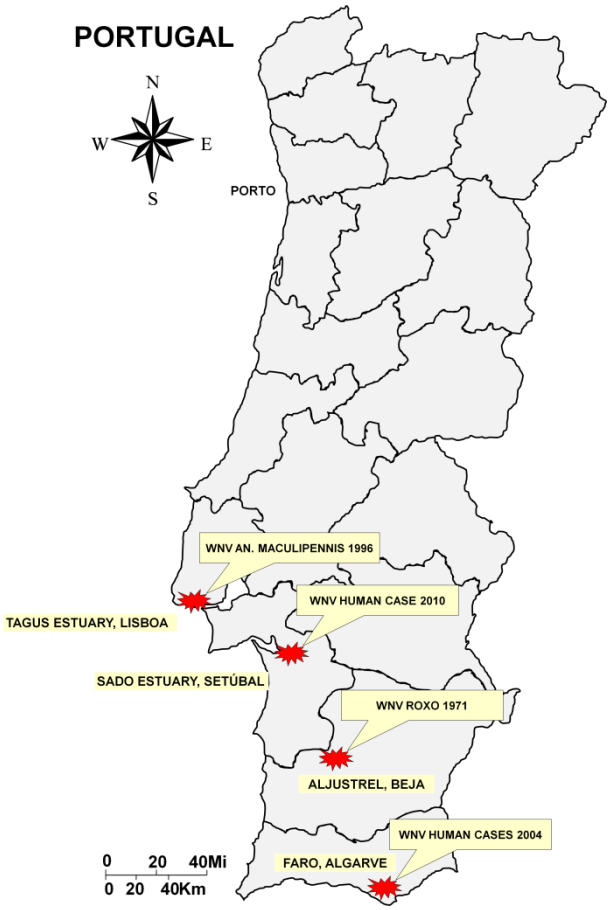


Figure 8: West Nile virus occurrences in Portugal.

1.2 MOSQUITOES AS DISEASE VECTORS

Mosquitoes are insects from order Diptera, family Culicidae, and are the most important medical and veterinary group of arthropods (Figure 9). More than 3,500 species and subspecies are distributed throughout the world where stagnant water occurs except in places permanently frost. Most species inhabit the tropical and subtropical regions, where temperatures and high humidity favour the rapid development of immature stages and survival of adults (Bates, 1954).

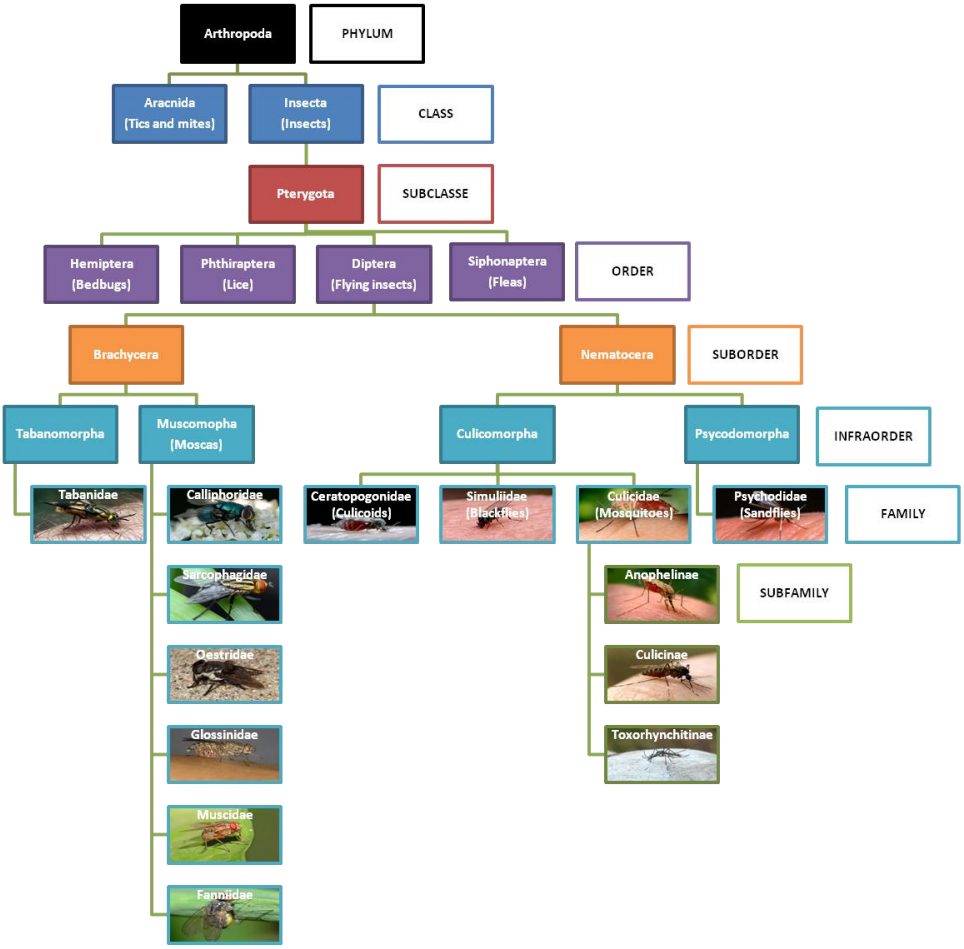


Figure 9. Systematic of arthropod vectors with interest in medicine and veterinary sciences.

Mosquitoes are classified into three subfamilies, Anophelinae, Culicinae, and Toxorhynchitinae and most species are distributed in three groups, commonly known as anophelines, culicines, and aedines, the latter referring to the tribe Aedini of the subfamily Culicinae (Knight and Stone, 1977).

Like other Diptera, mosquitoes have complete metamorphosis, *e.g.* larval and pupal stages are anatomically different from adults, occupying different habitats and with other

dietary requirements. The adult insect emerges from the pupa, which does not feed unlike the larvae (Snodgrass, 1959). The immature stages of the mosquitoes live in water and species have different criteria for breeding site selection. Some common examples of immature stages habitats are ponds, ditches, puddles of rain water, marshes, cavities in trees, trunks or rocks, and a variety of artificial containers related to human activities such as tires, irrigation tanks and small containers (Silver, 2008).

As other dipterans, adult mosquitoes have only one pair of wings and are good flying insects. They feed on fluids and, as all primitive groups of Diptera, have elongated body and wings. Adult females have mouthparts adapted for piercing and sucking the blood of vertebrate animals, which is responsible for the transmission of infectious agents between vertebrate hosts and makes mosquitoes the insects with more pages in the history of medical entomology literature (Spielman and D'Antonio, 2001).

Regarding the age-old relationship between humans and mosquitoes is surprising that only in the second half of the nineteenth century mosquitoes were identified as human disease vectors. In 1876 Sir Patrick Manson found that the nematode responsible for human lymphatic filariasis was transmitted by mosquitoes. This discovery has named him "the father of medical entomology" and signed the beginning of a productive period in medical sciences, with the discovery of other mosquito-borne diseases: malaria in 1898, YF and dengue in 1900 and 1902, respectively (Eldridge, 1993).

CLASSIFICATION OF CULICIDAE

The family Culicidae has three subfamilies, in which 18 genera are the most representative (Table 5). Culicidae systematic is complex and has been continuously subject to updates from the beginning of the first taxonomic revisions (Edwards, 1932; Stone *et al.*, 1959). The first world catalogue of the family Culicidae includes 1,400 species grouped in 30 genera (Edwards, 1932). In 1977, the most comprehensive catalogue of Culicidae was published, *A Catalog of the Mosquitoes of the Word* (Knight and Stone, 1977), which has been updated in three supplements (Knight, 1978; Ward, 1984; Ward, 1992) comprising the last review, a total of 3,209 valid species arranged in 71 subgenera, 26 genera and 10 tribes. Today this catalogue is maintained electronically by the Walter Reed Biosystematics Unit in Washington DC (URL: http//wrbu.si.edu) and has 3,528 valid species distributed among 43 genera (Harbach and Howard, 2010).

Table 5. Classification of Culicidae.

Subfamily	Tribe	Most representative genera
Anophelinae	Anophelini	*Anopheles*
		Bironella
		Chagasia
Culicinae	Aedini	*Aedes*
		Haemagogus
		Ochlerotatus
		Psorophora
	Culicini	*Culex*
		Deinocerites
	Culisetini	*Culiseta*
		Coquillettidea
	Mansoniini	*Mansonia*
	Orthopodomyiini	*Orthopodomyia*
	Sabethini	*Sabethes*
		Trichoprosopon
		Wyeomyia
	Uranotaeniini	*Uranotaenia*
Toxorhynchitinae		*Toxorhynchites*

(After Knight and Stone, 1977).

Mosquito species with public health importance are classified within Anophelinae and Culicinae subfamilies. The family Toxorhynchitinae does not feed on blood; however larvae are predators of aquatic organisms, including other mosquito larvae, and thus may be considered important for the biological control of other species (Collins and Blackwell, 2000). The tribe Sabethini includes many tropical and subtropical species of the Old and New World, some of which immature stages are unknown.

This thesis adopts the classification of Harbach and Howard (2010) with two exceptions, namely *Stegomyia aegypti* and *Stegomyia albopicta* where it remains the previous terminologies *Aedes aegypti* and *Aedes albopictus*, respectively.

MEDICAL AND VETERINARY IMPORTANCE

Mosquitoes are vectors of viruses, protozoan and nematodes (Table 6). These microorganisms can switch between a parasitic phase with and a free living phase, and also switch between vertebrate and invertebrate hosts, in this case the mosquito. The need for blood meal make adult mosquitoes susceptible to pathogens and parasites of vertebrate hosts and lead to transmission of these zoonotic agents to other vertebrates, although transmission of a particular organism depends on many aspects of mosquito physiology and ecology (Clements, 2000).

Table 6. Most representative mosquito-borne pathogens.

Pathogen/ Disease	Host		Primary vector
	Natural	Accidental	
Virus			
Eastern equine encephalitis	Birds	Humans, horses	*Coquillettidea* perturbans
West equine encephalitis	Birds	humans, horses	*Culex tarsalis*
Venezuelan encephalitis	Mammals	Humans	*Cx. pipiens*
Dengue	Humans	Humans	*Aedes aegypti, Ae. albopictus*
Japanese encephalitis	Pig	Humans	*Cx. tritaeniorhynchus*
Saint Louis encephalitis	Birds	Humans	*Cx. pipiens, Cx. nigripalpus*
Yellow fever	Primates	Humans	*Ae. aegypti, Ae. albopictus*
La Crosse encephalitis	Rodents	Humans	*Ae. triseriatus*
West Nile fever/ encephalities	Birds	Humans, horses	*Cx. pipiens*
Apicomplexa			
Human malaria	Humans		*Anopheles gambiae*
Avian malaria	Birds		*Culex* spp.
Filarial nematodes			
Wuchereria bancrofti	Humans		*Culex* spp., *Mansonia* spp.
Brugia malayi	Cats	Humans	*Culex* spp., *Mansonia* spp.
Dirofilaria immitis	Dogs	Humans	*Culex* spp., *Aedes* spp.

(Adapted from Eldridge, 2005).

The first evidence of a human parasite developing in an insect was reported by Manson in 1877 in China, who discovered that mosquitoes served as intermediate hosts of the nematode *Wucheria bancrofti* (Eldridge, 1993). In 1897 Ross observed oocysts of malaria plasmodium in the stomach wall of *Anopheles* engorged mosquitoes and later established the life cycle "bird - culicine mosquitoes - bird" of the avian malaria plasmodium (Ross, 1898[a, b]). However, before these findings mosquitoes were considered active agents in the YF virus transmission, but only in 1900 was experimentally demonstrated by Walter Reed the transmission of YF virus from an infected person to an uninfected person by a mosquito (Tan and Ahana, 2010). In the twentieth century there was a great advance in knowledge and appropriate technologies designed to monitor the main agents of disease transmitted by mosquitoes.

From the second half of the twentieth century the term arbovirus has been used to name a group of taxonomically diverse viruses transmitted by arthropod vectors (Kuno and Chang, 2005) of which YF, dengue, JE and WNV cause more serious morbidity and mortality.

TRANSMISSION CYCLE

In biological transmission, the *extrinsic incubation period* (EIP) corresponds to the period of time in the vector from the ingestion of an infected blood meal until the successful transmission of the agent to another host. During this period the virus infects the cuboidal epithelium stomach cells and spreads in the vector hemolymph to other tissues and organs, including the salivary glands. The length of EIP is inversely proportional to temperature, but also depends on the vector species and the virus. Despite the presence of an immune system, mosquito vectors once infected can transmit a virus throughout his life (Girard *et al.*, 2004).

Vector competence is determined by the natural biological barriers of the vector to a particular etiologic agent, and varies with species and also inside the same species. Vectors and diseases are associated, *e.g.* dengue and *Aedes aegypti*, which reflects the interspecific variation of the vector barriers to infection and viral dissemination (Hardy, 1988). The intraspecific variation explains the global distribution of YF that does not match the distribution of the vector *Ae. aegypti*. This species is abundant in Asia, where there is no endemic YF. It seems that the Asian variant is less relevant to the transmission of YF that variants from Africa, South America and the Caribbean (Black *et al.*, 2002).

Vector capacity represents the total ability of a species to transmit a pathogen in a given place and in a specific time period. The size of the vector population, its longevity, the number and duration of the gonotrophic cycle, and the seasonal feeding behaviour and activity affect the vectorial capacity of a given population (Figure 10).

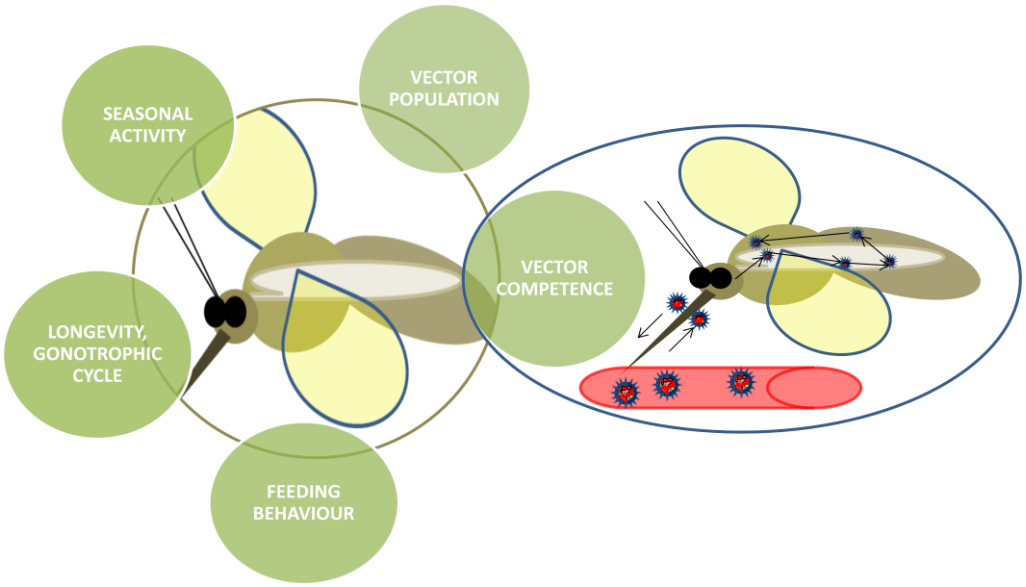


Figure 10. Vector capacity to transmit a pathogen depends on more ecological variables than intrinsic vector competence.

Although there may be several competent species to transmit a specific agent, only those which maintain the natural cycle of transmission are considered *primary vectors*. Four criteria are necessary to identify a species as the primary vector: 1) Spatial and temporal association with the vertebrate host and blood meals under natural conditions, 2) Detection of naturally infected specimens in nature, 3) vector infection by feeding on a viremic host, 4) experimental studies on vector competence under controlled conditions (Kent, 2009).

BIOECOLOGY

Mosquito life cycle comprises four stages: egg, larva, pupa and adult mosquito. The female lays 50 to 500 eggs per egg-laying, depending on the species and physiological status of females (Figure 11). Eggs are laid on water or in damp which will later be flooded (Clements, 2000). Embryogenesis occurs immediately after oviposition and larvae hatch one day to a week later depending on the temperature. Aedini eggs are resistant to desiccation and can diapause for several months. A combination of physic-chemical external stimuli and species specific innate tendencies are determinant for oviposition site selection, which is a key factor in mosquito distribution (Bentley and Day, 1989; Picket and Woodcock, 1996).

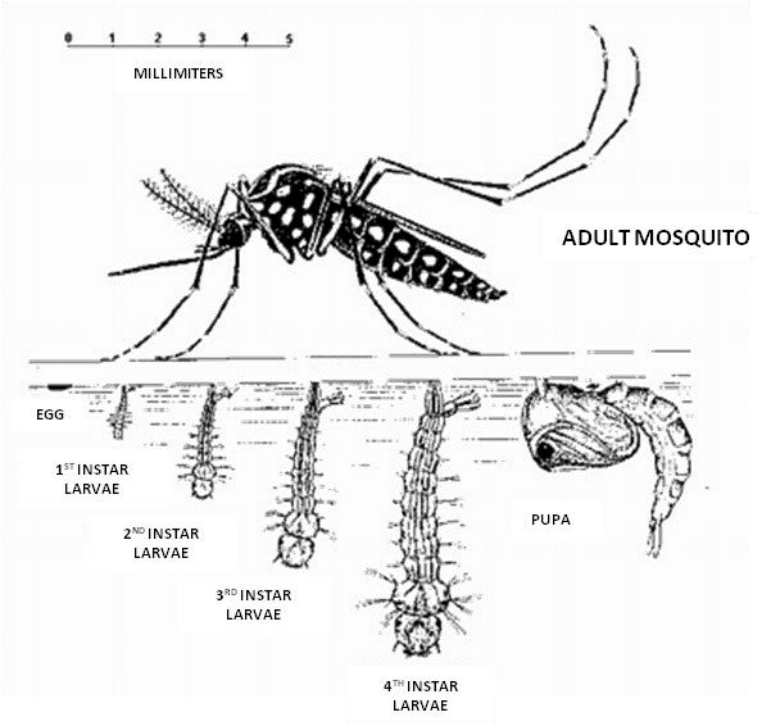


Figure 11. Life cycle of the mosquito (Adapted from Clements, 2000).

IMMATURES - LARVAE AND PUPAE

Larvae are fully adapted to aquatic life. They have four development stages during which moult three times (Figure 11). Two main features determine larval lifestyle: the use of atmospheric oxygen; and the feeding on organic particles in suspension or in the sediment of aquatic systems (Silver, 2008). The subfamilies Culicinae and Toxorhynchitinae present a pair

of spiracles in the last modified abdominal segment, the respiratory siphon, from which a network of respiratory tracheas extend to all body parts. Only two genera of the family Culicinae, *Coquillettidea* and *Mansonia* are exception to this rule, living permanently underwater since they have piercing respiratory siphons which attach to the root tissue of some plants that store atmospheric air. The Anophelinae larvae spiracles are flat on the dorsal surface of the last segment, which does not exhibit the morphologic characteristics of the respiratory siphon. Larvae feed on bacteria, algae and diatoms, but also on particles of decomposing organic matter. The larvae of Toxorhynchitinae are predators of small invertebrates as well as other mosquito larvae. The time required for full larval development depends on various factors, being the most important the water temperature and the food availability (Clements, 2000).

In the pupa the head and thorax are together, forming the cephalothorax which contains the appendices corresponding to the antennae, mouthparts and legs (Figure 12). The abdomen ends in two spade-shaped structures that serve as propellers. An air bubble trapped between the two structures ensures the natural buoyancy of the pupae. The mesothoracic spiracles open into two broad respiratory trumpets, which ensure gas exchange. Pupae do not feed, and in this stage the replacement of several larval organs by adult organs occurs by the multiplication of undifferentiated embryonic cells. Adults can emerge in one or two days if the temperature is favourable (Clements, 2000).

Figure 12. Immature stages of the mosquito *Culex pipiens*. A: Hatching of eggs (560X); B: 4th instar larva in a drop of water (150X); C: Pupa, lateral view (350X); D: Pupa, dorsal view (300X).

Mosquitoes explore a wide variety of aquatic habitats for larval development, with most species of mosquitoes breeding only in freshwater. Some species are able to occupy highly polluted aquatic environments, *e.g.* septic tanks, and others are adapted to very saline environments, *e.g.* brackish tidal marshes. Breeding sites can be categorized as 1) permanent, *e.g.* lakes, ponds and reservoirs, or 2) temporary, *e.g.* flood plains, animal drinkers, abandoned containers, tires, irrigation tanks, puddles of rain, sewage treatment tanks, and rice fields. Most species breed in temporary breeding sites. Permanent aquatic systems are usually deep and open and do not provide protection against natural predators such as fish and insect

larvae that are commonly present in such habitats. It is rare to find immature mosquitoes in running water systems. Some species are found in extremely small containers, as the leaves of plants belonging to the family Bromeliaceae and mollusc shells (Clements, 2000; Silver, 2008).

The species adapted to freshwater habitats have to restrict the intake of water and minimize loss of ions through the production of very dilute urine to maintain the osmotic balance to minimize the adverse effects of the difference in osmolarity. In addition larvae absorb ions through four (rarely two) structures at the end of the abdomen, the anal papillae. Larvae of brackish water must be able to maintain ionic balance from freshwater environments to saline environments. Under conditions of brackish water, where loss of water and ion concentration are problematic factors the larvae increase the water intake and excrete excess ions from the hemolymph by Malpighian tubes (Bradley, 1987). Although the species adapted to saline environments can live in freshwater environments, for reasons of inter-specific competition, are rarely found in these habitats (Silver, 2008).

ADULTS - IMAGO

Under natural conditions the male mosquitoes are first to emerge (Figure 13). Mating occurs close to the breeding sites of the immature stages after female emergence. The males are sensitive to the specific tone produced by the wing beats of females and attracted for mating. Many species form male swarms of ten to thousands of individuals over the breeding sites. The matting occurs by juxtaposition of the genitals and the semen is stored in two or three spermathecae, and is available to fertilise several lots of eggs at the time of oviposition. Once inseminated, females become refractive (Clements, 2000; Silver, 2008).

Figure 13. Adult forms of *Aedes aegypti* (80X).

As an energy source both sexes require carbohydrates generally in the form of flowers or fruit nectar. The carbohydrates are stored in the ventral diverticulum, which has the form of

a closed bag connected to the alimentary canal in the thorax region. This reserve of sugar is being continuously used by adult mosquitoes (Day, 1954). Anophelinae and Culicinae females need protein intake for egg development, which is obtained from blood of vertebrate hosts. Toxorhynchitinae only feed on nectar.

There is a degree of host preference, and some species are highly specific (Tempelis, 1975). Body temperature, carbon dioxide and the volatile compounds released from vertebrate hosts, stimulate the sensory receptors of the antennae and maxillary palps of females, which became responsive (Bowen, 1991; Cardé, 1996). During the byte the female releases saliva that contains numerous polypeptides with anticoagulant activity (Ribeiro *et al.*, 1985; Ribeiro, 1987). The salivary glands are critical in the transmission of pathogens, as these agents are injected into the vertebrate host with saliva during the blood meal. Receptors located in the abdomen suppress blood intake when the stomach is fully extended (Gwadz, 1969). Diuresis can occur during the blood meal removing water and salts such as sodium and potassium. After a few hours cells of the stomach secrete a chitinous material that is organized in a fine network that will envelop the ingested blood, the peritrophic matrix. This is important since it represents a barrier to pathogens and parasites that complete their development in the mosquito host. Digestion of the blood occurs within the peritrophic matrix in the stomach and is completed in two or three days, depending on temperature. Amino acids derived from ingested proteins are reconstituted, transported to the ovary and incorporated into oocytes. The process of oocyte maturation is not continuous, and periodic blood meals are necessary for new batches of mature oocytes (Clements, 2000). Some female mosquitoes, called autogenic, have the ability to develop a batch of eggs from lipo-protein reserves from the larval stage, without the need to perform a blood meal to the first oviposition. The autogeny is a genetic characteristic of some species or biotypes of some species, *e.g. Culex pipiens*, and its expression in nature is complex and variable.

Eggs may be individually placed on the water surface, as in the case of *Anopheles*, or grouped into a raft as in *Culex* sp. and *Culiseta* sp. and the larvae will hatch in a suitable habitat. The gonotrophic cycle of the female is restarted again. In a single gonotrophic cycle several blood meals can occur (Briegel and Horler, 1993; Scott *et al.*, 1993). The activities of adult mosquitoes, emerging, mating, blood meal and oviposition occur at different times of day, which varies among species (Silver, 2008).

SAMPLING METHODS

The interest in the ecology of mosquitoes and the development of appropriate methods of sampling began in mid-nineteenth century, after the first discovery that

mosquitoes can be vectors of pathogens to humans and pets. The abundance of mosquito populations is the most important criterion for most ecological studies that evaluate strategies to control or estimate the *vector capacity* (Silver, 2008). The estimation of larval abundance is often subject to bias in aquatic systems of large dimensions, *e.g.* lakes, due to limitations of the sampling and heterogeneous distribution of the larvae, usually clustered together.

To estimate adult population abundance specific traps with or without bait attractive are required. Data interpretation may also be subject to bias due to the unpredictability of environmental factors, the innate tendency of species, and particularly the physiological condition of the mosquito population, *e.g.* baited traps do not attract gravid females.

COLLECTING IMMATURES

Eggs can be sampled in the wild or using artificial devices (Gold, 2008). In both cases it is necessary to consider that mosquito oviposition strategies generally fall into four categories: 1) single eggs on the water surface (*Anopheles, Toxorhynchites, Sabethes, Wyeomyia*); 2) egg rafts on the water surface (*Culex, Coquillettidea, Culiseta*); 3) eggs in the vegetation under the water surface (*Mansonia*); 4) eggs isolated on wet surfaces that are intermittently flooded (*Aedes, Ochlerotatus, Psorophora*). Egg sampling in the natural habitat is usually associated with larvae and pupae collection. It should be taken into account the diversity of breeding sites used by mosquitoes and knowledge of the geographic region. Several tools to collect volumes of water containing eggs and other immature stages can be used, such as spoons, dippers, mesh nets, strainers.

Otherwise, ovitraps may capture pregnant female or only retain the eggs. They consist of plastic water containers, *e.g.* a bucket or other materials such as bamboo, rubber, metal, where water with organic matter or an attractive infusion is placed (Figure 14). The colour and contrast are important to attract gravid females of some species more effectively. The contrast black/ white or black/ red is usually used to capture various species of *Aedes* eggs. The ovitraps should be weekly monitored in the study site. This method is often used for invasive species surveillance as *Aedes aegypti* and *Aedes albopictus* (Marques *et al.*, 1993). The advantage of

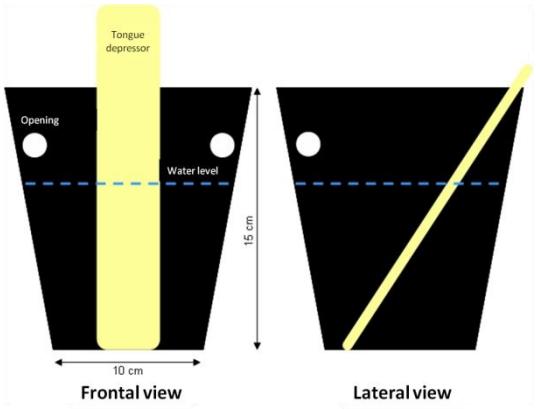


Figure 14. Handmade ovitrap.

ovitraps is the applicability in the field and the sensitivity to the presence of some mosquitos' species, even when relative abundance is low (Focks, 2003).

The standard device for larvae and pupae collection is the dipper. Many other materials may be used, as stated above for egg collection (Figure 15).

Figure 15. Image of a dipper, commonly used in larvae collection.

COLLECTING ADULTS

Several sampling methods and different traps can be used for adult mosquitoes (Silver, 2008). Sex, species and physiological state, *e.g.* female without blood meal, engorged or pregnant, determine the result of sampling, and it is necessary to adapt the methodology and the objectives of the study. Resting collections or host collections with vacuums and baited traps for overnight collections are the most used methods. A combination of methods may be carried out to optimize collection. In most mosquito species the adults are inactive during the day, resting in dark, cool and moist places. The adult's mosquito peak of activity is at dawn and dusk although some species, *e.g.* *Aedes aegypti*, have a constant activity throughout the day. Populations at rest are found in the vegetation, within homes, stables and underground locations and can be sampled with the use of vacuum. Resting collections often provide a more representative sample of the population, with males and females in various physiological stages. For mosquito species with crepuscular and nocturnal activity CDC (Centers for Disease Control) light traps baited with CO_2 are often used (Figure 16).

Figure 16. CDC light trap baited with CO_2.

THE STUDY OF MOSQUITOES IN PORTUGAL

In Portugal, the study of Culicidae fauna began in 1901 with Sarmento and France (Sarmento and France, 1901), but only in 1931 a monograph with the description of 21 species was published (Braga, 1931). The medical epidemiologist Francisco Cambournac continued the study of the Portuguese culicid fauna (Cambournac, 1938; Cambournac, 1943; Cambournac, 1976) from 1930. Between 1977 and 1988 the Portuguese mosquito fauna was systematically studied by Ribeiro and colleagues (Ribeiro *et al.*, 1977; Ribeiro *et al.*, 1977-78; Ribeiro, *et al.*, 1982; Ribeiro *et al.*, 1983; Ramos, 1983-84; Ribeiro *et al.*, 1985; Ribeiro *et al.*, 1985b; Ribeiro *et al.*, 1989). As a result of this continued work a list of 40 species of mosquitoes of Portugal describing 10 Anophelinae and 30 Culicinae was published in 1988 (Ribeiro *et al.*, 1988). In 1999, Ribeiro and Ramos published an identification key of the mosquitoes of mainland Portugal, Azores and Madeira, which was updated with five species, totalizing 45 species and subspecies distributed in 15 genera and seven subgenera (Ribeiro and Ramos, 1999). Some of the species listed in this publication have a limited distribution, are sporadic or considered only for their potential presence (Ramsdale and Snow, 1999), as is the case of the species *Aedes aegypti*, which is not found in Portugal since 1956 (Costa *et al.*, 1956), and *Ae. albopictus*, an invasive species never detected in Portugal. In 2005 was published an update of the mosquito distribution of some mosquito species in mainland Portugal (Almeida *et al.*, 2005).

In 2005, *Ae. aegypti* was detected in the district of Funchal, Madeira, probably introduced with the international transport of goods (Margarida *et al.*, 2006).

CHAPTER 2

SPECIES COMPOSITION AND DISTRIBUTION OF MOSQUITOES

2.1 SPECIES COMPOSITION AND DYNAMICS OF ADULT MOSQUITOES OF SOUTHERN PORTUGAL

RESEARCH ARTICLE

Osório, H.C., Amaro, F., Zé-Zé, L., Moita, S., Labuda, M. & Alves, M.J. (2008) Species composition and population dynamics of adult mosquitoes of southern Portugal. *European Mosquito Bulletin*, 25: 12-23.

ABSTRACT

An adult mosquito surveillance program was established at Ria Formosa and the Sado Estuary, Portugal during 2005 and 2006. Climatic data were recorded throughout the study by the nearest climate station. A total of 114,928 female mosquitoes were captured with ten CDC light-traps and were identified as *Anopheles algeriensis* Theobald, *An. maculipennis s.l*. Meigen, *Coquillettidia richiardii* Ficalbi, *Culex pipiens s.l*. Linnaeus, *Cx. theileri* Theobald, *Cx. univittatus* Theobald, *Culiseta annulata* Schrank, *Cs. longiareolata* Aitken, *Ochlerotatus caspius* Pallas, and *Uranotaenia unguiculata* Edwards. The most abundant species were *Oc. caspius* and *Cx. pipiens s.l.* The temporal dynamics in both years and localities was established for these species and significant differences in distribution patterns were observed. Some aspects of their bio-ecology are discussed, as well the importance of weather variables in disease transmission dynamics due to vector abundance.

INTRODUCTION

Mosquito surveillance is a prerequisite for an effective and efficient mosquito control program. The seasonal distribution of mosquitoes depends on climatic conditions which reflect individual survival strategies in response to climatic variation (Santos *et al.*, 2002). Flood and drought can trigger mosquito outbreaks by creating breeding sites for mosquitoes whose dry eggs remain viable and hatch in still water. These severe climatic changes can also reduce the number of natural mosquito predators, resulting in elevated numbers of adult mosquitoes. Flood and drought are reoccurring events and climate change data point to an increase in both the frequency and intensity of these events in Portugal (Epstein, 2000; Santos *et al.*, 2002).

The dynamics of changing mosquito populations provides significant information for evaluating risk potential for the transmission of mosquito-borne diseases (Service, 1993; Ryan *et al.*, 2004). Countries with a temperate climate, such as Portugal, which anticipate important ecological changes due to future climate conditions, face a significant increase in risk for mosquito-borne disease outbreaks.

Studies of Portuguese mosquitoes was started by Sarmento and França in 1901 and Jorge and Sarmento in 1906 (Ribeiro *et al.*, 1988; Almeida *et al.*, 2005), however only 21 species were listed in a comprehensive monograph in 1931 (Braga, 1931). At present, a total of 45 species and subspecies are included in the identification keys of the mosquitoes of Portugal, including Madeira Islands and the Azores Archipelago. Although *Stegomyia aegypti* (L.) has not been found in mainland Portugal since 1956, and *St. albopictus* Skuse has not been yet recorded, both species are included in the keys to Portuguese mosquitoes because of their potential introduction (Ribeiro and Ramos, 1999). Their presence in neighbouring Mediterranean countries is sufficient reason to establish surveillance and control programs for mosquito species in Portugal (Gratz, 2004; Aranda *et al.*, 2006).

The aim of this current study was to determine species composition and abundance of potential arboviral vectors, thus providing updated information on the diverse mosquito fauna and climatic factors that influence their seasonal variation.

MATERIALS AND METHODS

Study areas

The investigation was conducted in the Sado Estuary, Setúbal, and Ria Formosa, Faro, two different geographic areas in southern Portugal (Figure 17). Both are wetlands and bird sanctuaries, receiving more than 20,000 over-wintering aquatic birds per year. Ria Formosa, with a total area of 18,400 ha is a coastal lagoon characterized by marshlands, salt-marshes, small islands, dunes and beaches. Fishery, aquaculture and salt-works are the most important human activities, but industrial complexes related to the fishing industry are also found. Tourism is particularly abundant during the summer season. The Sado Estuary with 24,632 ha has a multitude of habitats, including those mentioned for Ria Formosa, as well as farming areas with several hectares of rice-fields and reed plantations. The north part is densely populated and industrialized. The climatological data were recorded by the nearest available climate station to the geographical location of Sado Estuary (Setúbal, 38º31'N08º54´W) and Ria Formosa (Faro, 37º01'N07º58'W).

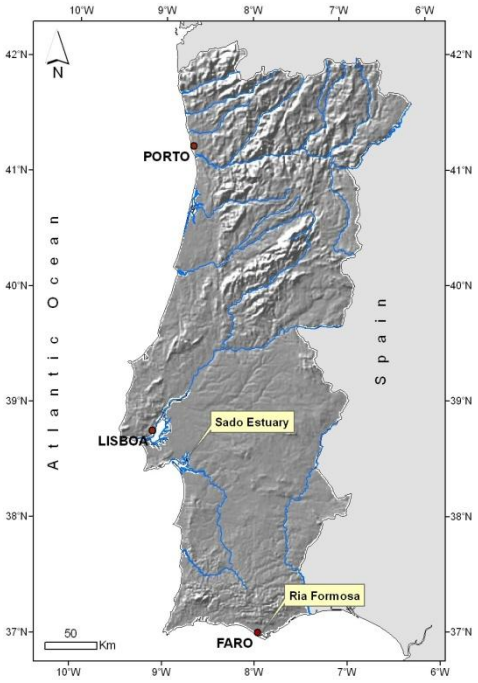


Figure 17. Study areas in Portugal.

Mosquito sampling

Mosquitoes were collected from June through September in 2005 and from May until October in 2006. Collections were performed 3-4 nights per month at each area. Mosquitoes were collected overnight, covering sunset and sunrise periods, with ten CDC (Centers for Disease Control and Prevention) light-traps baited with carbon dioxide. The traps were set at the same collection sites throughout both study years. Each morning the traps were taken to the laboratory and mosquitoes were transferred to net cages under controlled temperature and humidity conditions. After 3-5 days the mosquitoes were removed from the cage with an electric aspirator and anesthetized in a 4ºC refrigerator. Only females were selected from the total sample and identified to species according to the identification keys of Ribeiro and Ramos (1999) and Schaffner *et al.* (2001). All mosquitoes were stored at -80ºC until further use.

Estimation of mosquito numbers

When the mosquito numbers were too high for conventional counting, the total sample was weighed (Sartorius Basic BA 210 S), and 3 sub-samples, each representing 10% of the total weight, were identified and counted. The total number of females by species for the total sample was then calculated. Mosquito abundance was inferred by calculation of the number of collected females per trap night (*fptn*) and all analyses of mosquito population abundance were done with this collection index.

Diversity analysis

Shannon's diversity index (*H*) was used to characterize species diversity in both study sites. Shannon's index accounts for both abundance and distribution of the species present. The proportion of species (*i*) relative to the total number of species (p_i) is calculated and then multiplied by the natural logarithm of this proportion ($\ln p_i$). The resulting product is summed across species and multiplied by -1 [$H= -\sum_{i=1} (P_i \ln P_i)$]. Shannon's equitability (E_H) can be calculated by dividing *H* by H_{max} [(where $H_{max} = \ln S$, the total number of species in the community (richness)]. Equitability assumes a value between 0 and 1 with 1 being complete evenness (Rosenweig, 1995).

RESULTS

Climatic data

The years 2004 and 2005 were characterized by levels of precipitation well below normal, classified as extremely dry years. Moreover, the annual precipitation was the lowest value recorded since 1931. Average annual maximum air temperature in 2005 was the second highest since 1931, with average annual minimum air temperature below normal, after being above normal for 18 consecutive years (data from Institute of Meteorology).

The year 2006 was the fifth warmest in mainland Portugal since 1931, with an average annual mean air temperature of 16.04°C, 1.05 °C above the 1961-1990 norm. The summer of 2006 (June, July and August) was the fifth warmest since 1931 (after 2005, 2004, 2003 and 1949) and the autumn (September, October and November) was the third warmest (after 1997 and 1983), recording the highest average annual minimum air temperature since 1931. The average minimum air temperature recorded in October, 2006 was the highest since 1931 and in November was the second highest, below that of 1983 (data from Institute of Meteorology).

The winter of 2005/ 2006 (December, January and February) was very dry. The spring (March, April and May) was classified as dry, even though March was very rainy; as a

consequence, drought conditions that began at the end of 2004 were over by March, 2006. The summer was rainy and by autumn the third highest value of monthly precipitation was recorded since 1931 (after the autumns of 1960 and 1965).

Species composition and abundance

An estimated 114,928 female mosquitoes, representing 6 genera and 10 species, were collected at both study areas over 32 collection nights in 2005 and 36 nights in 2006 (Table 7). In both years, the mosquito density at Sado Estuary was higher than at Ria Formosa, with only 316 and 13,362 mosquitoes collected at Ria Formosa and c. 63,187 and 38,063 at Sado Estuary in 2005 and 2006 respectively (Table 7).

Table 7. Adult female mosquitoes collected at Sado Estuary and Ria Formosa in 2005 (June-September) and 2006 (May-October) in order of abundance.

	2005			2006			Total
	Sado Estuary	Ria Formosa	Sub-total	Sado Estuary	Ria Formosa	Sub-total	
Species	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)	Nº (%)
Oc. caspius	62,941 (99.6)	88 (27.9)	63,029 (99.3)	33,537 (88.1)	12,439 (93.1)	45,976 (89.4)	109,005 (94.8)
Culex pipiens	218 (0.4)	144 (45.6)	362 (0.6)	4,432 (11.6)	744 (5.6)	5,176 (10.1)	5,538 (4.8)
Cx. theileri		55 (17.4)	55 (<0.1)	74 (0.2)	158 (1.2)	232 (0.5)	287 (0.3)
Cx. univittatus	10 (<0.1)	17 (5.4)	27 (<0.1)	10 (<0.1)	1 (<0.1)	11 (<0.1)	38 (<0.1)
Cs. longiareolata	8 (<0.1)	12 (3.8)	20 (<0.1)	9 (<0.1)	5 (<0.1)	14 (<0.1)	34 (<0.1)
An. maculipennis	10 (<0.1)		10 (<0.1)		3 (<0.1)	3 (<0.1)	13 (<0.1)
An. algeriensis					4 (<0.1)	4 (<0.1)	4 (<0.1)
Cq. richiardii					3 (<0.1)	3 (<0.1)	3 (<0.1)
Cs. annulata					3 (<0.1)	3 (<0.1)	3 (<0.1)
Ur. unguiculata				1 (<0.1)	2 (<0.1)	3 (<0.1)	3 (<0.1)
Sub-total	63,187	316	63,503	38,063	13,362	51,425	114,928

The overall mean number of females per trap per night (*fptn*) was 2.5 times higher in 2005 than in 2006. At Ria Formosa a mean number of 111.7 *fptn* was collected in 2006, compared with 4.2 fptn in 2005. At Sado Estuary the mean number of *fptn* decreased from 782.9 in 2005 to 200.9 in 2006 (Figure 18).

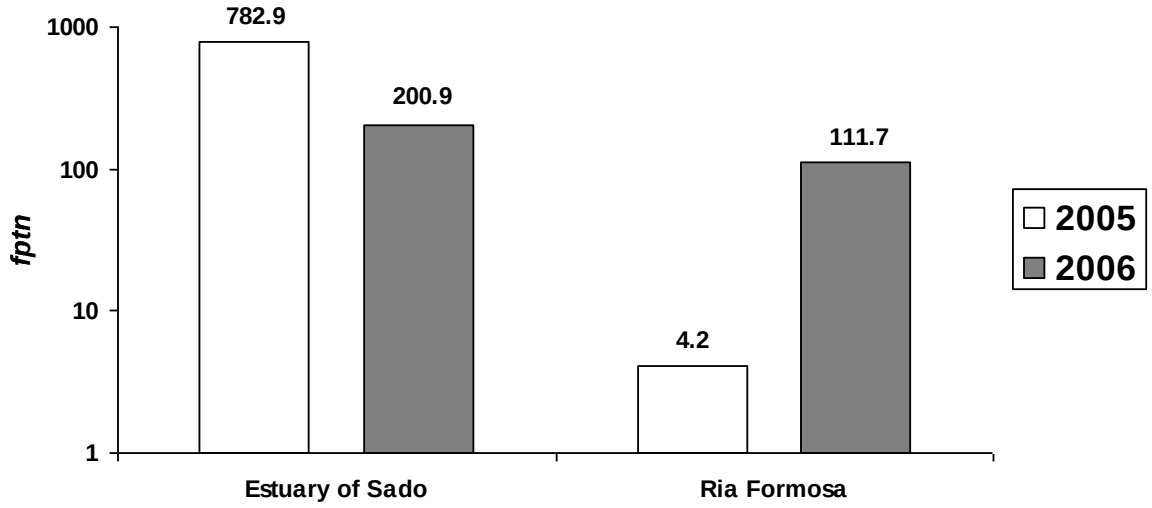


Figure 18. Overall mean number of females collected per trap night (*fptn*) at Sado Estuary and Ria Formosa in 2005 and 2006 (logarithmic scale).

In terms of species composition, *Anopheles algeriensis*, *An. maculipennis s.l.*, *Coquillettidia richiardii*, *Culex pipiens s.l.*, *Cx. theileri*, *Cx. univittatus*, *Culiseta annulata*, *Cs. longiareolata*, *Ochlerotatus caspius*, and *Uranotaenia unguiculata* were collected over both years. *Ochlerotatus caspius* was always the most collected species followed by *Culex pipiens* s.l., representing 94.9% and 4.8%, respectively, of the total collection (Table 7). Other mosquito species were collected in low numbers. *Culex theileri* was not found at Sado Estuary in the first collection year and in 2006 it was present at both sites, but in higher numbers at Ria Formosa (158 specimens). *Anopheles algeriensis*, *Cq. richiardii*, *Cs. annulata* and *Ur. unguiculata* were collected only in 2006 at Ria Formosa, except *Ur. unguiculata*, with one identified specimen from Sado Estuary (Table 7). *Stegomyia albopicta* (Skuse) and *St. aegypti* (L.) were not detected in this study.

Species diversity and evenness

Considering diversity (*H*) and evenness data (E_H) of collected mosquito species, 2006 had higher index values (*H*= 0.158, E_H= 0.158) than 2005 (*H*= 0.022, E_H= 0.028) (Table 8). This indicates that a greater number of species was present, with individuals in the population being distributed more equitably among these species. Throughout the collection season, population diversity and evenness (*H*=0.152, E_H= 0.152) were higher in Ria Formosa than in the Sado Estuary (*H*=0.086, E_H= 0.101), particularly during 2005 (*H*= 0.565, E_H= 0.808).

Table 8. Species diversity and evenness at Sado Estuary (SE) and Ria Formosa (RF) in 2005 and 2006 (*number of species in parentheses).

		2005	2006	2005 and 2006
Shannon's Diversity Index (*H*)	SE	0,011 (5)*	0,164 (6)	0,086 (7)
	RF	0,565 (5)	0,127 (10)	0,152 (10)
	SE and RF	0,022 (6)	0,158 (10)	0,095 (10)
Shannon's Equitability (*EH*)	SE	0,017 (5)	0,211 (6)	0,101 (7)
	RF	0,808 (5)	0,127 (10)	0,152 (10)
	SE and RF	0,028 (6)	0,158 (10)	0,095 (10)

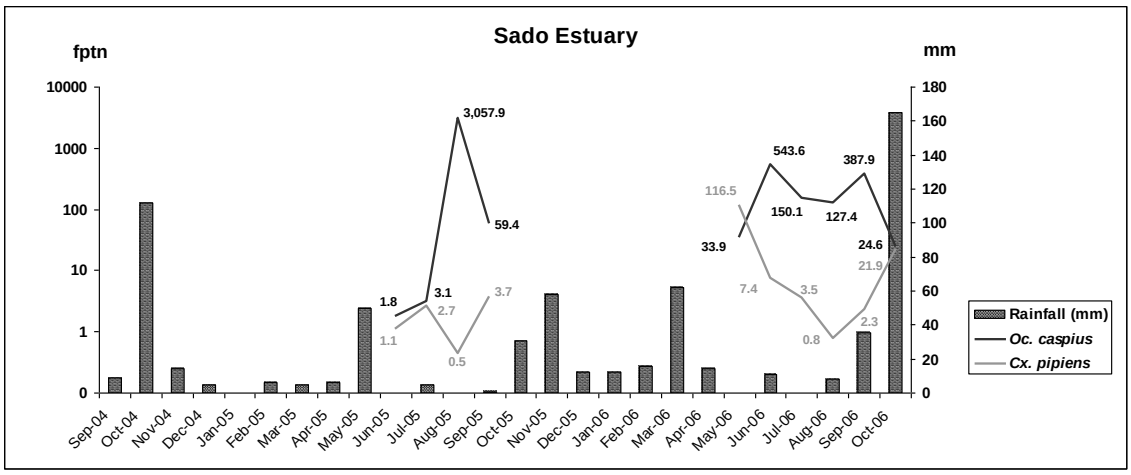
Temporal distribution of the most abundant species

When we considered the two most abundant species, *Oc. caspius* and *Cx. pipiens s.l.*, the *fptn* varied considerably over time. An inverted fluctuation of abundance was observed in both study areas (Figure 19 and Figure 20). The peak density of each mosquito occurred at different month over consecutive years and a distinct distribution pattern of the abundance of *Oc. caspius* and *Cx. pipiens s.l*. was noted.

In August 2005, *Oc. caspius* had one peak at Estuary of Sado (mean *fptn* 3,057.9), whereas in 2006, two peaks were clearly detected, one in June and another in September (Figure 19). Regarding *Cx. pipiens s.l.* at Sado Estuary, it was the most abundant mosquito species during the month of July (mean *fptn* 2.7) and September (mean *fptn* 3.7) of 2005, before and after the peak of *Oc. caspius*. In August 2005, an average of 0.5 *fptn* was collected, corresponding to the lowest value of the sampled year.

In 2006, the peaks of *Cx. pipiens s.l*. were in May and October (Figure 19). At Ria Formosa, a similar distribution of the population was observed, with a similar abundance peak in August (mean *fptn* 4.2). In June no *Oc. caspius* specimens were recorded. In 2006 the abundance of both species increased from May to June and *Oc. caspius* continued to increase with a peak in August and another in October. Conversely, *Cx. pipiens* s.l. abundance decreased in July and reached another abundance peak in September (Figure 20).

A



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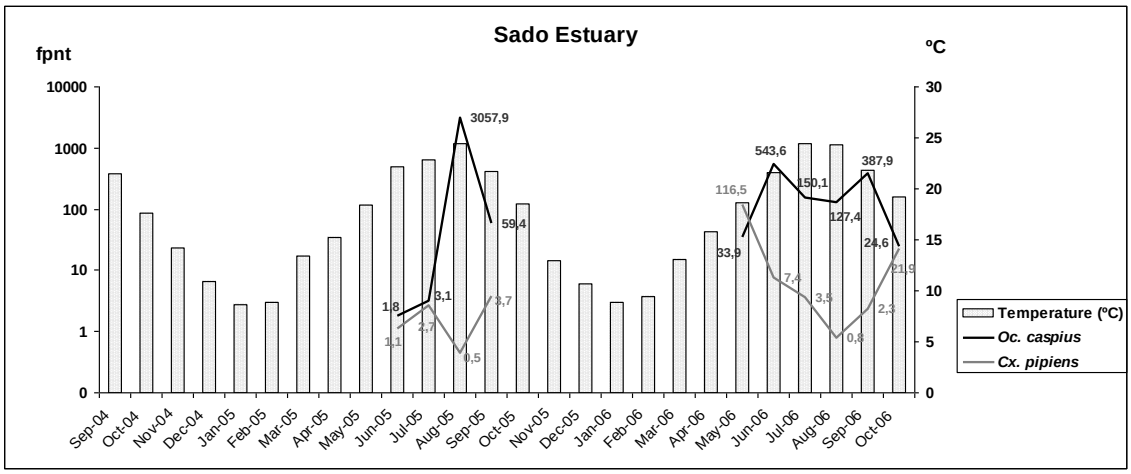

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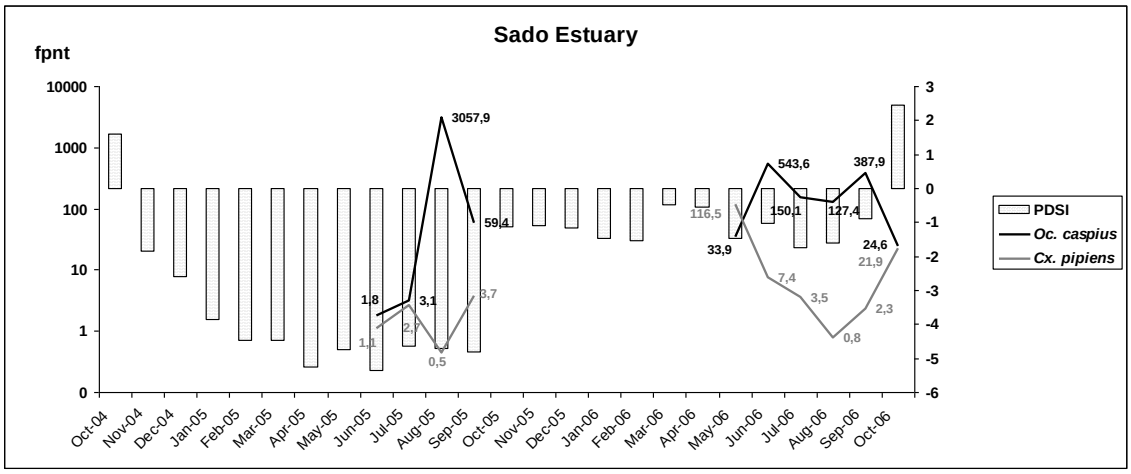
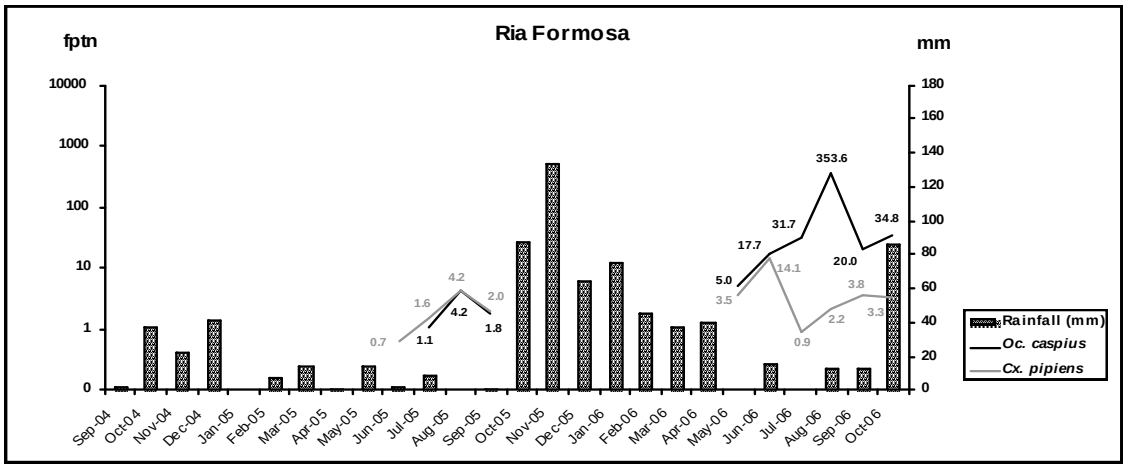


Figure 19. Number of females of the most abundant species collected per trap night (*fptn*) at Sado Estuary in 2005 and 2006 (logarithmic scale) in relation to A) seasonal rainfall, B) average temperature and C) Palmer Drought Severity Index.

A



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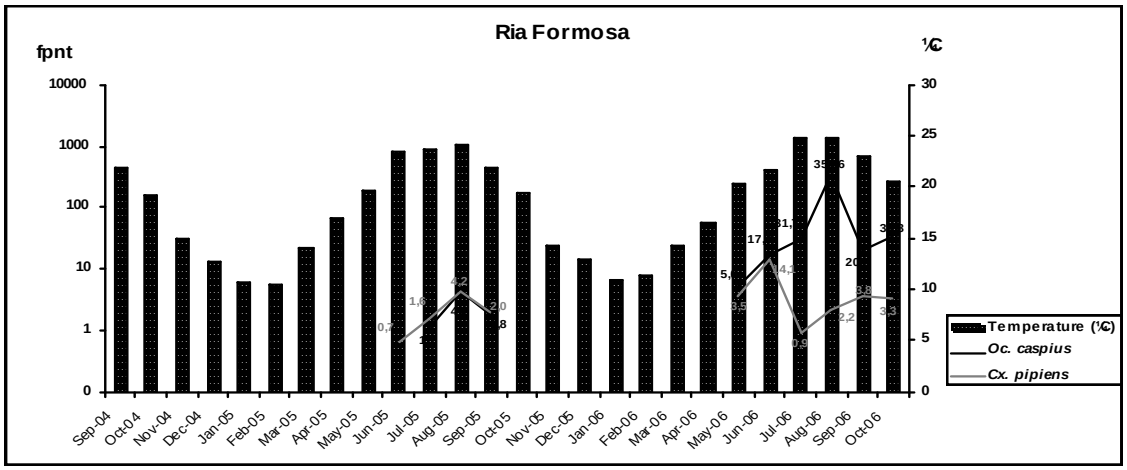

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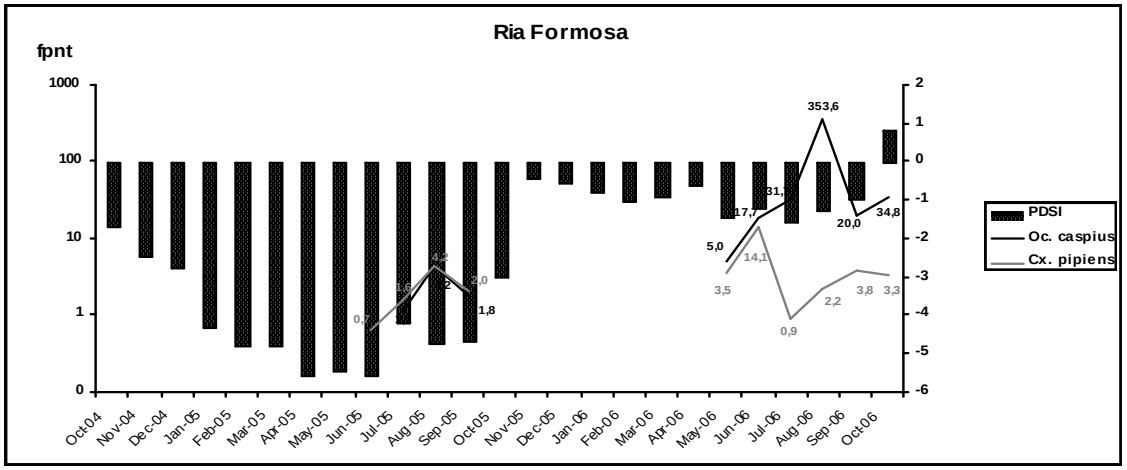

Figure 20. Number of females of the most abundant species collected per trap night (*fptn*) at Ria Formosa in 2005 and 2006 (logarithmic scale) in relation to A) seasonal rainfall, B) average temperature and C) Palmer Drought Severity Index.

DISCUSSION

Trapping with only type of trap does not accurately represent the mosquito fauna of individual study areas. Indeed, sampling efficacy differs according to trap type, mosquito species, gonotrophic condition of the female mosquitoes, and the geographic location where the species are collected (Kline *et al.*, 2006). In this study, only CDC light traps were used and, consequently, this may have biased the mosquito species captured. Moreover, the mosquitoes were operated overnight, covering sunset and sunrise periods, and some species which tend to be diurnally active, namely aedines, may have been misrepresented.

The results indicate that the mosquito diversity and evenness observed in 2006 was higher than in 2005, reflecting the effects of drought. The Ria Formosa supported a wider spectrum of mosquito species than the Sado Estuary, which was only apparent in 2006, when 10 mosquito species were identified. The indices of diversity decreased in Ria Formosa from 2005 to 2006 because the abundance of the most common species in that area was higher and the distribution of individuals in the population among the species was affected. The species collected in low numbers in Ria Formosa in 2006, namely *An. algeriensis*, *Cq. richiardii*, *Cs. annulata* and *Ur. unguiculata*, were not detected at all in 2005. It is also possible that these species had not reached high enough adult densities for detection with CDC light-traps. Likewise, *St. albopicta* and *St. aegypti* were not detected in this study. However, considering the high vector potential for arbovirus transmission to humans, surveillance for these species should continue, albeit with a more comprehensive use of sampling methods.

The obtained results show the dominance of two mosquito species in both study sites - *Oc. caspius* and *Cx. pipiens s.l*. In Portugal, *Oc. caspius* is an outdoor active human biting mosquito occurring in large numbers, primarily along the costal belt. Although females bite all warm-blooded vertebrates, they are highly anthrophophilic, and are often responsible for nuisance biting, even in towns some distance away from larval sites. *Culex pipiens s.l.* is a common mosquito in Portugal. This species demonstrates great morphological variation and ecological plasticity. Two groups resulting from ecological selection are established in Europe: the *pipiens* group, typically ornithophilic, anautogenous, eurygamous and rural, with a diapause at the adult stage, and the *molestus* group, where females bite all warm-blooded vertebrates but are mostly anthropophilic, autogenous, stenogamous, and urban, with continuous growth (homodynamic). These species are not genetically isolated but are the result of ecological selection (Ribeiro *et al.*, 1988; Schaffner *et al.*, 2001).

Based on these results, the temporal dynamics of *Oc. caspius* and *Cx. pipiens s.l.* were different in both years and in both study areas, except in Ria Formosa 2005, where a similar

monthly distribution was observed. The fluctuations in time and space reflected the changes in the climatic conditions between 2005 and 2006, an extreme drought year in Portugal, and the specific ecological characteristics of the Sado Estuary and the Ria Formosa. In 2005 the total number of collected females was low in Ria Formosa, which suggests an unavailability of breeding sites. In contrast, within the Sado Estuary, one massive abundance peak of *Oc. caspius* (mean *fptn* 3057.9) was observed, probably a result of extreme drought. In terms of bio-ecology, *Oc. caspius* is a multivoltine species and the generations follow one after the other in rhythm with the flooding of individual sites. *Ochlerotatus caspius* also over-winters in the egg stage, which is laid individually at the base of vegetation tufts, and several immersion and desiccation cycles are needed to induce egg hatching (Schaffner *et al.*, 2001). As a result any drastic change in the natural dynamics of the aquatic habitats will thoroughly influence the bio-ecology of *Oc. caspius*.

It is likely that mosquito populations will increase under future climate scenarios (Eritja *et al.*, 2005). If this occurs, higher arbovirus prevalence rates will follow (Hubálek and Halouzka, 1999; Jourdain *et al.*, 2007[a,b]). However, several factors are required for a virus, such as West Nile Virus, to emerge as a cause for human epidemics (Zeller and Schuffenecker, 2004). Since climatic change is one of the most important factors, and may determine both the occurrence and distribution of arbovirus within future reservoirs (Reiter, 2001), an appropriate mosquito surveillance programme during periods of potential virus transmission should be established which can predict potential mosquito-borne disease outbreaks in humans. We are currently utilizing flavirus-specific RT-PCR and viral isolation techniques from these mosquito collections to enhance surveillance activities established for the the Sado Estuary and Ria Formosa.

2.2. MOSQUITO SPECIES DISTRIBUTION IN MAINLAND PORTUGAL 2005-2008

RESEARCH ARTICLE

Osório, H.C., Amaro, F., Zé-Zé, L., Pardal, S., Mendes, L., Ventim, R., Ramos, J.A., Nunes, S., REVIVE Workgroup & Alves, M.J. (2010) Mosquito species distribution in mainland Portugal 2005-2008. *European Mosquito Bulletin*, 28: 187-93.

ABSTRACT

This work presents an update of the distribution and relative abundance of the mosquito fauna collected within several surveillance programs and projects, in mainland Portugal, over 2005-2008. A total of 48 localities belonging to 13 districts were surveyed all over the year, mainly from May to October. The adult mosquitoes were collected with CDC light traps baited with CO_2 and the larvae collected with deepers. More than 150,000 adult mosquitoes and 3,000 larvae belonging to 16 species were identified at the National Institute of Health/ Centre for Vectors and Infectious Diseases Research. The three most collected species were *Ochlerotatus caspius, Culex pipiens*, and *Cx. theileri*. The most widespread species was *Cx. pipiens*, followed by *Culiseta longiareolata*. Some species as *Cx. hortensis* and *Cx. laticinctus* were sampled only as larvae. The composition and abundance of the different mosquito species varied through the geographical locations. In this report the collection methods, mosquito fauna composition and distribution are discussed.

INTRODUCTION

An essential component of a mosquito-borne disease control program includes a comprehensive mosquito surveillance plan that identifies the geographical distribution and relative abundance of potential vector populations (Ryan *et al.* 2004). In Portugal, as in other European countries, important ecological changes due to future climate conditions are expected (Santos *et al.* 2002). Portugal is currently recognized as a high risk country for the introduction of *Aedes albopictus* Skusa (Almeida *et al.* 2008), a mosquito vector of several arboviruses, owing to its presence in the neighboring Mediterranean countries (Aranda *et al.* 2006, Dalla Pozza and Majori 1992). Furthermore *Aedes aegypti* L., another invasive species that was present in mainland Portugal until 1956 (Ribeiro and Ramos 1999), after which it was no longer detected, was first reported in 2005 in Madeira Island, Portugal (Margarida *et al.* 2006).

Up to date a total of 40 mosquito species, 10 anopheline and 30 culicine, have been reported in mainland Portugal (Ribeiro *et al.* 1988). *Culex pipiens* s.l. L., *Anopheles maculipennis* s.l. Meigen, *Cx. theileri* Theobald, *An. claviger* s.l. Meigen and *Culiseta longiareolata* Macquart were found to be the most widespread mosquitoes throughout the country in a survey carried out over 2001-2004 (Almeida *et al.* 2008).

In the framework of arboviruses surveillance programs the National Institute of Health has been studying the mosquito fauna of several Portuguese provinces to acknowledge the potential infection risk to the human population. The data included herein provide information about the distribution and abundance of the collected mosquito species over the period 2005-2008. The aim of this report is to contribute with updated information on the mosquito fauna and abundance of potential arboviral vectors in mainland Portugal.

MATERIAL AND METHODS

Study area and surveillance programs

A total of 48 localities belonging to 13 districts were surveyed over 2005-2008, mainly from May to October (Table 9). In 2005 and 2006 mosquitoes were collected from the districts of Setúbal and Faro. Both geographic areas are wetlands and bird sanctuaries, characterized by marshlands, salt-marches, small islands, dunes and beaches, as well as rice fields and reed plantations. In 2007 the survey took place only in the district of Faro, with five localities being studied. All of these sites are coastal wetlands rich in avifauna and show a multitude of habitats as described above. In 2008 the study was extended to 13 districts of mainland Portugal, where 48 localities were surveyed. In each locality one to 10 collection sites were set.

The ecological characteristics of these collection sites exhibit a wide variation, ranging from rural to urban habitats.

Table 9. Summary of collections.

	2005	2006	2007	2008
Period	May-October		June-October	January-December
CDC Trap-night (*n*= 1299)	320	440	80	459
Adult mosquitoes (*n* = 169,586)	63,503	58,733	11,566	35,784
Larva collections	-	-	-	143
Larvae (*n* = 3,337)	-	-	-	3,337
Most collected species (%)	*Oc. caspius* (99.3) *Cx. pipiens* (<1) *Cx. theileri* (<1)	*Oc. caspius* (78.6) *Cx. pipiens* (19.4) *Cx. theileri* (1.6)	*Oc. caspius* (66.9) *Cx. pipiens* (22.6) *Cx. theileri* (4.7)	*Oc. caspius* (36.2) *Cx. theileri* (35.4) *Cx. pipiens* (25.0)
Genera / species (*n* = 6 / 16)	3/ 6	6/ 10	5/ 10	6/ 14
Localities / Districts	2/ 2	2/ 2	5/ 1	48 / 13

Mosquito collections

Adult mosquitoes were caught with CDC light traps baited with CO_2 for a minimum of 12-hour-periods covering sunset to sunrise. The traps were set at the same collection site over 2 to 3 nights at monthly intervals. Mosquitoes were knocked down on a 4ºC refrigerator and identified under a stereomicroscope, on a chill table, according to the identification keys of Ribeiro and Ramos (1999) and Schaffner *et al.* (2001). Larvae were collected with a deeper in the same localities and districts of adult mosquitoes collection, transported to the laboratory and identified using the same identification keys.

RESULTS

Over 2005-2008 a total of 169,586 adult mosquitoes and 3,337 larvae were caught in 1,299 trap-nights and 143 positive larvae surveys (Table 9). The most collected species during 2005-2008 was *Ochlerotatus caspius* Pallas, mainly in 2005 when this species achieve 99% of total mosquito collection, followed by *Cx. pipiens* s.l. and *Cx. theileri*. In 2008 the number of collected mosquitoes of the three most captured species was more alike and a total of 14 species were reported. In this year 13 districts of mainland Portugal were included in the surveillance program and the collection period was carried throughout the year (Table 9).

During the surveillance period a total of 16 species belonging to six genera were identified (Table 10), representing 40% of the 40 known species in mainland Portugal (Ribeiro and Ramos, 1999).

Table 10. Mosquito species collected in Portugal over 2005-2008 in order of adult abundance (A- adults; L- larvae).

	Districts (*n*= 13)	Adults (A) / Larvae (L)	Adults (*n*= 169,586) (%)	Larvae (*n*= 3,337) (%)	TOTAL (*n*=172,923) (%)
Ochlerotatus caspius	8	A	129,907 (76.6)	0 (0)	129,907 (75.1)
Culex pipiens s.l.	13	A / L	23,302 (13.7)	1651 (49.5)	24,953 (14.4)
Cx. theileri	9	A / L	14,201 (8.4)	22 (1.3)	14,223 (8.2)
Oc. detritus s.l.	2	A	615 (0.4)	0 (0)	615 (0.3)
Cx. perexiguus	8	A / L	517 (0.3)	66 (2.0)	583 (0.3)
Anopheles algeriensis	4	A	495 (0.3)	0 (0)	495 (0.2)
Culiseta longiareolata	9	A / L	212 (0.1)	1258 (37.7)	1,470 (0.9)
An. maculipennis s.l.	6	A / L	136 (0.1)	4 (0.1)	140 (0.1)
Uranotaenia unguiculata	2	A / L	90 (0.1)	1 (<0.1)	91 (<0.1)
Cs. annulata	6	A	72 (<0.1)	0 (0)	72 (<0.1)
Coquillettidea richiardii	3	A	30 (<0.1)	0 (0)	30 (<0.1)
An. claviger s.l.	3	A	7 (<0.1)	0 (0)	7 (<0.1)
An. plumbeus	1	A	1 (<0.1)	0 (0)	1 (<0.1)
Cx. modestus	1	A	1 (<0.1)	0 (0)	1 (<0.1)
Cx. hortensis	2	L	0 (0)	40 (1.2)	40 (<0.1)
Cx. laticinctus	2	L	0 (0)	295 (8.8)	295 (0.1)

The overall number of mosquitoes varied considerably among collection sites, though the most collected species were *Oc. caspius* (129,907 specimens), *Cx. pipiens* s.l. (24,953 specimens) and *Cx. theileri* (14,223 specimens). *Cx. pipiens* s.l. was the most widespread species, being present in all sampled districts, followed by *Cx. theileri* and *Cs. longiareolata*, which were reported in 9 districts (Table 10; Figure 21).

Considering the less common species, *An. algeriensis* Theobald, *Uranotaenia unguiculata* Edwards and *Oc. detritus* s.l. Pallas were only found in the southernmost regions of Portugal. The district of Portalegre was the upper limit for *An. algeriensis* collections and the district of Setúbal for *Ur. unguiculata* and *Oc. detritus* s.l.. *An. maculipennis* s.l. was reported in the central district of Coimbra as also in southernmost regions of Setúbal and Faro and *An. claviger* s.l. was collected in three districts throughout the country. *Cx. perexiguus* Theobald and *Cs. annulata* Schrank appeared in eight and six districts, respectively. *Coquiletidia richiardii* Ficalbi was found in Coimbra, Setúbal and Faro districts. The rarest collected species were *An. plumbeus* Stephens, Coimbra, and *Cx. modestus* Ficalbi, Faro, with just one detected specimen (Table 10; Figure 21).

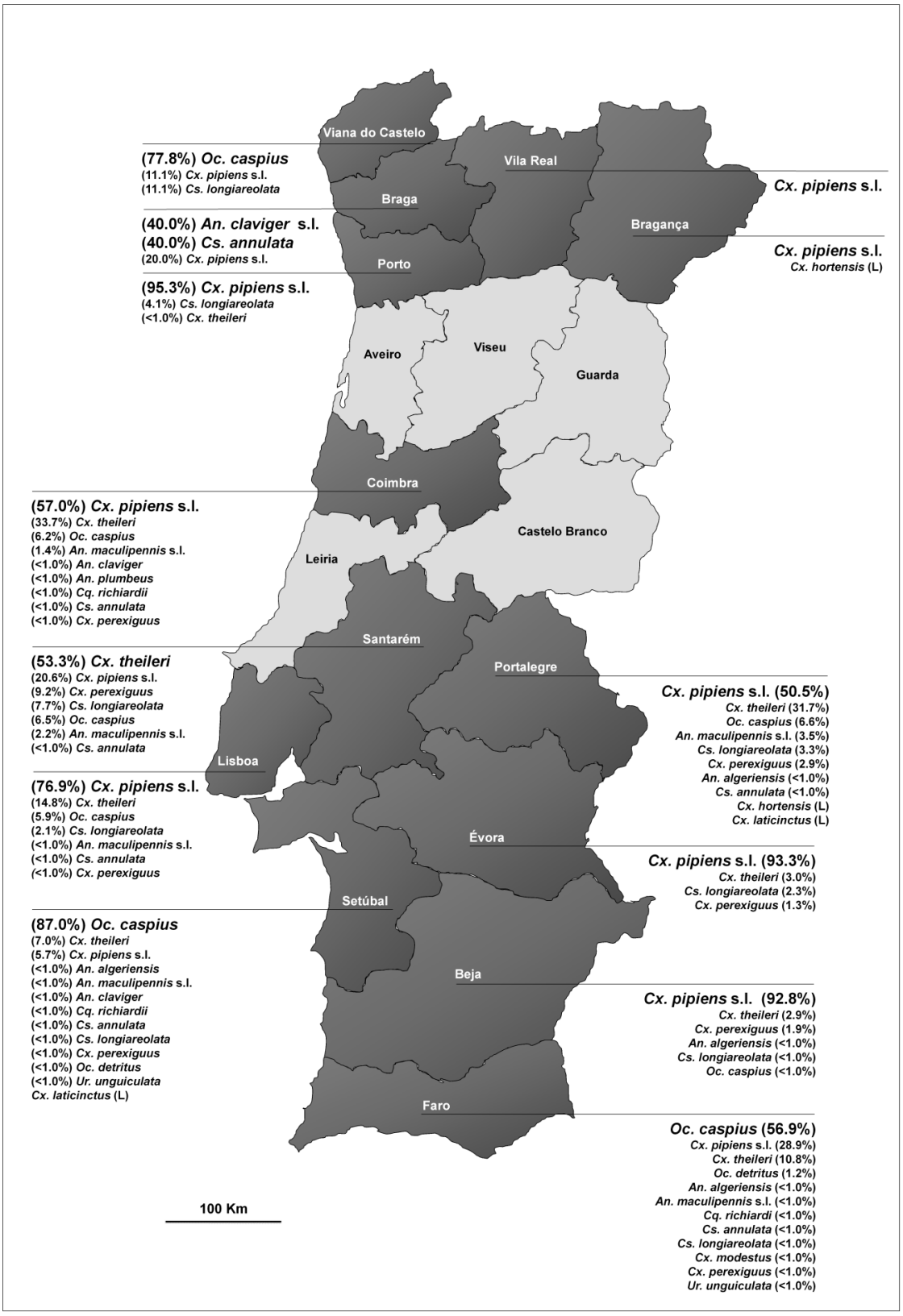

Figure 21. Relative abundance by district of the adult mosquitoes collected in mainland Portugal over 2005-2008 (L- species collected only as larvae).

In this study, *Ae. albopictus* was not detected, as well as *Ae. aegypti*, although it has been reported since 2005 in Madeira island (Margarida *et al.* 2006).

Considering the larvae, the most collected species were *Cx. pipiens* s.l., 1,651 specimens and *Cs. longiareolata*, 1,258 specimens (Table 10). Both species were found in a vast number of different breeding sites and throughout all districts surveyed. *Cx. hortensis* Ficalbi and *Cx. laticinctus* Edwards were only collected as larvae. *Cx. hortensis* larvae only appear in the inner districts of Portalegre and Bragança (Figure 21).

DISCUSSION

Ochlerotatus caspius, Cx. pipiens s.l. and *Cx. theileri* were the most abundant species found in this survey and they were also the most widespread mosquitoes throughout the country, as was also the case of *Cs. longiareolata* and *Cx. perexiguus*, captured in more than eight of the surveyed districts. *An. maculipennis* s.l. and *An. claviger* s.l., considered two abundant species in Portugal, were collected in low number in this survey with the CDC traps. This is a result of using only one collection method for adults, the CDC light traps baited with CO_2, which may misrepresent the relative numbers of the captured species and consequently the mosquito fauna of individual study areas. These results are in agreement with previous surveys where was observed that *An. maculipennis* s.l. seldom appeared in CDC traps, although this species comprise one of the most collected mosquitoes resting inside animal shelters (Almeida *et al.* 2008).

Ochlerotatus detritus s.l. was found in the district of Setúbal and Faro, where collections were carried in estuarine regions. This is in agreement with previous studies that show that this mosquito is common in wetlands and coastal habitats (Ribeiro *et al.* 1988).

Anopheles algeriensis was only reported once in western Algarve, in 1982, where few larva and adults were collected (Ramos *et al.* 1982). Our data confirm the presence of this species not only in Algarve, but also in the north most districts of Beja, Setúbal and Portalegre.

Uranotaenia unguiculata was considered to be a very rare mosquito species in Portugal. A male from a pupa, was recorded for the first time in the southern district of Beja (Ramos *et al.* 1977-78) and two additional male specimens were recorded in 1991, also in the South of the country, in the districts of Beja and Faro (Ramos *et al.* 1992). The first record of a female was in 2004, from Almancil, also in Faro district (Almeida *et al.* 2005). In this study 90 specimens of *Ur. unguiculata* were captured in the district of Faro and in the north most district of Setúbal, which shows that *Ur. unguiculata* is not as rare as reported and is widespread throughout the south region of mainland Portugal.

Culiseta annulata, *Cq. richiardii*, *An. claviger*, *An. plumbeus* and *Cx. modestus* were the adult mosquitoes collected in lesser number (<0.1%), the last two with just one identified specimen.

Larvae collection allowed the detection of two more species, *Cx. hortensis* and *Cx. laticinctus* that were not found in CDC adult traps.

Aedes albopictus and *Ae. aegypti* were not detected, but these are considered the most widespread and invasive mosquito species, vectoring several arboviruses, as yellow fever, dengue and West Nile (Flaviviridae), as well as chikungunya (Togaviridae). The recent establishment of these species in distinct and wider geographic regions makes the development of appropriate mosquito surveillance programs imperative (Gratz 2004, Eritja *et al.* 2005).

In summary *Oc. caspius*, *Cx. pipiens* and *Cx. theileri* were the most abundant mosquitoes. These species are recognized as vectors of arboviruses, such as West Nile virus (Lundström 1999) and may constitute a public health threat. Furthermore, the proximity to migratory bird sanctuaries increases the risk of involvement in arbovirus transmission cycles. *Cx. pipiens* s.l., as well as *Cx. perexiguus*, were already found positive for West Nile virus in the southern district of Faro (Parreira *et al.* 2007), and due to its abundance and distribution may contribute to establishment of local virus circulation in Portugal.

CHAPTER 3

VECTOR-HOST INTERACTION

3.1 HOST FEEDING PATTERNS OF *CULEX PIPIENS* AND OTHER POTENTIAL MOSQUITO VECTORS OF WEST NILE VIRUS COLLECTED IN PORTUGAL

RESEARCH ARTICLE

Osório, H.C., Zé-Zé, L. & Alves, M.J. (2012) Host feeding patterns of *Culex pipiens* and other potential mosquito vectors (Diptera, Culicidae) of West Nile Virus collected in Portugal. *Journal of Medical Entomology*, 49 (3): 717-21.

ABSTRACT

The host blood feeding patterns of mosquito vectors affects the likelihood of human exposure to zoonotic pathogens, including West Nile Virus (WNV). In Portugal data are unavailable regarding the blood feeding habits of common mosquito species including *Culex pipiens* L., which is considered the primary vector of WNV to humans. The sources of blood meals in 203 blood-fed mosquitoes of nine species, collected from June 2007 through November 2010 in 34 Portuguese counties, were analyzed by sequencing cytochrome-b partial fragments. *Culex pipiens* was the most common species collected and successfully analyzed (*n* = 135/ 78). In addition, blood-fed females of the following species were analyzed: *Ochlerotatus caspius* Pallas (*n* = 20), *Cx. theileri* Theobald (*n* = 16), *Anopheles maculipennis* s.l. Meigen (*n* = 10), *Culiseta longiareolata* Macquart (*n* = 7), *Aedes aegypti* L. (*n* = 6), *Cx. perexiguus* Theobald (*n* = 3), *Cs. annulata* Schrank (*n* = 3), and *Oc. detritus* Haliday (*n* = 3). The *Cx. pipiens* mosquitoes fed predominantly on birds (*n* = 55/ 78, 70.5%) with a high diversity of avian species used as hosts, though human blood was identified in 18 specimens (18/ 78, 23.1%). No significant differences were found between the host feeding patterns of blood fed *Cx. pipiens* collected in residential and non-residential habitats. The occurrence of human derived blood meals and the presence of a mix avian-human blood meal accordingly suggest this species as a potential vector of WNV. Therefore, in Portugal, *Cx. pipiens* may play a role both in the avian-to-avian enzootic WNV cycle and in the avian-to-mammal transmission. In this context, the identity of *Cx. pipiens*, (considering the forms *molestus* and *pipiens*) and the potential consequence on feeding behaviour and WNV transmission are discussed.

INTRODUCTION

The host feeding patterns of mosquitoes are driven by several factors including innate tendencies, host availability and abundance, host defensive behaviors, and flight behavior and feeding periodicity of mosquitoes (Clements 1999). Different vector species have different host feeding patterns, which may expose humans to multi-host zoonotic vector-borne pathogens (Kilpatrick *et al.* 2007, Molaei *et al.* 2008). The emergence, dispersion, and maintenance of a vector-borne pathogen are affected by the efficiency of transmission, which depends on the convergence in time and space between competent vectors, competent vertebrate hosts, and the pathogen. Therefore the blood feeding behaviors of mosquito vectors play a critical role in the transmission and maintenance of vector-borne pathogens in natural systems (Kent 2009).

Culex pipiens L. is one of the most ubiquitous mosquito species in the world (Farajollahi *et al.* 2011) and based on our surveillance results obtained from 2005 throughout 2010, it is one of the most frequent and widespread collected mosquitoes in residential and non-residential areas in Portugal (Osório *et al.* 2010). It consists of two forms, denoted *molestus* and *pipiens*, that are morphological indistinguishable but exhibit important behavioral and physiological differences and occupy different habitats in the northern regions of Europe, underground and aboveground, respectively (Vinagradova 2000). In some regions of Portugal physiological/ behavioral and genetic data provide evidence for the sympatric occurrence of both forms (Gomes *et al.* 2009).

The *Cx. pipiens* complex represents the primary enzootic vector of WNV in Europe (Hubalek and Halouzka 1999) and northeastern and north-central United States (Apperson *et al.* 2004, Molaei *et al.* 2006) and is also considered a potential epidemic vector (Hamer *et al.* 2008). However, since the detection of WNV in North America in 1999 (Anderson *et al.* 1999) more than 60 mosquito species have tested positive for WNV (Centers for Disease Control, West Nile virus homepage) and several species have also been implicated as potential secondary vectors because of their local abundance, vector competence in the laboratory (Turell *et al.* 2005), and frequent reports of infection with WNV in nature (Andreadis *et al.* 2004, Apperson *et al.* 2004).

In Portugal WNV was isolated from three mosquito species, *Anopheles maculipennis* s.l. Meigen, *Cx. perexiguus* Theobald, and *Cx. pipiens* (Filipe and Pinto 1972, Parreira *et al.* 2007), although the precise role that each species plays in the enzootic transmission among birds or epidemic transmission to humans is not presently clear. To our knowledge this is the first study on feeding behavior of wild mosquitoes collected in Portugal. Regarding

transmission of WNV, we assessed the blood feeding patterns of some representative mosquito species in Portugal, namely *Aedes aegypti* L. (Madeira Island), *Anopheles maculipennis* s.l. Meigen, *Cx. perexiguus* Theobald, *Cx. theileri* Theobald, *Culiseta annulata* Schrank, *Cs. longiareolata* Macquart, *Ochlerotatus caspius* Pallas, *Oc. detritus* Haliday, and particularly *Cx. pipiens*. The role of this species on WNV transmission is questioned considering the presence of the forms *pipiens* and *molestus* and their host preference.

MATERIALS AND METHODS

Mosquito collection and species identification

Mosquitoes were collected nationwide from a variety of urban, peri-urban, and rural environments in the framework of the Portuguese National Program of Vector Surveillance - REVIVE (Alves *et al.* 2010). Trap sites included non-residential areas such as rice fields, marsh fields, water treatment plants, parks, and farms and residential areas in urban habitats (more than 411.5 inhabitants/ km^2 in 2010, http://www.ine.pt). A total of 43 sites in 34 counties belonging to 13 districts in mainland Portugal were surveyed during the 2007-2010 mosquito seasons, which in Portugal generally last from April to October. In 2010, blood-fed mosquitoes were collected in residential areas of Câmara de Lobos and Funchal in the Autonomous Region of Madeira Island from April to November. Baited (CO_2) Centre for Disease Control and Prevention (CDC) light traps (John W. Hock Company, USA) were used according to Osório *et al.* (2010). In addition, battery powered aspirators were used to sample *Aedes aegypti* L. mosquitoes, particularly in the Funchal and Câmara de Lobos residential areas. Mosquitoes with fresh or visible blood remnants were identified to species and individually transferred into 1.5-ml micro-tubes and stored frozen at -80ºC until used for blood meal analysis (Ribeiro and Ramos 1999).

DNA isolation from blood-fed mosquitoes

Mosquito abdomens were removed with the aid of a stereomicroscope and reserved for blood meal analysis. Each mosquito was dissected individually on a new microscope slide by using flame sterilized forceps to avoid cross contamination. DNA was isolated from the abdominal contents of blood-fed mosquitoes individually using Qiagen DNeasy Blood & Tissue Kit (Germany), according to the manufacturer's recommendation.

Blood meal analysis

To identify the vertebrate host on which the mosquitoes had fed, PCR-based blood meal analyses were performed using a primer sequences for the cytochrome b previously published (Molaei *et al.* 2006, Ngo and Kramer 2003, Cicero and Johnson 2001, Sorenson *et al.* 1999). All DNA templates were initially screened with avian-specific primers pairs *a* (5'-GAC TGT GAC AAA ATC CCN TTC CA- 3' and 5'- GGT CTT CAT CTY HGG YTT ACA AGA C- 3') and mammalian-specific primer pairs *c* (5'- CCA TCC AAC ATC TCA GCA TGA TGA AA- 3' and 5'- GCC CCT CAG AAT GAT ATT TGT CCT CA-3'). If the above set of primers failed to yield any PCR product, three additionally universal primer sets were used, mammalian-a (5'- CGA AGC TTG ATA TGA AAA ACC ATC GTT G- 3' and 5'- TGT AGT TRT CWG GT CHC CTA- 3'), mammalian-b (5'- GCG TAC GCA ATC TTA CGA TCA A- 3' and 5'- CTG GCC TCC AAT TCA TGT GAG- 3'), and avian-b (5'- CCC TCA GAA TGA TAT TTG TCC TCA- 3' and CCT CAG AAK GAT ATY TGN CCT CAK GG- 3'). In some cases these last primers were used to solve ambiguous sequences. PCR and cycling conditions were as described by Molaei *et al.* (2006). High Fidelity PCR Master (Roche, Germany) was used for all PCRs according to the manufacturer recommendation. Amplicons were visualized by electrophoresis in a 1.5% agarose gel with GelRed (0.5-1 µg/ml) and purified using Jet Quick-PCR Purification Kit (Genomed, Germany) as described by the manufacturer. The purified DNA fragments were directly sequenced in an ABI automated DNA capillary sequencer (Applied Biosystems, USA) by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences were annotated by using BioEdit software (Ibis biosciences, Carlsbad CA) and identified by comparison to the GenBank DNA sequence database (National Center for Biotechnology Information available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi). Positive identification and host species assignment were by sequence similarity. Sequences that did not meet the criteria were reported as unknown. A blood meal was classified as mixed if two different species were identified in two separate PCRs from the same template or when chromatograms from each PCR demonstrated double-nucleotide peaks. In addition, positive controls of known-origin blood were processed and correctly identified with the above procedure. Species selected as controls included human (*Homo sapiens*), cow (*Bos taurus*), pig (*Sus scrofa*), and lizard (*Lacerta dugesii*).

RESULTS

Blood meal sources were successfully identified by DNA sequencing from 131 (64.5%) of 203 field-collected mosquitoes with visible blood meals, representing nine species of five

genera. Of the remaining blood-fed mosquitoes either PCR amplification was negative or the sequencing results were inconclusive. In total, 32 species of vertebrates were identified, including 23 birds and nine mammals (Table 11).

Table 11. Blood meals by host class for mosquitoes' species collected in Portugal, during 2007, 2008, 2009 and 2010 mosquito season.

Species	Mammalian-host	(N)	Avian-host	(N)	Mixed blood-meal	(N)
Aedes aegypti	*Homo sapiens*	(6)				
Anopheles maculipennis s.l.	*Bos taurus*	(5)	*Anser cygnoides*	(1)		
	Capra hircus	(1)	*Gallus gallus*	(1)		
	Homo sapiens	(2)				
Culiseta annulata	*Bos taurus*	(1)				
	Homo sapiens	(1)				
	Ovis aries	(1)				
Cs. longiareolata	*Homo sapiens*	(1)				
Culex perexiguus	*Homo sapiens*	(1)				
	Ovis aries	(1)				
Culex pipiens	*Homo sapiens*	(18)	*Annas platyrhyncos*	(1)	*Homo sapiens/ Bos taurus*	(1)
	Ovis aries	(3)	*Bradypterus*	(1)	*Homo sapiens/ Turdus*	(1)
			Carina moshata	(1)		
			Cyanopica cooki	(1)		
			Delichon urbica	(1)		
			Falco tinnunculus	(1)		
			Galerida cristata	(1)		
			Gallus gallus	(30)		
			Hyppolais polyglotta	(1)		
			Laurus fuscus	(1)		
			Meleagris gallopavo	(1)		
			Miliaria calandra	(1)		
			Nycticorax nycticorax	(1)		
			Nymphicus hollandicus	(1)		
			Parus caeruleus	(1)		
			Passer domesticus	(1)		
			Serinus canaria	(1)		
			Sylvia borin	(1)		
			Sylvia communis	(2)		
			Sylvia melanocephala	(1)		
			Turdus merula	(5)		
Cx. theileri	*Bos taurus*	(1)	*Morus bassanus*	(1)	*Homo sapiens/ Bos taurus*	(1)
	Canis familiaris	(1)				
	Felis catus	(1)				
	Homo sapiens	(3)				
	Ovis aries	(1)				
	Sus scrofa	(2)				
Ochlerotatus caspius	*Canis familiaris*	(3)				
	Capra hircus	(4)				
	Equus caballus	(1)				
	Homo sapiens	(8)				
	Rattus rattus	(1)				
	Sus scrofa	(1)				
Oc. detritus	*Bos taurus*	(1)				
	Ovis aries	(1)				

Culex pipiens blood meals, which comprised 78 of the total blood meal sources sampled, were identified most commonly as avian (*n* = 55/ 78, 70.5%), and less commonly, but not infrequently, as mammals (*n* = 21/ 78, 26.9%) in which humans were prevalent, (*n* = 18/ 21, 85.7%), and sheep (*Ovis aries*) was the second mammalian host detected (Table 12). Chicken (*Gallus gallus*) was the most common bird species identified for *Cx. pipiens* (*n* = 30/ 55, 54.6%), followed by common blackbird (*Turdus merula*) (*n* = 5/ 55, 9.1%), and several other bird species frequent in Portugal. Mix blood meals were detected in two specimens of *Cx. pipiens*, one including the blood of two mammal hosts, human and bovid, and the other including human and avian blood from common blackbird (Table 11; Table 12). In relation to residential/ non-residential habitats, no significant difference was detected in *Cx. pipiens* blood feeding behavior (χ^2 test, $P > 0.3$).

All the other species examined, *Ae. aegypti*, *An. maculipennis* s.l., *Cs. annulata*, *Cs. longiareolata*, *Cx. perexiguus*, *Cx. theileri*, *Oc. caspius* and *Oc. detritus* fed primarily on mammals. Humans represented 100% (*n* = 6/ 6) of all the blood meals from *Ae. aegypti* and was also the most common blood source identified in *Oc. caspius* Pallas (*n* = 8/ 18, 44.4%), which fed exclusively on mammals, as *Cs. annulata*, *Cs. longiareolata*, *Cx. perexiguus* and *Oc. detritus*. For *An. maculipennis* s.l., ten different avian and mammalian blood sources were identified including two blood meals from human. In one out of 11 tested specimens of *Cx. theileri* a mixed blood meal consisting of human and bovid blood was detected (Table 12).

Table 12. Hosts of *Culex pipiens* collected from residential and non-residential areas of Portugal, during 2007, 2008, 2009 and 2010 mosquito season.

Habitat	Host									
	Avian		Mammal			Mix			Total	
			Human		Sheep	Avian/ Human		Bovid/ Human		
	N	(%)	N	(%)	N (%)	N	(%)	N (%)	N	(%)
Residential	10	(66.7)	5	(33.3)	0 (0)	0	(0)	0 (0)	15	(19.2)
Non-residential	45	(71.4)	13	(20.6)	3 (4.8)	1	(1.6)	1 (1.6)	63	(80.8)
Total	55	(70.5)	18	(23.1)	3 (3.9)	1	(1.3)	1 (1.3)	78	(100)

DISCUSSION

In this study, six species (*Ae. aegypti, Cs. annulata, Cs. longiareolata, Cx. perexiguus, Oc. caspius, Oc. detritus*) demonstrated a exclusive mammophilic fed behavior. The remaining analyzed species, *An. maculipennis* s.l., *Cx. pipiens* and *Cx. theileri* also fed on birds. Considering WNV epidemiology, these latest are the important species, with an ability to circulate and amplify WNV in nature and transmit the virus to humans. West Nile virus was

isolated for the first time in Portugal from *An. maculipennis* (Filipe and Pinto 1972). The avian and human blood meals detected in this species support that it may play a role in WNV epidemiology.

Culex pipiens is considered primarily ornithophilic (Apperson *et al.* 2004, Turell *et al.* 2005), although it is clear from the data presented here that the blood meals analyzed were not exclusively from birds. The results show that this species fed predominantly on avian hosts (70.5%) including some key bird species that can maintain WNV transmission, in particular the common blackbird, house sparrows, and common kestrel (Hayes *et al.* 2005, Zeller and Schuffenecker 2004). This behavior supports the speculated significance of the role of *Cx. pipiens* in enzootic transmission of WNV noted in previous studies (Molaei *et al.* 2006, Hamer *et al.* 2008, 2009). However, 23.1% of all blood meals were human-derived, which points to *Cx. pipiens* not only as a potential enzootic vector but also as a potential bridge vector of WNV to humans. The mixed human/ common blackbird blood meal confirms the presence of individuals from *Cx. pipiens* population readily feeding on both birds and humans, which is a necessary condition for epidemic transmission (Kilpatrick *et al.* 2005) and reinforces *Cx. pipiens'* role in WNV epidemiology.

Regarding the *Cx. pipiens* identified with human blood meals, 27.8% (5/ 18) were collected from residential areas as backyards of homes in urban habitats and 72.2% (13/18) in mosquitoes collected in farms, natural parks, culture field crops, and other rural/ peri-urban areas where humans are not, in any way, the most abundant hosts. *Culex pipiens* was previously shown to be influenced by host availability (Hamer *et al.* 2009). However, in this study no significant differences were found between the proportions of avian/ human blood fed *Cx. pipiens* in both residential/ non-residential habitats, although there was a small difference in variety of avian hosts used. Therefore our finding of a high frequency of human feeding by *Cx. pipiens* mosquitoes could not be explained by collecting blood-fed mosquitoes in immediate proximity to human habitation. Some studies indicate that the innate host preference of many mosquito species is modulated by the spatial and temporal abundances of potential hosts and by regional differences in host reproductive cycles and other behaviors (Savage *et al.* 2007, Richards *et al.* 2006); however, despite the limited number of samples in this study, it seems that there is no correlation between choice and availability of hosts. For example, the *Cx. pipiens* specimen that fed on a house sparrow was collected from a dog kennel. In this way the results from this study support a clear innate host preference by *Cx. pipiens* which seemingly can be modulated by spatial and temporal factors that were not evaluated. This can be supported considering that the two forms of the species *Cx. pipiens*, *molestus* and *pipiens*, which occupy different habitats (underground vs aboveground) in

northern Europe, can occur aboveground as sympatric populations in southern Europe (Kothera *et al.* 2010, Byrne and Nichols 1999, Huang *et al.* 2008). It has also been suggested that the differential introgression of *molestus* genes into the *pipiens* form may induce a more opportunistic biting behavior in the latter (Gomes *et al.* 2009). Hybrids between *molestus* and *pipiens* are considered epidemiologically significant since hybrids can readily feed on both avian and mammalian hosts, including humans. This opportunistic biting behavior may potentiate the role of *Cx. pipiens* as a bridge vector for the transmission of arboviruses such as WNV from their amplification hosts to humans (Fonseca *et al.* 2004). Further investigation focusing on genetic data to characterized *Cx. pipiens* population in relation to feeding habit is warranted.

3.2 SYMPATRIC OCCURRENCE OF *CULEX PIPIENS* BIOTYPES *PIPIENS*, *MOLESTUS* AND THEIR HYBRIDS IN PORTUGAL, WESTERN EUROPE: FEEDING PATTERNS AND HABITAT DETERMINANTS

RESEARCH ARTICLE

Osório, H.C., Zé-Zé, L., Amaro, F., Alves, M.J. (2013) Sympatric occurrence of *Culex pipiens* (Diptera, Culicidae) biotypes *pipiens*, *molestus* and their hybrids in Portugal, Western Europe: feeding patterns and habitat determinants. *Medical and Veterinary Entomology*, accepted for publication (PubMed PMID: 23786327).

ABSTRACT

Culex (Culex) pipiens has two recognized biotypes, *pipiens* and *molestus* that differ in physiology and behaviour, which may influence their vectorial capacity for West Nile virus (WNV). Our goal was first to determine the presence of *Cx. pipiens* populations in 31 locations of Portugal, but also to analyse their host-feeding preferences and habitat determinants. Molecular identification of *Cx. pipiens* forms and their hybrids was performed in 97 females and the blood meal sources identified in 59 analysed engorged specimens. In total, 61.9% specimens were identified as *Cx. pipiens f. pipiens*; 20.6% as *Cx. pipiens f. molestus*; and 17.5% as hybrids forms. *Culex pipiens f. pipiens* fed preferentially on birds, and *Cx. pipiens f. molestus* on humans. Hybrids forms fed mostly on birds, but human blood meals were common. Regarding the habitat, *Cx. pipiens f. pipiens* and hybrid forms were positively correlated with peri-urban habitats. Concluding, our results confirm the sympatric presence of *Cx. pipiens* biotypes from 14 of 31 studied locations. Peri-urban areas were common habitat to all biotypes and may represent hybridization zones. The feeding preference and the sympatric distribution of the *Cx. pipiens* biotypes observed in Portugal favor the epizootic circulation of WNV and the occurrence of disease outbreaks of WNV.

INTRODUCTION

Four species are currently recognized in the *Culex pipiens* complex, being *Cx. pipiens* Linnaeus 1758 and *Cx. quinquefasciatus* Say 1823 the most abundant and ubiquitous in temperate and tropical regions, respectively. Further *Cx. pipiens* has two recognized sub-species, *Cx. p. pipiens*, originally distributed from Northern Europe to South Africa and *Cx. p. pallens* in temperate Asia (Knight, 1978). *Cx. p. pipiens*, which we will herein refer to as *Cx. pipiens*, represents the primary enzootic and epidemic vector of West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) in Europe and northeastern and central United States (Farajollahi *et al.*, 2011). In Portugal this is one of the most widespread and abundant mosquito vectors (Osório *et al.*, 2010).

Culex pipiens has two different forms or biotypes: *pipiens* and *molestus*. Whereas *Culex pipiens f. pipiens* diapause (heterodynamic), requires a blood meal to lay eggs (anautogeny) and is unable to mate in confined spaces (eurygamous), *Cx. pipiens f. molestus* does not diapause and remains active during winter (homodynamic), can oviposit without a blood meal (autogeny) and mates in confined spaces (stenogamous) (Harbach *et al.*, 1984; 1985). The combination of stenogamy and autogeny allows *Cx. pipiens f. molestus* to occur in underground and confined areas in urban settings. Indeed in UK, Russia and North America the forms *molestus* and *pipiens* occupy different habitats underground and aboveground, respectively (Byrne & Nichols, 1999; Vinogradova, 2000; Huang *et al.*, 2008). This physical discontinuity of habitats does not promote gene flow between *molestus* and *pipiens* and genetic isolation seems to occur (Chevillon *et al.*, 1995). In northern Europe *pipiens* is the ubiquitous form of the biotype complex and *molestus* was only reported from Germany and the United Kingdom from underground breeding sites (Byrne & Nichols, 1999; Weitzel *et al.*, 2009). Detection of hybrid forms was only recently reported in the Netherlands (Reusken *et al.*, 2010). On the other hand, in southern Europe autogenous and anautogenous populations were observed co-occurring in the same habitats and hybrid genetic forms of *Cx. pipiens f. molestus* and *Cx. pipiens f. pipiens* have already been described in two populations (Fonseca *et al.*, 2004; Gomes *et al.*, 2009).

The taxonomic status and phylogeny of these biotypes remains controversial. Previous studies have suggested that *pipiens* and *molestus* represent two distinct genetic entities and that the underground *molestus* populations in northern Europe were most likely derived from southern *molestus* populations that have dispersed and colonized underground habitats (Kent *et al.*, 2007; Weitzel *et al.*, 2009). In another hypothesis *molestus* form is believed to derive

from surface *pipiens* populations that have undergone local adaptation to underground conditions (Byrne & Nichols, 1999).

Regarding public health, hybrids between *molestus* and *pipiens* forms have been considered of great epidemiological importance (Fonseca *et al.*, 2004). The different vectorial capacities of the biotypes are due to their potentially different feeding preferences with *Cx. pipiens f. pipiens* considered mainly ornithophilic, *Cx. pipiens f. molestus* mainly mammophilic, and hybrids having opportunistic behaviour readily feeding on both avian and mammalian hosts, including humans. This opportunistic biting behaviour may thus potentiate the role of *Cx. pipiens* as a bridge vector for the transmission of WNV from their amplification hosts (birds) to humans (Hamer *et al.*, 2008). However, conclusive evidences for *Cx. pipiens* mosquitoes host feeding preference are still lacking.

In this study we have used a rapid PCR-based assay based in polymorphisms in the flanking region of microsatellite locus CQ11, which allows discrimination of pure forms and putative hybrid populations of *Cx. pipiens* (Bahnck & Fonseca, 2006). Further regarding WNV epidemiology we have assessed the blood-feeding patterns of *pipiens*, *molestus* and hybrid forms of *Cx. pipiens* specimens and also analysed habitat determinants.

MATERIAL AND METHODS

Mosquito collection

Larvae collection

Collections of larvae were performed in a well and in a rice field from Palmela (38.567495, -8.726578), and in a pen underground sewage from Santiago do Cacém (38.021185, -8.673363). The pen and the well larvae were successfully colonized and five larvae and five females from each colony were used as controls in diagnostic PCR's.

Adult collection

Adult specimens were collected from May to October 2007-2011 from 31 locations in Portugal (Figure 1) using CDC light-traps (John W. Hock Co., Gainesville, FL) and battery power aspirators as described in Osório *et al.* (2010).

Adult collection habitats were classified in urban versus peri-urban areas according to human density, the first with more than 411.5 inhabitants per km^2 (data from 2010; http://www. ine.pt). The peri-urban areas were further categorized as: 1) peri-urban/ farm, where farming activities take place but there are no dwellings in vicinity; 2) peri-urban/ dwelling, in which collection was performed indoors in a residential area of a small town or

village (<411.5 inhabitants per km^2) in rural setting. Urban areas were further classified as: 1) urban/ dwelling, for collections performed indoors; 2) and urban/ public areas, in which mosquitoes were collected in residential urban settings, the most frequent being local urban gardens, playgrounds, urban parks, and warehouses. Further, sylvatic habitat was considered for collections performed in forests or natural parks where human activities are limited.

The mosquitoes were morphologically identified according to Ribeiro & Ramos (1999). *Culex pipiens* with fresh or visible blood remnants were individually transferred into 1.5-mL microtubes and stored frozen at -80ºC until used for molecular analysis.

DNA extraction

The DNA of blood fed mosquitoes was extracted from abdominal contents using DNAeasy Blood & Tissue kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer´s recommendations. Each mosquito was dissected individually on a new microscope slide by using sterilized forceps to avoid cross contamination.

Larvae and non engorged females were completely processed for DNA extraction.

Genetic analyses

The PCR assay described by Bahnck & Fonseca (2006) was used to detect a size polymorphism in the flanking region of the CQ11 microsatellite of *Cx. pipiens*, which display a PCR product of 190 bp for the *pipiens* form, 260 bp for *molestus* form, and hybrids exhibiting both amplicons (190 bp/ 260 bp). Briefly, the primers sequences used in this multiplex PCR were as follows: pipCQ11R 5'–CATGTTGAGCTTCGGTGAA–3' and molCQ11R 5'–CCCTCCAGTAAGGTATCAAC–3' as form specific reverse primers and CQ11F2 5'–GATCCTAGCAAGCGAGAAC–3' as forward primer. Conditions for PCR amplification were as follows: 94^{o}C for 5 minutes and then 40 cycles of 94^{o}C for 30 seconds, 54^{o}C for 30 seconds, and 72^{o}C for 40 seconds, concluding with a final 5 minute extension at 72^{o}C. Amplification products were run on a 2% agarose gel with GelRed (0.5 – 1 µg/ mL; Biotarget, Portugal) and discrimination of *pipiens* and *molestus* specific DNA fragments was easy in a single PCR for most samples. However, in case of doubt or negative electrophoresis, separate PCRs with each set of primers were run until unambiguously identification of each form. Samples were considered DNA degraded when PCRs also failed in blood meal analyses. Specific amplification products from *molestus* and *pipiens* were purified using Jet Quick-PCR Purification Kit (Genomed, Germany) as described by the manufacturer. The purified DNA fragments were directly sequenced in an ABI automated DNA capillary sequencer (Applied Biosystems, USA) by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Sequences were annotated by using BioEdit software (Ibis biosciences, Carlsbad CA) and confirmed by comparison to the GenBank DNA sequence database (National Center for Biotechnology Information available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In order to discard the potential presence of *Cx. torrentium*, a closely related species morphologically very similar to members of the *Cx. pipiens* complex, a PCR assay based in polymorphisms in the second intron of the acetylcholenesterase (ACE-2) was applied to all samples (Smith and Fonseca, 2004).

Blood meal analyses

To identify the vertebrate hosts PCR-based blood meal analyses were performed using primers sequences for the cytochrome-b previously published (Molaei *et al.*, 2006). Primers sequences, PCR cycling conditions, electrophoresis, and sequencing of purified DNA fragments and species assignment were performed as described by Osório *et al.* (2012). In summary, all DNA templates were screened with avian-specific primer pairs and mammalian specific primer pairs with High Fidelity PCR Master (Roche, Manheim, Germany) according to manufacturer's recommendation. Amplicons were visualized by electrophoresis in a 1.5% agarose gel with GelRed (0.5-1 µg/ml) and purified using Jet Quick-PCR Purification Kit as described by the manufacturer. The purified DNA fragments were directly sequenced in an ABI automated DNA capillary sequencer as described above. Cytochrome-b sequences were analysed and identified as described for microsatellite sequences.

Data Analyses

A one-way Generalized Linear Model (GLM) with binomial distribution and a logistic link function was performed to test whether the selection of different hosts differed among biotypes, with Host as binary dependent variable (proportion of species feeding on Bird vs. Mammal/human) and Biotype as dependent variable.

To test whether the distribution of species was associated with the habitat type a multinomial logistic regression was performed with Biotype as dependent variable with three categories (Pipiens, Hybrid and Molestus) and Habitat as independent variable considering only Peri-urban/ farm, Peri-urban/ dwelling and Urban/ dwelling habitats (the ones with N≥11), since sample size guidelines for multinomial logistic regression indicate a minimum of 10 cases per independent variable (Schwab, 2002). All statistical analyses were performed with software R, version 2.15.2 (R Core Team, 2012). Multinomial logistic regression was performed with mlogit library (Croissant, 2012).

RESULTS

The developed insectary colonies of field-caught *Culex pipiens* larvae collected from the well and from the pen underground sewage revealed the characteristic traits that define the *molestus* form (Vinogradova, 2000). Both larvae populations were easily colonized and showed autogenic behaviour with egg raft production for two generations without blood meal supplying. Male insemination behaviour was observed in colonies. By contrast the larvae population collected from the rice field was not able to grow and establish a colony in the same insectary conditions as the above mentioned populations. Additionally in this attempt no insemination behaviour was observed in males. Genetic microsatellite analyses confirmed the phenotypic alternative traits of *molestus* and *pipiens* forms.

Overall, 97 morphologically identified *Culex pipiens* were molecularly typed by PCR at the biotype level (Table 13). Of the total analysed specimens 60 (61.9%) were identified as *pipiens* form, 20 (20.6%) as *molestus* form, and 17 (17.5%) as hybrid form. The sympatric presence of *Cx. pipiens* biotypes was observed from 14 of 31 studied locations in Portugal (Figure 22; Table 14). *Culex torrentium* was not identified by PCR in this study.

Table 13. Frequencies of *Culex pipiens* forms *pipiens, molestus* and hybrids determined by PCR directed to the flanking region of the CQ11 microsatellite and blood meal sources determined by PCR with universal primers set to cytochrome-b of vertebrate hosts.

BIOTYPE	N	BLOOD MEAL		HABITAT [1]				
		BIRD	HUMAN	PU/ D	PU/ F	S	U/D	U/ PA
Hybrid	17	8	4[†]	5	9	1	2	0
Molestus	20	2	7	1	8	0	10	1
Pipiens	60	33	5	13	37	3	5	2
TOTAL	97	43	16	19	54	4	17	3

[1] PU/ D: Peri-uban/ dwelling; PU/ F: Peri-urban/ farm; S: Sylvatic (non-residential); U/ D: Urban/ dwelling; U/ PA: Urban/ public areas
[†] One blood meal identified as sheep

Blood meal sources were successfully identified from 59 (60.8%) of the 97 blood-fed *Cx. pipiens* (Table 14). Of the remaining mosquitoes, either PCR amplification was negative or the sequencing results were inconclusive. In total, 43 blood meals were identified as avian (72.9%) and 16 (27.1%) as mammalian, in which humans prevail (15/16, 93.8%).

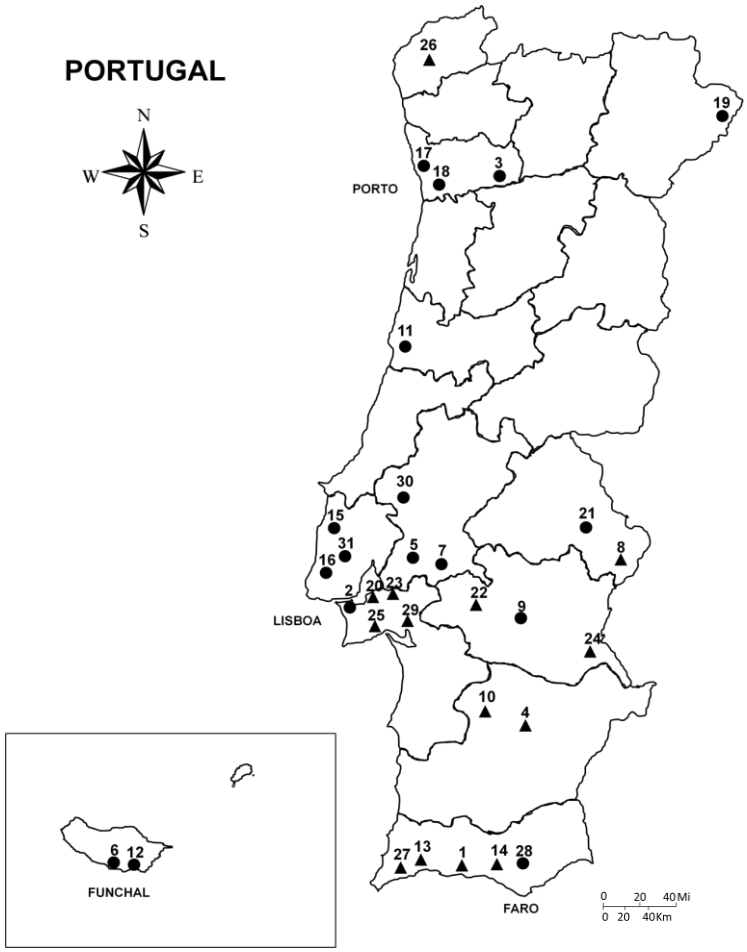

Figure 22. Adult mosquito collections sites (●). Sympatric presence of *Culex pipiens* biotypes are marked as (▲).

The proportion of bites in birds and mammals was significantly different between the biotypes, with *Cx. pipiens f. pipiens* feeding preferentially on birds (33/38, 86.8%) in comparison with *Cx. pipiens f. molestus* ($p<0.01$, Table 15) and with *Cx. pipiens* hybrids ($p<0.05$, Table 15). *Culex pipiens f. molestus* fed mainly on humans (7/9, 77.8%) while *Cx. pipiens* hybrids fed on humans/ sheep (4/12, 33.3%), and birds (8/12, 66.7%) although no significant differences were detected in relation to their blood-feeding behaviour (Table 13 and 15).

Table 14. Mosquito collection sites and frequencies of *Culex pipiens* considering 1) the collection habitat, 2) biotype molestus, hybrid and pipiens, and 3) blood meal host.

SITES [1]	GPS DATA	HABITAT [2]	BIOTYPE (N)	HOST (N)
1. Albufeira	37.104764, -8.326950	PU/ F	Hybrid (1)	Sheep (1)
			Pipiens (5)	Bird (3); Human (1)
2. Almada	38.684129,-9.150026	U/ D	Molestus (1)	Human (1)
3. Baião	41.163583, -8.036117	U/ D	Molestus (1)	Human (1)
4. Beja	38.036869, -7.872350	PU/ F	Hybrid (1)	Bird (1)
			Molestus (2)	Not determined
			Pipiens (3)	Bird (1)

Table 14. (continuation)

5. Benavente	38.977905,-8.835555	PU/ F	Hybrid (1)	Bird (1)
6. Câmara de Lobos	32.645417, -16.97225	U/ D	Molestus (2)	Human (1)
7. Coruche	38.962681, -8.530556	U/ D	Pipiens (1)	Bird (1)
8. Elvas	39.002778, -7.179722	S	Hybrid (1)	Not determined
			Pipiens (3)	Bird (3)
9. Évora	38.570580, -7.893118	U/PA	Pipiens (2)	Bird (1)
10. Ferreira do Alentejo	38.118050, -8.173200	PU/ F	Hybrid (2)	Bird (2)
			Molestus (3)	Human (1)
			Pipiens (12)	Bird (5)
11. Figueira da Foz	40.163317, -8.861833	U/PA	Molestus (1)	Human (1)
12. Funchal	32.646083, -16.90886	U/ D	Molestus (3)	Human (2)
13. Lagos	37.123678, -8.694489	PU/ D	Hybrid (1)	Not determined
			Pipiens (1)	Bird (1)
14. Loulé	37.045786, -8.052344	PU/ D	Hybrid (2)	Bird (1); Human (1)
			Pipiens (2)	Human (1)
15. Lourinhã	39.243975, -9.329778	PU/ D	Pipiens (2)	Bird (1)
16. Mafra	38.931333, -9.333150	PU/ F	Pipiens (1)	Not determined
17. Maia	41.233317, -8.625933	U/ D	Molestus (2)	Not determined
18. Matosinhos	41.197250, -8.680017	PU/ D	Pipiens (1)	Not determined
19. Miranda do Douro	41.489833, -6.266667	PU/ F	Pipiens (1)	Not determined
20. Moita	38.670524, -8.977065	PU/ D	Hybrid (1)	Not determined
			Pipiens (1)	Not determined
21. Monforte	38.920709, -7.420618	U/ D	Pipiens (1)	Human (1)
22. Montemor-o-Novo	38.645556, -8.191667	PU/ F	Hybrid (1)	Bird (1)
			Pipiens (6)	Bird (4)
23. Montijo	38.705521,-8.972223	U/ D	Hybrid (1)	Bird (1)
			Pipiens (3)	Bird (2)
24. Mourão	38.343033, -7.374950	PU/ F	Hybrid (2)	Human (1)
			Pipiens (4)	Bird (3)
25. Palmela	38.640797, -8.932350	PU/ F	Molestus (2)	Not determined
			Pipiens (1)	Bird (1)
26. Ponte de Lima	41.764444, -8.578333	U/ D	Hybrid (1)	Human (1)
			Molestus (1)	Not determined
27. Portimão	37.152050, -8.622383	PU/ F	Hybrid (1)	Bird (1)
			Molestus (1)	Bird (1)
			Pipiens (4)	Bird (3); Human (1)
28. São Brás de Alportel	37.176028, -7.894750	PU/ D	Pipiens (1)	Bird (1)
29. Setúbal	38.557756, -8.763400	PU/ D	Molestus (1)	Bird (1)
			Pipiens (2)	Human (1)
30. Torres Novas	39.427833, -8.551408	PU/ D	Pipiens (3)	Bird (3)
31. Torres Vedras	39.083698,-9.332137	PU/ D	Hybrid (1)	Not determined

[1] See Figure 22.

[2] PU/ D: Peri-uban/ dwelling; PU/ F: Peri-urban/ farm; S: Sylvatic (non-residential); U/ D: Urban/ dwelling; U/ PA: Urban/ public areas.

Table 15. Summary of Generalized Linear Model used (with binomial distribution and a logistic link function) to test whether the selection of different hosts (Bird *vs.* Mammal/human) differed among biotypes, with Host as binary dependent variable (proportion of species feeding on Bird *vs.* Mammal/human) and Biotype as dependent variable.

BIOTYPE	ESTIMATE	STD. ERROR	T-VALUE	P-VALUE
Pipiens *vs.* Hybrid	-1.771	0.834	-2.124	0.034*
Pipiens *vs.* Molestus	-2.371	0.799	-2.968	0.003**
Molestus *vs.* Hybrid	0.600	0.770	0.780	0.436

Considering the collection habitat, the ratio of collected biotypes was different between different habitats ($P < 0.05$, χ^2 test). *Culex pipiens f. pipiens* and hybrids were positively correlated with peri-urban/ farm habitats (50/60, 83.3% and 14/17, 82.4%, respectively). The frequency of *Culex pipiens f. pipiens* was significantly higher in peri-urban/ farm locations compared with peri-urban/ dwelling ($p<0.05$, Table 16) and with urban/ dwelling ($p<0.01$, Table 16), whereas the frequency of hybrids was significantly higher in peri-urban/ farm locations compared with urban/ dwelling ($p<0.05$, Table 16). On the other hand no significant differences were found in *Cx. pipiens f. molestus* collected in urban (11/20, 55.0%) and peri-urban habitats (9/20, 45.0%), although the frequency of this biotype was marginally higher in urban/ dwelling habitat compared with peri-urban/ dwelling ($p=0.07$, Table 16).

Table 16. Summary of Multinomial Logistic Regression used to examine whether the distribution of biotypes was associated with habitat type, with Biotype as nominal dependent variable with three categories (Pipiens, Hybrid, Molestus) and Habitat as dependent variable considering Peri-urban/ farm, Peri-urban/ dwelling and Urban/ dwelling (N≥11).

BIOTYPE	HABITAT[1]	ESTIMATE	STD. ERROR	T-VALUE	P-VALUE
Pipiens	PU/ F *vs.* PU/ D	0.887	0.449	1.976	0.048*
	PU/ F *vs*. U/ D	1.735	0.626	2.770	0.006**
	PU/ D *vs*. U/ D	0.847	0.690	1.228	0.220
Hybrid	PU/ F *vs*. PU/ D	1.099	0.667	1.648	0.099
	PU/ F *vs*. U/ D	2.197	1.054	2.085	0.037*
	PU/ D *vs*. U/ D	1.099	1.155	0.951	0.341
Molestus	PU/ F *vs*. PU/ D	1.792	1.080	1.659	0.097
	PU/ F *vs*. U/ D	-0.154	0.556	-0.277	0.782
	PU/ D *vs*. U/ D	-1.946	1.069	-1.820	0.069

[1] PU/ D: Peri-uban/ dwelling; PU/ F: Peri-urban/ farm; U/ D: Urban/ dwelling.

DISCUSSION

Of all the molecular markers so far analysed (Crabtree *et al.*, 1997; Vinogradova & Shaĭkevich, 2005; Kent *et al.*, 2007), the locus CQ11 has been considered the most promising diagnostic marker obtained for *Cx. pipiens* genetic forms identification (Gomes *et al.*, 2009). This molecular assay was a valuable tool in conjunction with the ecological studies aiming at ascertaining the host preference and typical habitat towards the epidemiological significance of these forms. Our results provide molecular evidence for the sympatric occurrence of *Cx. pipiens f. molestus* and *Cx. pipiens f. pipiens* and their hybrids in 14 different areas of mainland Portugal and Madeira Island and show co-occurrence of these forms in urban and peri-urban range of different habitats.

Culex pipiens f. pipiens populations were significantly linked to peri-urban habitats, where a wide range of larvae breeding sites are likely to be found, accordingly to the behavioural traits of this form (Harbach *et al.*, 1985; Vinogradova, 2000). Farms include a range of sites where host availability and diversity is high, which may attract host seeking mosquito females of the surroundings. *Cx. pipiens f. pipiens* may also use dwellings on the ground as resting or diapause places and, therefore, it is not surprising to find *Cx. pipiens f. pipiens* indoors.

The considerable presence of hybrids in the peri-urban areas suggests that these urban borders may represent high level hybridization zones, where *molestus* and *pipiens* forms have conditions to co-occur and interbreed. Typically occupying underground and confined breeding habitats in peri-urban areas (Fonseca *et al.*, 2004; Medlock *et al.* 2005), *molestus* populations can accidentally or naturally take place in open and aboveground habitats (e.g. in case of discharge of sewage into open areas), and favourable breeding conditions for successful matting between *molestus* and *pipiens* may occur with emergence of hybrid forms.

Regarding feeding preference we have documented a significant association between *Cx. pipiens f. pipiens* and avian blood meals and, on the other hand, *Cx. pipiens f. molestus* and mammalian blood meals. Together without evidence of habitat segregation these results suggest an underlying genetic basis for mammalian versus avian host selection and are consistent compared with the scarce previous studies focusing on the feeding habits and population dynamics of *molestus* and *pipiens* forms (Kilpatrick *et al.*, 2007; Huang *et al.*, 2009). Accordingly, the analysed hybrids showed an opportunistic feeding behaviour and potentially the same ability to switch vertebrate hosts, biting mammals and birds, supporting that natural hybridization induces to a change in host preference. Hybrid forms can readily feed on avian and mammalian hosts and is possible that they exhibit intermediate physiological and

behavioural traits and that this flexibility in the phenotype would boost bridging transmission by these mosquitoes, which are competent to feed first on a viremic bird and later on a human. For this reason, hybrids between *molestus* and *pipiens* forms have been considered of great epidemiological importance (Fonseca *et al.*, 2004).

In conclusion, our results provide evidence for the sympatric occurrence of *Cx. pipiens f. molestus* and *Cx. pipiens f. pipiens* populations from several locations in Portugal. Natural hybridization seems to occur most intensely in peri-urban areas, which may represent high level hybridization zones, where favourable conditions for *molestus* and *pipiens* interbreeding occur. Our results support previous studies showing that *Cx. pipiens f. pipiens* is primarily ornithophylic and peri-urban, *Cx. pipiens f. molestus* is anthropophilic, appearing mainly associated, even if marginally, with urban habitats, and hybrid forms have opportunistic feeding behaviour. The different biotype feeding preference, which affects the vectorial capacity of vector mosquitoes, together with the observed sympatric distribution of the *Cx. pipiens* biotypes create suitable conditions for the epizootic circulation of WNV in Portugal and the occurrence of accidental human infections.

Further investigation on the genetic mechanisms influencing feeding on birds and mammals is warranted. This sort of studies will help clarify the role that *Cx. pipiens* plays in both enzootic and epidemic transmission of WNV.

CHAPTER 4

VECTOR COMPETENCE

4.1 VECTOR COMPETENCE OF *ANOPHELES ATROPARVUS* AND *CULEX PIPIENS F. MOLESTUS* MOSQUITOES FOR WEST NILE VIRUS

PRELIMINARY STUDY

Osório, H.C., Amaro, F., Zé-Zé, L., Alves, M.J. (2012) Vector competence of *Anopheles atroparvus* and *Culex pipiens* f. *molestus* mosquitoes for West Nile Virus.

ABSTRACT

As a step in determining the potential for endemic *Anopheles atroparvus* and *Culex pipiens f. molestus* transmitting West Nile Virus (WNV), evaluation of vector competence under laboratory conditions was conducted. Mosquitoes were exposed to virus by blood-feeding in soaked cotton pads with WNV titers ranging from $10^{2.5}$ to $10^{9.5}$ $CCID_{50s}$/mL. After 15 days extrinsic incubation mosquitoes were tested by PCR and cell culture to calculate infection, dissemination and transmission rates. All *An. atroparvus* mosquitoes showed to be refractory to infection, suggesting a minor role of this species in WNV epidemiology and ecology. However, *Cx. pipiens f. molestus* displayed vector competence for WNV with a transmission rate of 2.2%, indicating this species with close association with urban and peri-urban areas as likely to play an important role in the enzootic maintenance and local transmission of WNV.

INTRODUCTION

Vector competence characterizes the likelihood that a vector will transmit a pathogen after feeding on an infected host (Hardy *et al.*, 1983) and is essential to 1) incriminate a potential vector species; 2) to understand its role in transmission; and 3) to elucidate critical events in infection and dissemination (Brault, 2009; Anderson *et al.*, 2010). For successful transmission of a pathogen by a mosquito four important transmission barriers must come overcome, namely the mesenteronal infection barrier, mesenteronal escape barrier, salivary gland infection barrier and salivary gland escape barrier (Chamberlain and Sudia, 1961; Kramer *et al.*, 1981; Grimstad, 1985; Paulson *et al.*, 1989; Kilpatrick *et al.*, 2010).

The vector competence of colonized and field populations of mosquitoes for West Nile Virus (family Flaviviridae, genus Flavivirus, WNV), as other aspects of WNV amplification (mosquito feeding patterns, habitat association, and host/ reservoir competence) have lately received substantial attention and are well documented for numerous species in countries where WNV circulation has occurred (Kilpatrick *et al.*, 2010). Following its introduction in the United States, numerous mosquito species have been tested for experimental transmission (Goddard *et al.*, 2002) and a number of studies confirm that more than a few mosquito species are competent (Sardelis *et al.*, 2001; Goddard *et al.*, 2002; Turell *et al.*, 2005; Balenghien *et al.*, 2008; Jansen *et al.*, 2008; Tiawsirisup *et al.*, 2008; Mutebi *et al.*, 2012). Moreover it has been found that vector competence vary among species and genera (Turell *et al.*, 2005), days since feeding (Kilpatrick, 2008), strain of WNV (Moudy, 2007) and temperature during the extrinsic incubation period (Dohm, 2002), and significant differences of vector competence among mosquito populations of the same species (Vaidyanathan and Scott, 2007) suggest a genetic basis for vector competence (Hayes, 1984). In Europe few experimental assays in laboratory conditions have been performed and few mosquito species have been tested for susceptibility to transmit WNV (Balenghien *et al.*, 2008).

In Portugal WNV was first isolated from *Anopheles atroparvus* mosquitoes in 1971 (Filipe, 1972) and later from *Culex perexiguus* and *Cx. pipiens* populations collected in the province of Algarve (Parreira *et al.*, 2007). *Anopheles atroparvus* is member of the *An. maculipennis* complex of sibling species, and was the main vector of malaria in Portugal (Vicente *et al.*, 2011). Although primarily mammalophylic, it also feeds on birds (Osório *et al.*, 2012), raising the question of the importance of this species in the ecology and epidemiology of WNV. Furthermore, owing to virus isolation from this species we also question the potential vector competence of *An. atroparvus* with the same isolate WNV-Eg101 strain.

Culex pipiens mosquitoes are implicated as the most important vectors of WNV, together in epizootically circulation and human and horses infection cases, as high competence/ transmission rates for WNV has been demonstrated (Reiter, 2010[a]). In Portugal *Cx. pipiens* is one of the most widespread and abundant mosquito (Osório *et al.*, 2010). It has two genetic forms, namely *Cx. pipiens f. pipiens* and *Cx. pipiens f. molestus,* of which the latter was used in this study.

In this study evaluation of vector competence under appropriate conditions was conducted as a step in determining the potential for endemic *An. atroparvus* and *Cx. pipiens* transmitting WNV. To our knowledge no vector competence studies for arboviruses have already been performed with mosquito populations from Portugal. Our aim was primarily to determine the ability of the most abundant active mosquitoes in Portugal to serve as competent vectors of WNV that might be seasonally in Portugal by infected migrant birds.

MATERIAL AND METHODS

Experimental design and data analyses

Infection, dissemination and transmission rates for *Anopheles atroparvus* and *Culex pipiens* f. *molestus* were determined by calculating the percentage of specimens with virus in torsos, legs plus wings, and salivary glands after 15 days extrinsic incubation of mosquitoes fed overnight in blood-soaked cotton pads with WNV titers ranging from $10^{2.5}$ to $10^{9.5}$ $CCID_{50s}$/mL. Only mosquitoes that fed to repletion were used in this study (Pilitt and Jones, 1972). For that mosquitoes were individually dissected and tested by RT-PCR with specific Flavivirus/ WNV primers. Positive mosquito infections were confirmed by virus recovery achieve by cell culture.

For each species infection rates were calculated as the percentage of infected bodies per the total number fed on the infectious blood meal and tested. Dissemination rates were calculated as the percentage of mosquitoes that contained virus in the legs plus wings per the total number tested. Finally, transmission rates were expressed as the percentage of tested mosquitoes with virus present in their salivary glands.

Mosquitoes

Both species were colonized in the insectary of the Centre for Vectors and Infectious Diseases Research Dr. Francisco Cambournac (CEVDI)/ National Institute of Health (INSA) maintained at 24-28ºC, 40-80% relative humidity (RH), and 12:12 light:dark photoperiod. First generation *An. atroparvus* were used in infection assays. The performed insectary colonies of field-caught *Culex pipiens f. molestus* larvae revealed the characteristic traits that define the *molestus form*: autogenic behaviour with egg raft production for two generations without

blood meal supplying. Further genetic analyses by PCR assay was used to positively confirm the *molestus* biotype (Bahnck and Fonseca, 2006). In this case, third generation mosquitoes were used in this study. Mosquitoes used in experiments were 4-5 days postecdyses and were starved for 24 hours prior to infection assay.

Virus

The strain Roxo WNV-Eg101 originally isolated in 1971 from *Anopheles maculipennis* in the region of Alentejo, district of Beja, Portugal (Filipe, 1972), was used during all infection experiments. The virus was passed four times in Vero-E6 cells (African green monkey kidney) before it was used in the infection experiments.

Mosquito infection

Mosquito infections took place in a physical containment biosafety level 3 (BSL3) at CEVDI/ INSA. Mosquitoes were exposed to virus by blood-feeding in soaked cotton pads with a virus suspension of washed defibrinated horse blood and 1% sucrose. To determine viral titer at the time of feeding, a sample of the blood/virus mixture was taken before and after mosquito exposure and stored at -80°C for later titration. Following feeding, blood-fed mosquitoes were sorted and place in batches of 25 in rearing cages within a growth cabinet (Bioquip, ref. 1452). Mosquitoes were maintained at 28°C, 70% to 75% RH, and 12:12 photoperiod for 15 days and provided 10% sucrose solution via soaked cotton pledged. After 15 days extrinsic incubation individual mosquitoes were dissected. Legs plus wings, torsos, and salivary glands were placed in separate tubes containing glass beads and 1.0 mL of Growth Medium (GM) supplied with 3% heat-inactivated fetal bovine serum (FBS) plus 50µg/ mL penicillin/ streptomycin, 50µg/ mL gentamicin, and 2.5µg/ mL Fungizone and stored at -80ºC until further use.

Virus assays

The blood/ virus mixture was titrated as 10-fold dilutions and inoculated onto 96-well microplates containing confluent Vero cell monolayers. After 7-day incubation at 37°C in 5% CO2, Vero monolayers were examined for cytopathic effects (CPE), and 50% endpoints were calculated (Reed and Meunch, 1938).

Torsos and legs plus wings were homogenized by impact and friction in mixer mill MM400 (Retsh, Germany) and filtered before inoculation onto duplicate 24-well microplates containing confluent Vero cell monolayers. After 7-day incubation at 37°C and 5% CO_2, Vero cells monolayers were stained with crystal violet stain and examined for CPE. Positive

mosquito infections were identified in wells that displayed CPE after 7 days post-inoculation with supernatant from the mosquito torso homogenate. A disseminated infection was confirmed by the presence of CPE in wells inoculated with the supernatant from the legs plus wings. Transmission was considered when CPE was detected in wells inoculated with the supernatant from the salivary glands. Virus recovery was achieved in all positive cell cultures.

DNA extraction and molecular analyses assays

Results were confirmed using RT-PCR to detect viral RNA in the saliva and body parts of the experimental mosquitoes. Viral RNA was extracted using Magcore HF16 Nucleic Acid Extraction Kit (RBCBioscience). A WNV-specific reverse transcriptase-polymerase chain reaction Super-script one step RT-PCR (Invitrogen) was used (Briese *et al.*, 2002). Confirmation was performed by 100% sequence similarity with WNV-Eg101. For that the positive samples by electrophoresis in a 1.5% agarose gel with GelRed (0.5-1 µg/ml) were purified using Jet Quick-PCR Purification Kit (Genomed, Germany) as described by the manufacturer. The purified DNA fragments were directly sequenced in an ABI automated DNA capillary sequencer (Applied Biosystems, USA) by using ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequences were annotated by using BioEdit software (Ibis biosciences, Carlsbad CA) and identified by comparison to the GenBank DNA sequence database (National Center for Biotechnology Information available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

A total of 45 *Cx. pipiens f. molestus* and 12 *An. atroparvus* were tested. The other mosquitoes initially used in the experiment did not survive, or do not fed on the infectious blood meal. Infection, dissemination and transmission were observed in one specimen of *Cx. pipiens f. molestus*, which was confirmed by cell culture virus recovery and RT-PCR (Table 17).

Table 17. Infection, dissemination and transmission rates of *Anopheles atroparvus* and *Culex pipiens f. molestus* species exposed to $10^{2.5}$ to $10^{9.5}$ cell $CCID_{50}$/ mosquito of WNV following 15-day extrinsic incubation period.

Species	Origin	Generation	% Infection [a]	% dissemination [b]	% transmission [c]
Anopheles atroparvus	38°01′0″N, 8°42′00″W	F1	0 (0 / 12)	0 (0 / 12)	0 (0 / 12)
Culex pipiens f. molestus	38°34′3″N, 8°53′57″W	F3	4.4 (2 / 45)	2.2 (1 / 45)	2.2 (1 / 45)

[a] Percentage of mosquitoes with virus in the body (number infected/ total number tested).

[b] Percentage of mosquitoes with virus in legs and wings (number infected/ total number tested).

[c] Percentage of mosquitoes with virus in salivary glands (number infected/ total number tested).

Another *Cx. pipiens* f. *molestus* mosquito body was positive by RT-PCR, but growth of virus in cell culture was not observed. All other tested mosquitoes were negative by both methods.

DISCUSSION

The knowledge of what species can in fact be involved in WNV circulation in a specific geographically location is essential to understand the WNV epidemiology and to predict the most vulnerable sites for WNV human cases (Vaidyanathan and Scott, 2007; Kilpatrick *et al.*, 2010; Richards *et al.*, 2010). *Cx. pipiens* is of particular interest to potential WNV transmission since its widely distributed in both rural and urban habitats and bird feeding behaviour is frequently documented (Apperson *et al.*, 2004; Turell *et al.*, 2005; Osório *et al.*, 2012). In the present study *Cx. pipiens f. molestus* displayed vector competence for WNV, despite relatively low compared with other *Cx. pipiens* population studies in the past (Dohm *et al.*, 2002; Goddard *et al.*, 2002). However some laboratories studies found *Cx. pipiens* populations relatively refractory to infection, with only 0-10% mosquitoes becoming infected and no more than 28% transmitting the virus (Kilpatrick *et al.*, 2010). Moreover, genetic evidences for vector competence were found in *Cx. pipiens* mosquitoes. The form *pipiens* and *molestus* differ in the ability to become infected by WNV, and susceptible mosquitoes had much higher probability of form *pipiens* ancestry (Kilpatrick *et al.*, 2010). Nevertheless, from the results obtained in the current study, coupled with its ornithophylic feeding behaviour and close association with urban and peri-urban areas it is predicted that this species could play a significant role in the potential transmission of WNV (Osório *et al.*, 2012). Variability in vector competence of *Cx. pipiens* mosquitoes has already been observed, not only over a spatial scale, but also over time scale, and implies that its contribution to the amplification of WNV may also vary. This evidence makes prediction of WNV transmission using surveillance data more changeling nowadays (Kilpatrick *et al.*, 2010). Beyond genetic ancestry influences of temperature and other environmental pressures may also alter susceptibility to infection (Hardy *et al.*, 1990; Reisen *et al.*, 1996). In this study, collection of *Cx. pipiens f. molestus* eggs and larvae was performed in April, corresponding to the end of the cold season, in which environmental factors may have affected the parental generation (Richards *et al.*, 2010).

The isolation of WNV from *An. atroparvus* in the past raises the question of the importance of this mosquito in its ecology and epidemiology. In our data *An. atroparvus* was refractory to infection. One reason may be the low number of *An. atroparvus* tested due the poor engorgement level of this species on the artificial system. This species bites bird and mammal vertebrates and by feeding pattern it could be considered an important natural vector of WNV. (Osório *et al.*, 2012). By our knowledge *An. atroparvus* is associated with rural habitats where livestock production and rice cultures occur and in Portugal this mosquito is not considered a pestilent species (Osório *et al.*, 2010). Our results suggest that this species is

not susceptible to be infected by WNV, or infection rate was quite low to be detected with the number of specimens tested. However additional studies are needed in order to fully understand its role in the epidemiology of WNV.

In conclusion we have shown for the first time that *Cx. pipiens f. pipiens* collected in Portugal can become infected with and transmit WNV. By our results it was not possible to prove the infection susceptibility and vector competence of *An. atroparvus*, which suggest a minor role of this species in WNV epidemiology and ecology. Further study with larger sample sizes would further elucidate the most likely candidates for the maintenance of WNV in natural transmission cycles.

CHAPTER 5

DETECTION OF FLAVIVIRUSES IN MOSQUITOES VECTORS

5.1 DETECTION OF MOSQUITO-ONLY FLAVIVIRUSES IN EUROPE

RESEARCH ARTICLE

Calzolari, M., Zé-Zé, L., Růžek, D., Vázquez, A., Jeffries, C., Defilippo, F., Osório, H.C., Kilian, P., Ruíz, S., Fook, A.R., Maioli, G., Amaro, F., Tlustý, M., Figuerola, J., Medlock, J.M., Bonilauri, P., Alves, M.J., Šebesta, O., Tenorio, A., Vaux, A.G.C., Bellini, R., Gelbič, I., Sánchez-Seco, M.P., Johnson, N. & Dottori M. (2012) Detection of mosquito-only flaviviruses in Europe. *Journal of General Virology*, 93: 1215-25.

SUMMARY

The genus Flavivirus, family Flaviviridae, includes a number of important arthropod-transmitted human pathogens such as dengue viruses, West Nile virus, Japanese encephalitis virus and yellow fever virus. In addition, the genus includes flaviviruses without a known vertebrate reservoir, which have been detected only in insects, particularly in mosquitoes, such as cell fusing agent virus, Kamiti River virus, *Culex* flavivirus, *Aedes* flavivirus, Quang Binh virus, Nakiwogo virus and Calbertado virus. Reports of the detection of these viruses with no recognized pathogenic role in humans are increasing in mosquitoes collected around the world, particularly in those sampled in entomological surveys targeting pathogenic flaviviruses. The presence of six potential flaviviruses, detected from independent European arbovirus surveys undertaken in the Czech Republic, Italy, Portugal, Spain and the UK between 2007 and 2010, is reported in this work. Whilst the *Aedes* flaviviruses, detected in Italy from *Aedes albopictus* mosquitoes, had already been isolated in Japan, the remaining five viruses have not been reported previously: one was detected in Italy, Portugal and Spain from *Aedes* mosquitoes (particularly from *Aedes caspius*), one in Portugal and Spain from *Culex theileri* mosquitoes, one in the Czech Republic and Italy from *Aedes vexans*, one in the Czech Republic from *Aedes vexans* and the last in the UK from *Aedes cinereus*. Phylogenetic analysis confirmed the close relationship of these putative viruses to other insect-only flaviviruses.

INTRODUCTION

Diseases caused by arthropod-borne viruses (arboviruses) are increasingly being reported from all over the world (Weaver and Reisen 2010). Many pathogenic arboviruses are transmitted by mosquitoes and belong to the genus *Flavivirus* such as dengue viruses, West Nile virus (WNV), yellow fever virus, Japanese encephalitis virus. The emergence of these viruses in recent years has led many countries to develop targeted surveillance of mosquito populations which involves capturing of mosquitoes and detection of viral nucleic acid by molecular techniques. These surveys have led to reports of several sequences with close similarity to a group of flaviviruses associated exclusively with mosquitoes, named mosquito-only flaviviruses (MOFs).

This group includes cell fusing agent virus (CFAV), a virus isolated in 1975 from an *Aedes aegypti* cell line (Stollar and Thomas 1975) and other viruses directly isolated from field-collected mosquitoes, including Kamiti River virus (KRV) isolated from *Ae. macintoshi* collected as larvae and pupae in Kenya (Sang *et al.* 2003, Crabtree *et al.* 2003), *Culex* flavivirus (*Cx*FV) and *Aedes* flavivirus (*Ae*FV) derived from *Culex* and *Aedes* mosquitoes in Japan (Hoshino *et al.* 2007, Hoshino *et al.* 2009), Quang Binh virus from *Cx. tritaeniorhynchus* sampled in Vietnam (Crabtree *et al.* 2009), Nakiwogo virus from *Mansonia africana* caught in Uganda (Cook *et al.* 2009), Calbertado virus from *Cx. tarsalis* sampled in Canada and Northern America (Bolling *et al.* 2011, Tyler *et al.* 2011, Pabbaraju *et al.* 2009). A sequence detected in Thailand related to this virus group, is present in the GenBank database (Wang Thong virus, AY457040)**.** Further reports include one strain of CFAV isolated in Puerto Rico (Cook *et al.* 2006), one detected in Mexico (Espinoza-Gómez *et al.* 2011), and *Cx*FV strains from different *Culex* species sampled in Guatemala (Morales-Betoulle *et al.* 2008), Mexico (Farfan-Ale *et al.* 2009), Uganda (Cook *et al.* 2009), Iowa (Blitvich *et al.* 2009), Chicago (Newman *et al.* 2011), Trinidad and Texas (Kim *et al.* 2009), and Colorado (Bolling *et al.* 2011).

The MOFs have unique characteristics, and this group of viruses could represent a primordial form of flaviviruses with replication restricted to mosquitoes and unable to infect vertebrate cells (Cook and Holmes 2006, Cammisa-Parks *et al.* 1992). This has been demonstrated by the exclusive isolation of these viruses from insect cell culture (Bolling *et al.* 2011, Hoshino *et al.* 2007, Hoshino *et al.* 2009, Sang *et al.* 2003, Crabtree *et al.* 2003, Crabtree *et al.* 2009, Cook 2009, Kim *et al.* 2009). Furthermore, the numerous unsuccessful attempts to grow or to isolate these viruses in vertebrate cell cultures (Bolling *et al.* 2011, Hoshino *et al.* 2009, Morales-Betoulle *et al.* 2008, Crabtree *et al.* 2009, Hoshino *et al.* 2007, Crabtree *et al.* 2003, Sang *et al.* 2003, Stollar and Thomas 1975) suggest that MOFs are unable to replicate in

vertebrates and do not represent a health risk for animals. The lack of a vertebrate host differentiates MOFs from other flaviviruses and raises the question of how these viruses persist in the environment. Laboratory studies have demonstrated the ability of KRV to infect *Ae. aegypti* mosquitoes via the oral route (Lutomiah *et al.* 2007). Vertical transmission of different MOFs from adult mosquitoes to their offspring has also been reported (Cook *et al.* 2006, Lutomiah *et al.* 2007, Bolling *et al.*, 2011). In addition, MOFs detection in mosquito males and immature stages has been observed (Bolling *et al.* 2011, Farfan-Ale *et al.* 2009, Hoshino *et al.* 2009, Hoshino *et al.* 2007, Crabtree *et al.* 2003, Sang *et al.* 2003).

Much of the ecology of MOFs is still largely unknown and the scarcity of knowledge on the life-cycle and characteristics of these viruses highlights the need for further study. Moreover, the abundance of reports worldwide of MOFs highlights the ubiquity of these viruses in different mosquito species, and suggests that many of these viruses have yet to be discovered. In this study we have collated the finding of MOFs in five independent mosquito surveys conducted across Europe. Wetland locations were targeted in each survey due to high mosquito abundance in these areas. Using the sequence data derived from these studies, we have then derived a phylogeny for these viruses in the context of the genus *Flavivirus*.

RESULTS AND DISCUSSION

A total of 817,240 mosquitoes were pooled and tested in five independent surveys (Figure 23). The most abundant species tested were *Cx. pipiens*, in Italy and Portugal, *Ae. caspius*, in Spain, *Ae. vexans* in the Czech Republic and *Ae. cinereus* in UK. Other species abundantly sampled were *Ae. caspius* and *Cx. theileri* in Portugal, *Cx. modestus, Cx. theileri* and *Cx. pipiens* in Spain, *Ae. caspius*, *Ae. vexans*, *Anopheles maculipennis* s.l. and *Ae. albopictus* in Italy, *Ae. rossicus* in the Czech Republic, and *Coquellettidia richiardii* and *Culiseta annulata* in United Kingdom (Table 18). Flavivirus positive reverse transcription-polymerase chain reaction (RT-PCR) mosquito pools were reported in all countries (Figure 23).

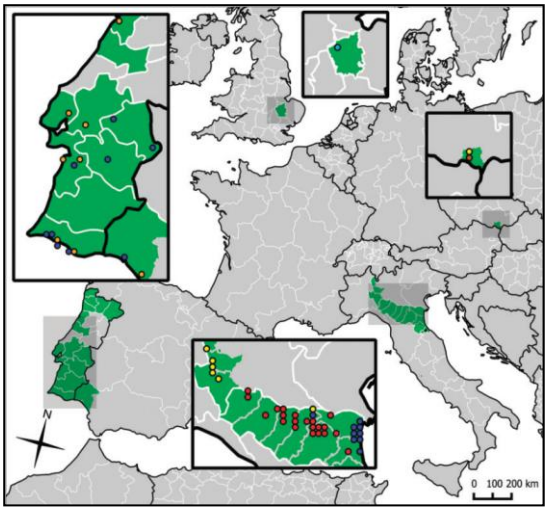

Figure 23. Location of surveyed areas (in green) and of positive detections in the surveys at 10 km resolution. Red, *Aedes* flavivirus; azure, *Aedes cinereus* flavivirus; yellow, *Aedes vexans* flavivirus (Italy and Czech Republic); brown, *Aedes vexans* flavivirus (Czech Republic); orange, *Culex theileri* flavivirus; blue, *Ochlerotatus* flavivirus.

Table 18. Total number of specimens (%) and pools collected for each mosquito species in the surveys in each country.

Mosquito species	Portugal 2007-2010		Spain 2007-2008		Italy 2007-2010		Czech Republic 2009		UK 2010	
	n (%)	Pools	n (%)	Pools	n (%)	Pools	n (%)	Pools	n (%)	Pools
Aedes albopictus					4219 (0.6)	353				
*Ae. cantans**									20 (8.5)	4
*Ae. caspius**	6625 (30.1)	166	23,927 (43.1)	1005	65,922 (9.0)	1086			10 (4.2)	2
Ae. cinereus					41 (<0.1)	15	150 (2.3)	3	96 (40.7)	20
*Ae. detritus**	462 (2.1)	16	830 (1.5)	155	79 (<0.1)	11				
*Ae. dorsalis**					13 (<0.1)	1				
*Ae. geniculatus**					674 (0.1)	28				
*Ae. punctor**					1 (<0.1)	1				
Ae. rossicus							800 (12.7)	16		
*Ae. sticticus**							500 (7.9)	10		
Ae. vexans					40,265 (5.5)	462	4200 (66.7)	84		
Aedes spp.									5 (2.1)	1
Anopheles algeriensis	278 (1.3)	7	129 (0.2)	40						
An. claviger	38 (0.2)	2							25 (10.6)	5
An. maculipennis s.l.	101 (0.5)	15	167 (0.3)	79	4510 (0.6)	122				
An. plumbeus			1 (<0.1)	1		12				
Anopheles spp.	27 (0.1)	1				10				
Coquillettidia richiardii	13 (<0.1)	3	1 (<0.1)	1		4			45 (19.1)	5
Culex modestus			9695 (17.4)	381	2471 (0.3)	90	350 (5.6)	7		
Cx. mimeticus	6 (<0.1)	1								
Cx. perexiguus	507 (2.3)	38	2330 (4.2)	237						
Cx. pipiens	10,098 (45.9)	322	8563 (15.4)	787	614,652 (83.8)	4300	300 (4.8)	6		
Cx. theileri	3759 (17.1)	117	9537 (17.2)	441						
Culex spp.			16 (<0.1)	5	15 (<0.1)	3				
Culiseta annulata	17 (<0.1)	5	119 (0.2)	64	4 (<0.1)	4			35 (14.8)	7
Cs. longiareolata	42 (0.2)	13	242 (0.4)	87						
Culiseta spp.			0 (0.0)		3 (<0.1)	3				
Uranotaenia unguiculata	13 (<0.1)	1	5 (<0.1)	4						
Total	21,986	707	55,562	3287	733,154	6505	6,300	126	236	47

*According to Savage and Strickman (2004), *Ochlerotatus* taxon was considered to be a subgenus of the genus *Aedes*.

The presence of surveyed flaviviruses was detected in Italian mosquitoes, with 29 West Nile virus-positive pools and 56 Usutu virus (USUV)-positive pools (Calzolari *et al.* 2010[a,b]). Moreover, three groups of sequences similar to MOFs were detected in Italy. One was detected in 32 pools of *Ae. albopictus* mosquitoes and showed a high identity with the sequence of *Ae*FV (GenBank ID: AB488408) isolated in Japan in 2009; the other two virus sequences had lower identity with already reported MOFs: one was detected in eight *Ae.*

caspius pools and in one *Cx. pipiens* pool, and the third sequence was detected in eight *Ae. vexans* pools. The sequence detections obtained in Italy from 2007 to 2009 have been reported previously (Calzolari *et al.* 2010[a,b]). In Portugal, two groups of sequences similar to MOFs were detected, one in *Culex* and *Aedes* species (21 sequences); and the second in *Cx. theileri* (11 sequences) mosquitoes, and were related to an insect flavivirus sequence detected in *Cx. fuscocephala* in Thailand (Wang Thong virus, GenBank ID: AY457040).

In Spain, two sequence groups were also detected in mosquitoes sampled, one from *Cx. theileri* mosquito (four sequences) and one from *Ae. caspius* mosquito (three sequences). West Nile virus and USUV were detected previously in *Cx. perexiguus* mosquitoes in 2008 and 2009, respectively, in the same geographic area (south-West Spain) but a different site (Vázquez *et al.* 2011[a,b]). In the Czech Republic two sequence types were detected (with two and three sequences), all in *Ae. vexans* mosquito pools. Finally, all the sequences derived from flavivirus-positive *Ae. cinereus* mosquito pools from the UK were identical (Figure 23; Table 19). The BLAST search performed in the GenBank database with the obtained amplicon sequences showed the highest identity rates with the MOF group (Figure 24).

Table 19. Characteristics of the reported MOFs with the reference to the mosquito species, number, period, area and environment of detection.

Virus	Mosquito species	Detections (GenBank)	Collection period	Collection years	Country	Collection area	Environment
Aedes Flavivirus	*Ae. albopictus*	32 (29)*	Jun- Oct	2008-2010	Italy	Pianura Padana	Floodplain
Ochlerotatus Flavivirus	*Ae. caspius* *Ae. detritus* *Cx. pipiens*	33 (22)†	Mar-Octo	2007-2010	Italy, Portugal, Spain	Lidi Ferraresi, Algarve, Alentejo, Andalusian	Inland and tidal wetland
Ae. vexans Flavivirus	*Ae. vexans*	9 (9)‡	Jul-Nov	2008-2009	Czech Republic, Italy	Pianura Padana, South Moravia	Inland wetland
Czech *Ae. vexans* Flavivirus	*Ae. vexans*	4 (4)§	Aug	2009	Czech Republic	South Moravia	Pond
Culex theileri Flavivirus	*Cx. theileri*	15 (9)#	Mar-Oct	2007-2010	Portugal, Spain	Alentejo, Algarve, Centro, Ribatejo Andalusian	Inland and tidal wetland
Ae. cinereus Flavivirus	*Ae. cinereus*	17||	Jun- Jul	2010	United Kingdom	Cambridgeshire	Inland wetland

* I: GQ477009, GQ477006, GQ477005, GQ477004, GQ477011, GQ477007, GQ477012, GQ477008, GQ477010, HQ441846, HQ441847, HQ441848, HQ441849, HQ441850, HQ441851, HQ441852, HQ441853, HQ441854, HQ441855, HQ441856, HQ441857, HQ441858, HQ441859, HQ441860, HQ441861, HQ441862, HQ441863, HQ441864, HQ441865.

† I:GQ476994, GQ476993, GQ476995, GQ476991, GQ476992, HQ441842, HQ441843, HQ441844, HQ441845; P: EU716415, EU716416, EU716417, EU716418, EU716419, EU716421, EU716422, EU716423, EU716424, HQ676618; SP: HU6086, HU4503, HU6404.

‡ I: GQ476996, GQ477001, GQ476997, GQ476998, GQ476999, GQ477000, GQ477002, GQ477003; CZ: JN802280.

§ CZ: JN802279, JN802281, JN802282 JN802283.

P: EU716420, HQ676619, HQ676620, HQ676621, HQ676623; SP: HU4212, HU4301, HU5659, HU5910.

|| Sequences availlable on request.

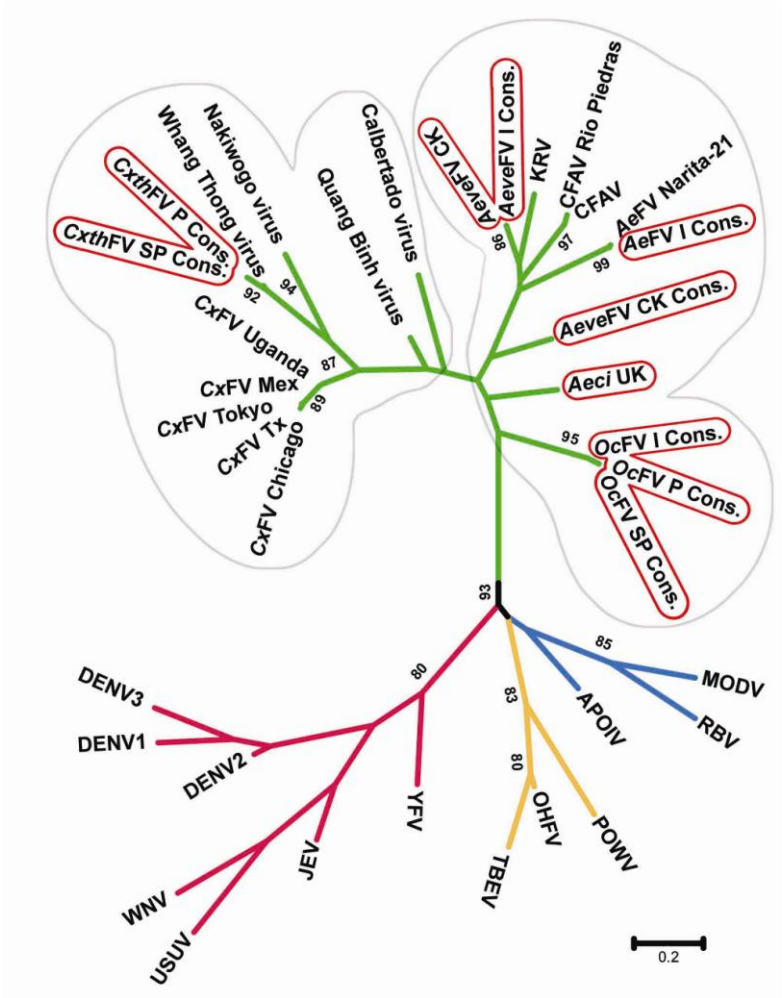

Figure 24. Molecular phylogenetic analysis using the maximum-likelihood method (Kimura two-parameter model) of the MOF sequences reported in this work (part of the *NS5* gene) and homologous GenBank sequences of other flaviviruses. The sequences from this work are circled in red; in grey are highlighted the *Aedes* and *Culex*-associated MOF sequences, on the right and left, respectively. The tree with the highest log likelihood (-2920.7) is shown. An initial tree for the heuristic search was obtained automatically by the maximum-parsimony method. A discrete γ distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter51.1014)]. The rate variation model allowed some sites to be evolutionarily invariable (+I0, 23.8% sites). The tree is drawn to scale, and bootstrap values (1000 replicate) of ¢80% are shown. Bar, number of substitutions per site. The analysis involved 36 nt sequences. There were a total of 155 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). Branches: green, mosquito-borne insect-only flaviviruses; blue, no-known vector; yellow, tick-borne flaviviruses; pink, mosquitoborne flaviviruses that can infect vertebrates. Abbreviations: CK, Czech Republic; I, Italy; P, Portugal; SP, Spain; Cons., consensus sequence.; *Ae*FV: *Aedes* Flavivirus, *Aeci*FV: *Aedes cinereus* Flavivirus, *Aeve*FV: *Aedes vexans* Flavivirus, *Cxth*FV: *Culex theileri* Flavivirus, *Oc*FV: *Ochlerotatus* Flavivirus, CK: Czech Republic, I: Italy, P: Portugal, SP: Spain, UK: United Kingdom, cons: consensus sequence; GenBank sequences (GenBank ID): *Ae*FV: *Aedes* flavivirus (AB488408), APOIV: Apoi virus (NC003676), Calberdado virus (EU569288), CFAV: cell fusing agent (NC001564, Rio Piedras: EU074056), *Cx*FV: *Culex* flavivirus (Tokyo: AB262759, Mex: EU879060, Tx: FJ502995, Chicago: GU990232, Uganda: GQ165808), DENV: Dengue virus (1: U88536, 2: NC001474, 3: NC001475), JEV: Japanese encephalitis virus (GQ902063), KRV: Kamiti River virus (AY149905), MODV: Modoc virus (AJ242984), Nakiwogo virus (GQ165809), OHFV: Omsk hemorrhagic fever virus (NC005062), POWV: Powassan virus (NC003687), Quang Binh virus (FJ644291), RBV: Rio bravo virus (NC003675), TBEV: Tick-borne encephalitis virus (DQ401140), USUV: Usutu virus (HM138707), Whang Thong virus (AY457040), WNV: West Nile virus (GU047875), YFV: Yellow fever virus (U54798).

The described sequence groups showed a high degree of identity within each group (Table 20). In some cases, variation detected in these viral sequences (even taking into consideration the small size of the amplified *NS5* sequence) appears to be very low; for example the 21 amplicons obtained from *Culex* and *Aedes* mosquitoes in Portugal were identical except for three polymorphisms in two sequences (PoMoFlavA95 and PoMoFlavR376, GenBank acession: EU716416 and HQ676618, respectively), particularly considering that the positive mosquito pools belong to two different genera and five species (*Cx. pipiens*, *Cx. theileri*, *Cx. perexiguus*, *Ae. caspius* and *Ae. detritus*).

Table 20. Number of differences and percentage identity (bold) in sequences detected in each country (a) and between consensus sequences from the different surveys (b) (based on 155 bp).

a)

Virus	Country	No. sequences	Identity (%)	No. diff.	Mean diff.
OcFV	I	8	100–96.8	0-5	1.86
	P	10	100–99.4	0-1	0.36
	SP	3	100–99.4	0-1	0.67
AeFV	I	32	100–98.1	0-3	0.83
AeveFV	I	6	100	0	-
	CZ	1	-	-	-
CxthFV	P	5	100–98.1	0-3	1
	SP	4	100–97.4	0-4	2
Czech AeveFV	CZ	3	100–97.4	1-4	2.67
Aedes cinereus FV	UK	17	100	0	-

The short sequences HQ441855, HQ441843, GQ477002 and GQ477003 were excluded from the analysis. I, Italy; P, Portugal; SP, Spain; CZ, Czech Republic; No. diff, number of differences in sequence; Mean diff., mean of differences for sequence.

b)

Virus		OcFV			AeFV	AeveFV		CxFV		Czech AeveFV	AeciFV
		I	P	SP	I	I	CZ	P	SP	CZ	UK
OcFV	I		**96.8**	**97.4**	**63.2**	**66.5**	**66.5**	**66.5**	**66.5**	**73.5**	**74.2**
	P	5		**99.4**	**61.9**	**67.1**	**67.1**	**67.1**	**67.1**	**72.3**	**74.2**
	SP	4	1		**61.3**	**66.5**	**66.5**	**66.5**	**66.5**	**71.6**	**73.5**
AeFV	I	57	59	60		**78.1**	**78.1**	**69.7**	**69.7**	**73.3**	**73.5**
AeveFV	I	52	51	52	34		**100**	**67.7**	**67.7**	**72.3**	**72.9**
	CZ	52	51	52	34	0		**67.7**	**67.7**	**72.3**	**72.9**
CxthFV	P	52	51	52	47	50	50		**100**	**65.2**	**66.5**
	SP	52	51	52	47	50	50	0		**65.2**	**66.6**
Czech AeveFV	CZ	40	40	41	41	42	42	52	52		**75.5**
AeciFV	UK	41	43	44	43	43	43	54	54	38	

Unexpectedly, several groups of sequences from different countries showed high identity and grouped closely within the phylogenetic analysis (Figure 24). In this analysis, the consensus sequences obtained from the different countries were compared with those available in the GenBank, and representative flavivirus sequences were used. The new sequences grouped with previously described MOFs diverge from other known flaviviruses, and were placed on six branches in the obtained tree, suggesting the presence of different MOFs in Europe (Figure 24). One sequence detected in Italy, Portugal and Spain, mainly from *Aedes* mosquitoes, was termed *Ochlerotatus* Flavivirus (*Oc*FV) in this study. A sequence detected in Portugal and Spain mainly from *Cx. theileri* mosquitoes, was termed *Culex theileri* Flavivirus (*Cxth*FV). A sequence detected in Italy from *Ae. albopictus* mosquitoes revealed high identity with the previously reported *Aedes* Flavivirus. One sequence detected in the Czech Republic and Italy from *Ae. vexans* mosquitoes, was termed *Aedes vexans* Flavivirus (*Aeve*FV). A second sequence detected in the Czech Republic was derived from *Ae. vexans* mosquitoes. Finally, one sequence detected in the UK from *Ae. cinereus* mosquitoes showed little identity with other MOFs and formed its own branch within the phylogenetic analysis (Figure 24; Table 20). As different protocols were utilized (Tabel 24), a portion of 155 base pairs of obtained sequences was aligned, producing a tree consistent with those obtained in other phylogenetic studies with longer sequences (Cook *et al.* 2011); this tree showed divergence between *Aedes*- and *Culex*-associated sequences (Figure 24), although this is weakly supported by bootstrap values. This divergence has been reported by Cook *et al.* (2012).

Integration of flavivirus sequences into the mosquito genome has been reported (Crochu *et al.* 2004, Katzourakis and Gifford 2010). To exclude the possibility that detected sequences were the result of integration into the mosquito genome, the nature of the sequences obtained was investigated. In the Portuguese study, total RNA extracts from most of the positive mosquito samples were submitted to specific PCR amplification without reverse-transcription. Specific amplification of total DNA extracts was also performed. No amplification was achieved using the same reaction conditions but omitting the reverse transcription step, or directly from total DNA extracts of mosquito macerates. In the Italian study the flavivirus-PCR was applied to total DNA extracted from two positive *Ae. albopictus* mosquito pools sampled in 2010 without any positive results.

In the Spanish study, samples prepared from positive pools were treated with RNase A before amplification and then were directly amplified without the previous reverse transcription step (Sánchez-Seco *et al.* 2010). RNase treatment resulted in a failure to amplify a flavivirus product, suggesting that the sequences obtained were most likely derived from RNA, probably of viral origin. In the Czech Republic samples the same result was obtained even

when the PCR was performed without reverse transcriptase, or despite RNase treatment, indicating the presence of the detected sequences in DNA-form. All samples were subsequently subjected to DNase treatment, which resulted in the confirmation that, in at least one sample, the detected sequence was in RNA-form only. These results did not indicate the presence of an integrated flaviviral sequence in a mosquito genome, as evidence of a reverse transcriptase mechanism was provided by CFAV infection in C6/C36 cell line (Cook *et al.* 2006, 2009), a surprising finding for a RNA virus that might replicate with a DNA intermediate.

For most of the PCR-positive mosquito pools, virus isolation in cell culture was attempted by inoculation of pooled or individual mosquito macerates in C6/36 cells and vertebrate cell lines (Table 21). Although, in this study, cell culture for several RT-PCR positive mosquito homogenates was attempted, the isolation procedures were unsuccessful. However, one MOF, related to *Oc*FV, detected in one mosquito pool collected in the southern region of Portugal in 2006, has previously been isolated in C6/36 cells (M. Niedrig, personal communication). The isolation of these viruses was also successful in Spain. Two detected MOFs were isolated in C6/36 cells from mosquitoes captured in 2002 and 2006. In both cases, the virus isolated developed a moderate cytopathic effect (CPE) and cell aggregation at 5-7 days post infection (Vasquez *et al.* 2011[b]).

These findings demonstrated the widespread presence of different MOFs in Europe, related to other MOFs isolated worldwide. However, there are a number of difficulties in characterization of MOFs, which can be challenging, particularly the problem of their isolation in cell cultures: the MOF CPE can be weak (Hoshino *et al.* 2009) or strain-dependent (Kim *et al.* 2009), or only visible after a number of blind passages. In addition, virus can go undetected by RT-PCR in cell culture medium during early passage after inoculation (Bolling *et al.* 2011).

The high sequence identity of the virus detected in Italian *Ae. albopictus* mosquitoes, previously detected in other studies in Italy (Roiz *et al.* 2009, Calzolari *et al* 2010[a]), compared with the *Aedes* flavivirus isolated in Japan strongly suggested that these two viruses are closely related. Japan is the probable origin of this mosquito's recent expansion to North America and Europe (Hawley *et al.* 1987, Rai 1991); thus it seems likely that the mosquitoes also brought the *Ae*FV with them. A further observation was that the mosquitoes from which other virus sequences were detected were collected in wetland ecosystems in the different countries (Table 18), although other environments were monitored in some surveys. These observations could be explained by the presence of environmental factors favoring MOF persistence, or by a MOF influence on the bionomic features of infected mosquitoes, as has been described for carbon dioxide sensitivity induced in mosquitoes by different viruses (Shroyer and Rosen 1983,

Vazeille *et al.* 1988); these influences could enhance mosquito adaptation to a particular ecosystem. Further experimental investigations will be required to support this hypothesis.

The widespread distribution of MOFs suggests their potential as a tool to prevent the transmission of pathogenic flaviviruses due to superinfection phenomena, as previously suggested (Blitvich *et al.* 2009, Crabtree *et al.* 2009). Alternatively, the enhancement of WNV transmission in mosquitoes inoculated simultaneously with *Cx*FV Izabal was reported in Honduras (Kent *et al.* 2010), indicating that the consequences of co-infection are not clear and require elucidation. Moreover, a positive association between *Cx*FV and WNV was reported in field collected mosquitoes (Newman *et al.* 2011), and in the 2009 Italian survey (Calzolari *et al.* 2010b), two *Ae. albopictus* pools sampled at the same site and week were positive for *Ae*FV and for Usutu virus, testifying persistence of both viruses in the environment.

Further studies are ongoing to achieve viral isolation of these flaviviruses through cell culture using mosquito cell lines to confirm the presence of viable viruses in these samples and to follow up their genetic characterization. This will involve evaluation of their potential to prevent or enhance the transmission of other pathogenic flaviviruses during co-infection.

The reported data suggests that MOFs have a broader geographical range in Europe than previously considered, with the probability that MOFs may exist in natural mosquito populations throughout the world.

MATERIALS AND METHODS

Survey areas.

In Italy, the survey area was the northern part of Emilia-Romagna, bounded on the north by the river Po and east by the Adriatic Sea, and a 91,000 ha Regional Natural Park sited in Lombardia region, `Parco Lombardo della Valle del Ticino´, that protects the Italian stretch of Ticino River. All these areas were in Pianura Padana, the most important Italian plain, characterized by an intensive agriculture and animal husbandry with scarce natural sites. All the monitored territories are densely populated and characterized by the abundant presence of villages, city and industrial areas (Figure 23).

In Portugal, in 2007, only the Algarve region in the south was surveyed. From 2008 the survey area was enlarged to cover most of the country. A wide range of ecosystems was surveyed from rural and urban habitats, including some airports and sea ports. In Algarve and North coastal areas, study sites were coastal wetlands rich in avifauna and show a multitude of habitats characterized by marshlands, salt-marshes, small islands, dunes and beaches. Fishery, aquaculture and salt-works as well as farming areas with several hectares of rice-fields and

reed plantation were the most important human activities, but industrial complexes related to the fishing industry were also found (Figure 23).

In the Czech Republic, the study was conducted at five sites in south-eastern Moravia in 2009. The localities included a lowland forest, a shore of a pond and a farmhouse. All these localities are characterized by high mosquito abundance. This region is characterized by a relatively warm and dry climate, and the surveyed area is endemic for several mosquito borne human pathogens, including West Nile virus (Hubálek *et al.* 2010) (Figure 23).

In Spain, mosquitoes were captured in the Guadalquivir marshes and adjoining wetlands, in the south-west of Spain and very near to Algarve in Portugal. Study areas were tidal marshes, freshwater marshes, coastal dunes and paddy fields in natural areas of the National Park of Doñana and Odiel Marshes Natural Park, with high diversity and abundance of sedentary and migratory birds, particularly waterfowl, herons, waders and gulls (Figure 23). In recent years, WNV has been repeatedly circulating in that area (Figuerola *et al.* 2007).

In the UK, all mosquitoes analysed were collected at Woodwalton Fen, a wetland area in Cambridgeshire. Habitats included flooded grasslands, reed-bed swamp habitat, as well as wet woodland and ditch/groundwater fed fen (Figure 23).

Mosquito collections.

Different traps baited with carbon dioxide were utilized for sampling mosquitoes (Table 21). All trap sites were georeferenced and worked at night from approximately 16:00 pm to 9:00 am.

Traps worked over different periods of the year in the different nations: from June to October in Italy and Portugal, from the beginning of April until the end of October 2009 in the Czech Republic, from March until the end of October in Spain and from April to October 2010 in the UK. Mosquitoes were identified to species level using morphological characteristics according to classification keys (Stojanovich and Scott 1997, Ribeiro and Ramos 1999, Schaffner *et al.* 2001, Becker *et al.* 2010, Severini *et al.* 2009, Kramář 1958, Encinas Grandes 1982, Snow 1990). *Ochlerotatus* taxon was considered as an *Aedes* sub-genus (Savage and Strickman 2004). Female mosquitoes were pooled according to date, location and species, with a maximum number of 10-200 individuals per pool.

The pooled mosquitoes were stored in tubes and frozen at -80°C. Specimens were macerated by different methods: in liquid nitrogen or at room temperature, by mechanical methods or by addition of medium and metallic beads and shaking the samples (Table 21). The homogenate was clarified by centrifugation; finally, aliquots were collected from ground samples and submitted to biomolecular analysis.

Virus survey.

RNA present in aliquots was extracted using different commercial products according to the manufacturer's instructions (Table 21). Samples were analyzed using different polymerase chain reactions protocol, targeted to a *NS5* gene fragment of flaviviruses (Briese *et al.* 1999, 2002; Johnson *et al.* 2010; Scaramozzino *et al.* 2001; Vázquez *et al.* 2011[c]).

PCR were performed on cDNA obtained by a reverse-transcription or by one-step PCR commercial kits according to the manufacturer's instructions (Table 21). Fragments obtained by *Flavivirus* genus PCR were sequenced by an automated fluorescence-based technique following the manufacturer's instructions. If necessary, a cloning passage was performed to obtain better quality sequences.

The obtained sequences were used to perform basic local alignment searches (BLAST) in the GenBank library to confirm the specificity of positive reaction and to estimate the degree of identity of detected strains (Altschul *et al.* 1997). The sequences obtained were aligned with available GenBank sequences and molecular phylogenetic analysis was performed by the maximum-likelihood method using MEGA5 program (Tamura *et al.* 2011). The Kimura two-parameter model was chosen between 24 different nucleotide substitution models for the lowest Bayesian information criterion value. The analysis involved 36 nucleotide sequences and a final dataset of 155 positions was utilized, eliminating all positions containing gaps and missing data (Figure 24).

Virus isolation was attempted starting from the remaining part of PCR positive mosquito homogenates using the C6/36 cell line (Igarashi *et al.* 1978) incubated at 28°C, or adapted to 33°C in Spain, and other vertebrate cell lines incubated at 37°C (Table 21).

Table 21. Materials and protocols used in the different surveys

Method	Italy	Portugal	Czechs Republic	Spain	UK
Traps	CDC modified traps (no light) CO_2baited (self produced)	CDC light traps CO_2 baited	CDC light traps CO_2 baited (BioQuip Products)	CDC light traps CO_2 baited (self produced)	Mosquito Magnet® baited by CO_2, heat, and Octenol attractant
Identification	Stojanovich & Scott 1997, Schaffner *et al.* 2001, Becker *et al.* 2010 Severini *et al.* 2009	Ribeiro & Ramos 1999, Schaffner *et al.* 2001	Becker *et al.* 2010 Kramář, 1958	Encinas Grandes 1982, Schaffner *et al.* 2001, Becker *et al.* 2010	Snow 1990, Schaffner *et al.* 2001
Maximum no. mosquitoes per pool	200 (Sutherland & Nasci 2007)	50	50	50	10
Storage	-80 °C polypropylene cryotube	-80 °C polypropylene cryotube	-80 °C glass tube	-80 °C polypropylene cryotube	-80 °C polypropylene cryotube
Grinding	Copper plated round balls and vortexing	Liquid nitrogen	TissueLyser II (Qiagen)	-	Scissors
Grinding medium	PBS	Minimal essential medium supplied with 10% SFB, streptomycin (0.1µg/ml) and fungizone (1 µg/ml)	PBS	Minimal essential medium supplied with 10% SFB	-
Centrifugation	4000 *g* for 3 min	12000 *g* for 2 min	14000 g for 10 min	10500 g for 5 min	-
RNA extraction	Trizol® LS Reagent (Invitrogen)	PureLink™ RNA Mini kit (Ambion)	Viral RNA Mini Kit (Qiagen)	Viral RNA Mini Kit (Qiagen)	MELT™ kit (Ambion) and Kingfisher 96 extraction robot (ThermoElectron)
DNA extraction	DNeasy® Kit (Qiagen)	-	-	-	-
Reverse transcription and PCR or one step RT-PCR	Random hexamer (Roche Diagnostics) and SuperScript® II	SuperScript™ One-Step RT-PCR (Invitrogen)	Titan One Tube RT-PCR Kit (Roche)	One Step RT-PCR (Qiagen)	Random hexamers (Invitrogen) reverse transcriptase (Promega)
PCR protocols	Scaramozzino *et al.* 2001	Briese *et al.* 1999, 2002	Scaramozzino *et al.* 2001	Vázquez *et al.* 2011b	Johnson *et al.* 2010
Purification	-	JETquick PCR Product Purification Spin Kit (Genomed)	Illustra GFX PCR and Gel Band Purification Kit (GE Healthcare)	Gel Extraction Kit (Qiagen) or using QIAquick PCR Purification Kit (Qiagen)	QIAquick gel extraction kit (Qiagen).
Cloning	-	-	CloneJet PCR Clonning Kit (Fermentas)	-	-
Sequencing	ABI-PRISM 3130 Genetic Analyzer (Applied Biosystems)	ABI PRISM 3130 Genetic Analyzer (Applied Biosystems)	ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems)	ABI PRISM 377 automated sequencer (Applied Biosystems)	ABI PRISM 3100 Genetic Analyzer (Applied Biosystems)
Isolation	C6/C36 cells	C6/36 and Vero E6 cells	C6/C36 cells	C6/36 cells, Vero or BHK-21	-

CHAPTER 6

DETECTION OF FLAVIVIRUSES ANTIBODIES IN BIRDS

6.1 SEROLOGICAL SURVEY FOR WEST NILE VIRUS IN WILD BIRDS IN PORTUGAL, 2004-2007

RESEARCH ARTICLE

Osório, H.C., Oliveira, A.L., Luz, T., Zé-Zé, L., Santos, N., Fevereiro, M. & Alves, M.J. (2012) Serological survey for West Nile Virus in wild birds in Portugal, 2004-2007. Submitted.

ABSTRACT

In this study we performed a retrospective serological survey of West Nile virus (WNV) in wild birds. A total of 612 avian serum samples from 29 species collected from 2004 to 2007 in Portugal were tested by ELISA. ELISA-positive serum samples were further tested for WNV neutralizing antibodies using micro virus-neutralization test (VNT) with WNV-Eg101 strain. Ten birds (1.6%) from three species (mallard, turtle dove and common blackbird) were positive by ELISA tests. Of these, one juvenile turtle dove collected in 2004 had high titer (1:256) neutralizing antibodies to WNV, which suggests local infection and viral circulation during 2004, when two linked human cases of WNV infection have occurred. Portugal has favourable ecological conditions for WNV re-emergence and turtle doves may play a role as bird hosts and should be considered as a target species in bird surveillance programs.

INTRODUCTION

West Nile virus (Flavivirus, Flaviviridae) is by far the most widely distributed arbovirus and currently is recognized as Europe's most important mosquito borne virus (Reiter, 2010[a]). In nature West Nile virus (WNV) is maintained in a cycle between birds and bird-feeding mosquitoes. Although many different species of mosquito are capable of upholding this cycle, the *Culex* species, *Cx. pipiens* and *Cx. modestus* in Europe, are recognized to play the largest role in natural transmission (Calistri *et al.*, 2010). Not all infected mosquitoes preferentially feed upon birds, which can lead to WNV transmission to other animals including humans. Humans (and horses) are incidental or "dead-end" hosts because viraemia is generally too low to infect mosquitoes (Rossi *et al.*, 2010). Birds have been implicated in spreading WNV during migratory events in Europe, Asia, Africa and the Middle East (Hubálek and Halouzka, 1999; Komar, 2003). In recent years outbreaks of WNV have been reported in several Mediterranean countries, with Greece reporting human cases in two consecutive years (Danis *et al.*, 2011[b]). In Portugal, WNV was demonstrated to be circulating in epizootic transmission since 1966/1967 (Filipe and Pinto, 1969; Filipe, 1974; Esteves *et al.*, 2005; Parreira *et al.*, 2007) and was first isolated in 1969 from *Anopheles maculipennis* (Filipe, 1972). No clinical cases were reported until the summer of 2004 when two tourists acquired WNV disease in the Southern province of Algarve (Connell *et al.*, 2004), after which WNV was detected in mosquitoes collected in the same region (Esteves *et al.*, 2005). Since then, positive serology has been detected every year in birds from zoological parks and horses in related serological studies (Barros *et al.*, 2011). In this study we report the results of a retrospective serological study for antibodies to WNV in several wild birds collected from 2004 to 2007 in Portugal in order to evaluate epizootic circulation of the virus during and after the human cases occurred in 2004 and to identify the bird species potentially involved in the introduction, amplification and spread of WNV.

METHODS

Birds were trapped for blood sampling using mist nets (operating from sunrise to sunset), captured in the nest, or trapped in baited traps (in the case on Anatidae) from several regions of Portugal. All birds, healthy at the time of sampling, were identified, aged, sexed, and released after the blood collection (Hoysak and Weatherhead, 1991). For each individual, a blood sample was drawn from the jugular or brachial vein (in small passerines by puncturing with miniature syringe needle and always less than 1% of body mass). The collected blood was transferred to a vial, or alternatively a Whatman paper card with a liquid retention of about 100 µL was imbibed and kept at ambient temperature (15-25ºC) until completely dry. Vials

with blood were centrifuged at 6000 rpm in an Eppendorf Mini spin centrifuge and paper cards were immersed in 1mL PBS overnight and also centrifuged to separate serum from blood clots. The sera were frozen at -80ºC until subsequent analysis.

Sera were firstly screened at 1:10 dilution for antibodies to flaviviruses by using a commercial competition Enzyme-linked Immunosorbent-assay (competition ELISA kit [IgM], ID-VET, Montpellier, France), according to manufacture instructions and an in-house indirect epitope blocking ELISA (Eb-ELISA) with monoclonal antibody MAb 3.1112G, according to Blitvich *et al.* (2003). Regarding the commercial competition ELISA from ID-VET the samples and controls to be tested were added to the microwells of a 96-well microplate coated with a purified extract of WNV. The WNV antibodies, if present in samples, form an antigen-antibody complex bounding to a pre-membrane protein (pr-E) of WNV. An anti-pr-E antibody peroxidase conjugate is added to the wells and it fixes to the remaining free pr-E-epitopes, forming an antigen-conjugate-peroxidase complex. A substrate solution is added and the resulting coloration depends on the quantity of specific antibodies present in the sample to be tested.

As regards Eb-ELISA, the inner of 96-well microtiter plates (Maxisorp Nunc) were coated with 100 μL of antigen prepared from infected cell monolayers diluted in carbonate-bicarbonate buffer (50 mM sodium carbonate, pH 9.6). Coated plates were incubated overnight at 4ºC. After blocking buffer (phosphate-buffered saline containing 5% skim milk) incubation, 50 μL of serum diluted 1:10 was added to each well and incubated for 2 h at 37ºC. Monoclonal antibody (MAb) 3.1112G (Chemicon International, cat. Nº MAB8152) was diluted in blocking buffer, added to each well and incubated for 1 h at 37ºC. The plates were washed with PBS 0.1% Tween 20 and the horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zimed Laboratories Inc, cat. Nº 61-6520) was added to each well and again incubated for 1 h at 37ºC. Equal volumes of ABTS (2,2'azino-bis[3-ethyl-benzthiazoline-6-sulfonic acid]) and peroxidase solutions from the ABTS Microwell peroxidase substrate system (Roche, ref. 1684302) were mixed, and added to each well. The optical density was determined with an automated plate reader (Bio-Rad Coda Automated EIA Analyzer – model 1575) at a wavelength of 405 nm. The percent inhibition of MAb binding was calculated. An inhibition value $\geq$ 30% was considered to indicate the presence of viral antibodies.

ELISA-positive sera were then tested for neutralizing antibodies using a micro virus-neutralization test (VNT) to WNV. These tests originally proposed by De Madrid and Porterfield (1969, 1974) and adapted by Hubálek *et al.* (1979) were conducted in the National Laboratory of Veterinary Research using WNV-Eg101 strain and performed as described by Barros *et al.* (2011). Briefly, replicates of twofold dilutions of heat inactivated serum samples were incubated with a constant amount (100 $CCID_{50}$) of WNV-Eg101 strain at 37ºC-5% CO_2, for 1 h in

96-well microplates. Vero E6 cells were added to each well and the plates were further incubated for 4 days. The wells were stained with crystal violet stain. Sera reactive with virus revealing no cytopathic effect at the 1:10 dilution at screening (corresponding to the 1:20 final dilution of the serum after mixing with the virus test dose), were considered positive (reciprocal titer ≥20). End points of serum replicates were then calculated using the Spearman-Kärber method (Käber, 1931). Controls included the virus test dose and its titration, positive and negative WNV reference sera (ID-VET, Montepellier, France; Vector Laboratories, Burlingame, CA, USA), and cells without virus for every sample at a dilution of 1:10 (control for cytotoxicity).

RESULTS

In total 612 wild birds belonging to 29 species were trapped and sampled (Table 22). Our results showed that ten examined serum samples (1.6%), representing three species of birds, mallard (N = 3), turtle dove (N = 4) and common blackbird (N = 3), were WNV positive as determined by the competition ELISA and/ or Eb-ELISA (Table 22 and 23).

Table 22. Species and number of wild birds (N) tested by ELISA (*Pos*- positive results) captured in Portugal from 2004 to 2007.

Common name (Species name)	2004	2005	2006	2007	TOTAL
	N/ Pos	N/ Pos	N/ Pos	N/ Pos	N/ Pos
Azure-winged Magpie (*Cyanopica cyanus*)			2/ 0		2/ 0
Bar-tailed godwit (*Limosa lapponica*)			3/ 0		3/ 0
Black Stork (*Ciconia nigra*)	14/ 0	19/ 0		1/ 0	34/ 0
Blue-and-white Mockingbird (*Melanotis hypoleucus*)			1/ 0		1/ 0
Cattle Egret (*Bubulcus ibis*)			1/ 0		1/ 0
Common Blackbird (*Turdus merula*)		26/ 0	28/ 3		54/ 3
Common Greenshank (*Tringa nebularia*)			5/ 0		5/ 0
Common Redshank (*Tringa totanus*)			20/ 0		20/ 0
Common Snipe (*Gallinago gallinago*)			8/ 0		8/ 0
Common Teal (*Anas crecca*)		9/ 0	16/ 0		25/ 0
Dunlin (*Calidris alpina*)			6/ 0		6/ 0
Grey Plover (*Pluvialis squatarola*)			9/ 0		9/ 0
House Sparrow (*Passer domesticus*)			3/ 0		3/ 0
Little Tern (*Sterna albifrons*)			1/ 0		1/ 0
Mallard (*Anas platyrhynchos*)		170/ 3	36/ 0	23/ 0	229/ 3
Nightingale (*Acrocephalus scirpaceus*)			1/ 0		1/ 0
Northern Shoveller (*Anas clypeata*)		2/ 0			2/ 0
Pied Avocet (*Recurvirostra avosetta*)			1/ 0		1/ 0
Red Knot (*Calidris canutus)*			3/ 0		3/ 0
Ringed Plover (*Charadrius hiaticula*)			4/ 0		4/ 0
Ruddy Turnstone (*Arenaria interpres*)			4/ 0		4/ 0
Ruff (*Philomachus pugnax*)			1/ 0		1/ 0
Song Thrush (*Turdus philomelos*)	20/ 0	24/ 0			44/ 0
Spotless Starling (*Sturnus unicolor*)			2/ 0		2/ 0
Spotted Redshank (*Tringa erythropus*)			1/ 0		1/ 0
Tufted Duck (*Aythya fuligula*)		2/ 0			2/ 0
Turtle Dove (*Streptopelia turtur*)	74/ 1	59/ 3			133/ 4
Wigeon (*Anas penelope*)		3/ 0	1/ 0		4/ 0
Wood Pigeon (*Columba palumbus*)		9/ 0			9/ 0
TOTAL	108/ 1	323/ 6	157/ 3	24/ 0	612/ 10

Two Eb-ELISA seropositive samples from mallard could not be tested by competition ELISA and VNT because of the small amount of sera. Overall, eight out of ten serum samples were positive by competition ELISA and four by Eb-ELISA. Out of these the samples B3, B4 and B6 revealed WNV antibodies with neutralizing ability by VNT. However, considering a 1:20 dilution as a titer cut-off point to estimate the results, only the turtle dove B4 collected in September 2004 in the south of Portugal (Figure 25) specifically reacted with WNV at titer 256 when examined by VNT (Table 23).

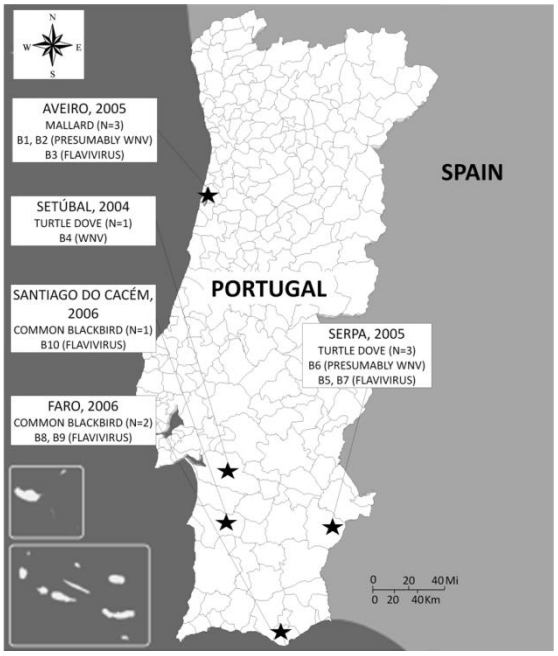

Figure 25. Geographic location of bird collection for the flaviviruses positive samples, 2004-2007.

Table 23. Summary of seropositive birds by ELISA assays and VNT captured in Portugal from 2004 to 2007.

	Species	Date	Age	ID-VET ELISA		Eb ELISA		VNT	DIAGNOSIS
				% S/ N	Result	% inhibition	Result	Titer	
B1	Mallard	Sep-05	Juvenile	NT	NT	51,9	+	NT	Presumably WNV
B2	Mallard	Sep-05	Juvenile	NT	NT	55,7	+	NT	Presumably WNV
B3	Mallard	Oct-05	Juvenile	34,6	+	<30	-	10	Flavivirus
B4	Turtle Dove	Sep-04	Juvenile	12,3	+	63,4	+	256	WNV
B5	Turtle Dove	Sep-05	Sub-adult	13,9	+	<30	-	<10	Flavivirus
B6	Turtle Dove	Aug-05	Adult	35,9	+	47,4	+	16	Presumably WNV
B7	Turtle Dove	Aug-05	Juvenile	30,1	+	<30	-	<10	Flavivirus
B8	Common Blackbird	Dec-06	ND	13,6	+	<30	-	<10	Flavivirus
B9	Common Blackbird	Aug-06	ND	25,3	+	<30	-	<10	Flavivirus
B10	Common Blackbird	Oct-06	ND	30,4	+	<30	-	<10	Flavivirus

NT = not tested (insufficient sample).
ND = not discriminated.
S/N = (Sample OD/ negative control OD) x 100

DISCUSSION

Despite not being a comparative study, three different serological approaches were used in the present study in order to survey wild birds of several species for WNV. ELISA provides a rapid, sensitive, and inexpensive screening test for the detection of avian antibodies to WNV (Calisher and Beaty, 1992). However, there are some limitations of this technique when screening samples from a wide variety of bird species, as different secondary (reporter) antibodies are required depending on the species to be tested. The ID-VET diagnostic kit is

designed to detect antibodies directed against pr-E containing an epitope common to Japanese Encephalitis (JE) antigenic complex. Thus is expected that this assay can also recognize other closely related specific antibodies produced by other flaviviruses of the same antigenic complex. In this context, we considered the eight positive samples, by the use of this ELISA, positive for antibodies against flaviviruses of the JE antigenic complex, which includes WNV together with Usutu, Kujin, Murray Valley Encephalitis and others (Kuno *et al.*, 1998).

The epitope blocking ELISA (Eb-ELISA) used in this study is directed to immunodominant epitopes on the NS1 protein of flaviviruses, which does not require the use of multiple reporter or capture antibodies, and has previously been shown to be an effective target to specifically identify Kunjin virus infections in sentinel chickens (Hall *et al.*, 1995). This assay as been exploited to screen thousands of serum samples from numerous avian species captured throughout the United States and showed to specifically identify infections with WNV when the most efficient monoclonal antibody MAb 3.1112G was used (Blitvich *et al.*, 2003). In our study, four samples were positive by Eb-ELISA (Table 23) and taking into account the displayed reports of high levels of sensitivity of Eb-ELISA, we considered these (B1, B2, B4 and B6) presumably positive for WNV (Blitvich *et al.*, 2003).

Besides the great sensitivity showed by Eb-ELISA, VNTs are more specific and discriminatory than other serological tests and usually used to confirm seropositive results by ELISA or hemagglutination-inhibition tests. However, VNTs for type-specific diagnosis are laborious, expensive and require live virus and for these reason are not ideal for large scale routine testing. For this reason only ELISA positive samples were screened by VNT. In our study, neutralization with the standard topotype Egyptian strain Eg-101 of WNV was used and three out of eight ELISA positive samples revealed antibodies with neutralizing ability. The sample B3 and B6 were positive at 1:10 and 1:16 dilution, respectively; however, considering a 1:20 dilution as a titer cut-off point to estimate the results the turtle dove B4 undoubtedly has been regarded as reacting specifically with the standard topotype Egyptian strain Eg-101 of WNV (titer 256). Notwithstanding flaviviruses can present a high degree of serological cross-reactivity even in neutralization assays (Filipe and Pinto, 1969; De Madrid and Porterfield, 1974; Garea González and Filipe, 1977; Calisher *et al.*, 1989; Weingartl *et al.*, 2003; Crill and Chang, 2004; Niedrig *et al.*, 2007; Hubálek *et al.*, 2008), and nowadays, diagnose of WNV infections in birds is getting more complex because closely related flaviviruses of the same antigenic group can co-occur in one area or migrating birds can be seropositive for a virus which is not present in a specific geographic location, making difficult to know which virus is responsible for the antibody production in birds (Weissenböck *et al.*, 2002; Weissenböck *et al.*,

2003; Linke *et al.*, 2007). In this context, the positive common blackbirds detected in our study can hypothetically suggest the presence of Usutu virus antibodies since this mosquito-borne flavivirus has been occasionally detected in Central Europe, UK, southwest Germany and northern Italy, particularly in common blackbirds (Weissenböck *et al.*, 2002; Buckley *et al.*, 2003; Becker *et al.*, 2012; Manarolla *et al.*, 2010).

Concerning the impact of bird migration on WNV epidemiology there are two important periods for pathogen introduction in Portugal that correspond to the biannual bird migration. During spring birds fly from Africa into the North hemisphere, potentially introducing pathogens in the Mediterranean wetlands, as well as in other European countries. In autumn birds return to Africa and may carry pathogens from Northern to Southern Europe (Jourdain *et al.*, 2007[a]). In European countries WNV activity is usually detected in late summer and at urban sites near wetlands where migratory birds and mosquitoes are concentrated at their highest densities (Rappole *et al.*, 2000; Rappole and Hubálek, 2003). The turtle dove is a migratory species that reproduces in Portugal (Equipa Atlas, 2008) and winters in Africa, but is not closely associated with wetlands. The high titer neutralizing antibodies to WNV in a juvenile bird of this species suggests local infection and epizootic viral circulation. In view of these results, turtle doves may play an important role as bird host for the WNV. Turtle doves became infected in Africa and arrive in Portugal with a sufficient level of viremia to infect vector mosquitoes during breeding season. Local competent mosquitoes of the species *Culex pipiens*, one of the most abundant in Portugal, amplify the virus with local avifauna that is not immunized against WNV and a high epizootic activity may occur (Osório *et al.*, 2010). In this scenario, suitable conditions for the occurrence of accidental human infections are set. Regarding the mallard this is mostly a resident species in Portugal (Rodrigues *et al.*, 2000), which uses wetland habitats, so in close contact with migratory waterbirds. Both the turtle dove and the mallard are competent reservoirs of WNV (Jozan *et al.*, 2003; Valiakos *et al.*, 2011[b]), but formal evidence for species incrimination on site will require a search for acute infection during bird migration periods (Barros *et al.*, 2011).

Concluding, our results support that WNV had circulated in wild birds in Portugal from 2004 to 2005, when viral activity in mosquitoes and human cases of infection were reported. Turtle doves may play an important role as bird hosts for the WNV and should be considered as a target species in bird surveillance programs. Regarding the risk for public health, identification of wild bird reservoirs is an important contribution for WNV epidemiological knowledge and should be encouraged.

CHAPTER 7

WEST NILE VIRUS CLINICAL CASE

7.1 WEST NILE VIRUS INFECTION IN PORTUGAL: CONSIDERATIONS ABOUT A CLINICAL CASE WITH FEVER AND A RASH

RESEARCH ARTICLE

Alves, M.J., Poças, J.M.D., Luz, T., Amaro, F., Zé-Zé, L., Osório, H.C. (2012) West Nile virus (*Flavivirus*) infection in Portugal: considerations about a clinical case with fever and a rash. *Revista Portuguesa de Doenças Infecciosas*, 8 (1): 46-51.

ABSTRACT

West Nile virus (WNV) is a flavivirus transmitted by mosquitoes and the etiologic agent of West Nile fever and neuroinvasive illness. The virus is maintained in nature in enzootic cycles involving ornithophilic mosquitoes as primary vectors, and some species of birds as primary reservoirs. West Nile virus presence in Portugal is well known emerging, sporadically, cases of infection in horses and humans. In 2010 a human case was identified in southern Portugal. This was the only human case detected by the National Institute of Health in the mosquito activity period in this year. In this case the patient had fever with very irregular hyperpyrexia, with peaks above 39ºC, occasionally chills, headache, myalgia, malaise and accentuated weakness, painful lymphadenopathy in the cervical region, as well as a diffuse erythematous rash on the trunk. The laboratory findings included IgM antibodies seroconversion against WNV.

INTRODUCTION

West Nile virus (WNV) is a virus of the genus *Flavivirus* (Family *Flaviviridae*) comprising about 70 virus, most of them transmitted by mosquitoes and ticks. Like other flaviviruses, WN virion is spherical with 40-60 nm in diameter. The single stranded positive RNA genome is surrounded by an icosahedral nucleocapsid enclosed by the lipid bilayer envelope originally from the host cell. Two genetic lineages are recognized, namely lineage 1 present in Europe, North America, Asia, Africa and Australia, and lineage 2 generally identified in sub-Saharan Africa and Madagascar (Hayes *et al.*, 2005). In 2010 WNV lineage 2 was identified for the first time in Europe in an outbreak occurred in Greece with 191 human cases and 32 deaths (CDC, 2002).

West Nile virus is maintained in nature in enzootic cycles involving ornithophilic mosquitoes as primary vectors, and some species of birds as primary reservoirs. Mosquitoes can incidentally transmit the virus to horses and humans. There are several reports of human infections associated with transplants and blood donations (CDC, 2002; Campbell *et al.*, 2002; Iwamoto, 2003; CDC, 2004). Many species of birds, including migratory birds, and mosquitoes, particularly of the genus *Culex*, have been identified as reservoirs and vectors of WNV respectively, which probably has contributed to the wide geographical distribution of the virus and hence the disease in animals and humans.

The incubation period of WNV normally ranges between three and 15 days after the bite of the mosquito vector. About 80% of human WNV infections are asymptomatic; the other can result in fever with sudden onset for two to five days, along with headache, myalgia, malaise, nausea and vomiting, sometimes with maculopapular or roseolar rash (Klein *et al.*, 2002). In 1% of infections neurotropic symptoms such as meningitis, encephalitis and myelitis, usually associated with high fever may occur. Other neurotropic signs include ataxia and extra pyramidal signs, polyradiculopathy, seizures and ocular neuritis (Filipe, 1973; Leis *et al.*, 2002). Muscle weakness is reported in most clinical presentations.

In Portugal there is evidence that WNV is maintained in epizootic cycles and has the capacity to infect humans sporadically and unexpectedly. The first studies on arboviruses (arthropod-borne viruses) were carried out since the 60s. In the first epidemiological survey, which included 1649 healthy blood donors, 0.5% persons were positive for WNV by serum neutralization test (Filipe, 1973). The lineage 1 *Roxo* WNV was isolated from mosquitoes of *Anopheles maculipennis* s.l. near the Roxo dam, in Aljustrel (Filipe *et al.*, 1972). In the same region seven horses with neutralizing antibodies for WNV were identified out of 24 horses that survived an outbreak of equine encephalomyelitis years earlier (Filipe and Pinto, 1969). At the

Centre for Vectors and Infectious Diseases Research of the National Institute of Health Dr. Ricardo Jorge (CEVDI/ INSA), routine laboratory diagnosis for arboviruses was established in 1996 (Alves and Filipe, 2003). West Nile virus was not identified in human samples until 2004, when two human cases related to tourists who would have remained in the region of Algarve were diagnosed (Connel *et al.*, 2004). At the same time a lineage 1 WNV was isolated from *Culex pipens* s.l. and *Cx. univittatus* mosquitoes collected in the same region (Esteves *et al.*, 2004). Within two years the Algarve Regional Health Authority and CEVDI/ INSA developed a surveillance program addressed to WNV vectors and an epidemiological survey in the region was conducted. Mosquitoes infected with WNV and other human cases were not reported.

In July 2010 a new human case of WNV infection was identified.

CASE STUDY

Clinical Outcome

On July 9, 2010, AMPCP, female, 55 years old, was observed in the emergency service (ES) of the Hospital São Bernardo (HSB), Setubal, with a clinical picture of fever, rash and polyadenopathies. She had a history of smoking (20 cigarettes/ day), hypertension treated with a thiazide diuretic for nearly a year, irregular episodes of ulcers of the oral mucosa (<3/ year and not accompanied by other complaints, including pathergy, red eye, arthralgia, arthritis, or venous thrombosis).

The fever had three weeks' duration and was characterized by very irregular hyperpyrexia, sometimes with chills and peaks above 39° C, which was maintained over time and did not recover with prescribed medication (analgesic, antipyretic and anti-inflammatory drugs). The patient complained of headache, myalgia, malaise and pronounced asthenia, accompanied by massive and painful lymphadenopathy in the cervical region that emerged week before as well as non-itchy diffuse erythematous rash with higher expression in the trunk, followed by desquamation of the skin hands.

Two days before turning to the ES the patient found a sharp decrease in the size of enlarged lymph nodes. The clinical observation highlights the presence of rash predominantly on the trunk, confluent, though evanescent, no hepatosplenomegaly, lymphadenopathy or fever, and the observation of the heart and lung was normal, as well as the neurological examination.

The patient resided in rural areas, and referred water intake from wells and regular contact with dogs and cats. Since the early summer that the patient was bitten by mosquitoes near her residence. Travel, particularly to tropical countries, in the last 12 months was denied.

The patient had consulted several doctors and performed diagnostic tests: autoantibody markers for vasculitis and connective tissue diseases all negative, normal complement, Widal and serology for Brucellosis also negative, hemogram (L: 21100 with N 80%, P 400000), ESR 16, serology (CMV: IgG and IgM negative, EBV: IgG and IgM -, HSV 1 e 2: IgG and IgM negative, HIV 1 and 2 negative) and chest radiography without significant changes. From the tests performed in SU the hemogram, glucose, renal and hepatic function, coagulation and blood gas normal, ionogram with hypokalemia (K 2.9 mEq / l) and CRP of 5.9 mg/ 100 ml.

Diagnostic tests were then requested to CEVDI/ INSA, thought of a possible zoonosis. The patient was discharged from Hospital and was treated with potassium chloride retard and kept under observation. Microbiological culture of blood was indicated in the case of new peaks of fever higher than 38^{o}C. A week later, in the external medical consultation, the patient had a clinical picture of mild fever (without hyperpyrexia over 38° C), asthenia and mild myalgia. The serology was negative for all zoonoses with exception of WNV showing IgM positive and negative IgG. The patient was kept at home with medical supervision, and the antihypertensive medication was changed from Indapamide to Cilazapril.

The following week the patience returned to hospital because of increasing headache and neck pain, and fever, which did not improve with Paracetamol. Clinical examination did not reveal any relevant changes at this time, except for a slight stiffness of the neck with terminal stiffness of the neck muscles. Cervical Computed Axial Tomography only revealed minor cervical degenerative changes and routine tests namely blood count, renal and hepatic function, ionogram, urine II, CRP and ESR, coagulation, without any changes.

Before the clinical worsening of the patient and the positive IgM serology for WNV, an lumbar puncture was performed and cerebrospinal fluid examination revealed a cyto-chemical (including ADA) normal. The microbiological examination and the serology for WNV, *Herpes simplex*, toxoplasmosis, EBV and HHV6, and PCR were all negative (the latter also including BK).

Two weeks after the first observation, the patient still had headache, sore throat, malaise, asthenia, although without fever or rash. Further analyses were required, and the blood-pressure was controlled with cilazapril and idanpamid.

In the observation carried out four weeks later there was an improvement in cervical complaints with less asthenia and malaise, no fever and without changes in the physical examination, including rash or lymphadenopathy. The patient remained under medical supervision at home, gradually returning to the exercise of her profession (Psychologist), prescribed only with the same anti-hypertensive therapy.

Three weeks later the patient was discharged with the clinical diagnosis of probable WNV autochthonous infection.

Laboratory tests in CEVDI / INSA

On July 12, in the laboratory of CEVDI/ INSA, whole blood and serum was collected for analysis of serological and nucleic acid detection of *Borrelia burgdorferi*, *Rickettsia conorii*, *Coxiella burnetii*, Dengue, Chikungunya virus and WN. Direct and indirect methods have shown to be negative for these agents except for IgM (positive, title 128) and IgG antibodies (suspect title 16) antivirus WN. The laboratory has requested a new sample to confirm seroconversion in IgM titres to WNV. On July 22 and August 6 a second and third whole blood sample was received. On July 27 a serum sample of cerebrospinal fluid was received. Table 24 presents a summary of results for WNV tests conducted over the outcome.

Table 24. Summary of WNV tests.

Date	12/07		22/07		06/08		27/07
Sample	**1.º serum and total blood**		**2.º serum**		**3.º serum**		**CSF**
Title	IgM	IgG	IgM	IgG	IgM	IgG	IgG+IgM
West Nile (IFA)	128	16	32	32	32	32	neg
Flavivirus (RT- PCR)	neg	neg	nd	nd	nd	nd	nd

IFA – Immunofluorescence assay; RT-PCR – *Reverse transcriptase Polymerase Chain Reaction*
nd – not done; neg - negative; sus - suspect.
Immunofluorescence positivity thresholds: IgG ≥32; IgM≥16

To exclude possible cross-reactions with other viruses of the genus flavivirus, the 1st and 2nd samples were also tested by IFA for Dengue, Yellow-Fever and Tick-borne Encephalitis (TBE) flavivirus. All samples were negative. The 1st and 2nd serum sample were sent to ENIVD-ECDC (European Network for Diagnostics of Imported Viral Diseases - European Centre for Disease Control), namely to the Robert Koch Institute, for plaque reduction neutralization test analysis (PRNT). Although positive by IFA, both were negative by PRNT.

Epidemiology

The patient lives in a rural area near a golf course and the estuary of a river into a natural park known to be on the migration route of birds. From 13-28 July adult mosquitoes were collected by CEVDI/ INSA in the area up to 1 km outside the house. Mosquito CDC (Centres for Disease Control and Prevention) light-traps baited with CO_2 were used in 15 night-traps. A total of 1825 adult mosquitoes from 11 species (*Anopheles claviger*, *Anopheles maculipennis* s.l., *Coquillettidea richiardii*, *Culiseta annulata*, *Cs. longiareolata*, *Cx. pipiens* s.l., *Cx. perexiguus*, *Cx. theileri*, *Ochlerotatus caspius*, *Oc. detritus* and *Orthopodomyia pulcripalpis*) were captured. The most abundant species was *Oc. caspius* (70%) followed by *Cx. pipiens*

(16%). Female mosquitoes were identified and 57 pools (with a maximum of 50 specimens) were tested for the presence of flaviviruses nucleic acid with RT-PCR. All pools were negative.

CEVDI/ INSA noticed an increase of requests for WNV diagnosis after a report from the Directorate of General Health has been published. However, no more human cases were identified.

WEST NILE VIRUS SURVEILLANCE IN PORTUGAL

Since 2008, Portugal has a Network for Vector Surveillance (REVIVE) that includes mosquitoes and transmitted flaviviruses. The REVIVE results from a collaboration of the Directorate of General Health, Regional Health Administrations and the National Institute of Health Dr. Ricardo Jorge. In this program the mosquitoes are collected twice a month in several municipalities of all health regions from May to October. The mosquitoes are then identified and assembled into pools for flavivirus screening. From 2008 to 2010, WNV was not detected in the 16,992 tested mosquitoes out of 77,710 collected in 44 municipalities of Portugal (Alves *et al.*, 2010).

DISCUSSION AND CONCLUSIONS

The detection of WNV in Portugal has been sporadic and unexpected, since apparently the virus occupies time and space localized ecological niches or is periodically introduced by migratory birds. In this case-study, after the detection of IgM antibodies anti-WN, vector surveillance measures were immediately adopted. The Culicidae fauna of the region (radius of 1 km) showed to be quite diverse (about 1/ 4 of the mosquito Portuguese species were identified). Some of the most abundant species are potentially vectors of WNV, but no infected mosquitoes were detected.

The WNV infection is a notifiable disease according to the European Commission decision of April 2, 2009 (EC, 2009). According to WNV infection case definition (EC, 2008), probable cases in which specific antibodies in serum are detected is confirmed by 1) WNV isolation; 2) WNV nucleic acids detection in the blood or cerebrospinal fluid; 3) IgM antibodies detection in cerebrospinal fluid; 4) high titre of IgM and IgG and neutralization evidence.

In this case the first sample for WNV diagnosis was collected three weeks after the onset of symptoms, which may explain the IgM decrease in four titles and the slight increase of IgG as well as the non-detection of nucleic acids in direct diagnosis, since five days is the average period of viraemia. The neutralization test (PRNT) is accepted as a confirmatory evidence for the presence of specific antibodies. However, has been recently shown by the *Quality External Assessment for West Nile "European Network for Imported Viral Disease*

Diagnostic" (unpublished results) that the techniques used in the few European laboratories that perform the PRNT may not detect both strains of WNV. In Portugal and Europe, only lineage 1 has been identified, however, the 2010 outbreaks in Europe were assigned to WNV lineage 2 (Sirbu *et al.*, 2010; Papa *et al.*, 2011; Kutasi *et al.*, 2011). Since in two consecutive samples the IgM title changed four times and neutralizing IgM was negative, this was considered a "probable case" according to case definition adopted by the European Commission. No more human cases were diagnosed in the laboratory throughout the season. However, in October and November 2010 two WNV infections in horses were reported just 4 km away from the residence of the human case identified (Barros *et al.*, 2011). These last two cases provide evidence of viral activity in the region, despite being low, since it was not detected in mosquitoes.

In summary WNV was identified in Portugal in 1969, 2004 and 2010 but the environmental events that led to the introduction or the appearance of these cases are still unknown. The epidemiological surveillance in vectors and reservoirs of WNV is crucial for taking control measures and to alert clinicians to new human cases.

CHAPTER 8

FINAL REMARKS AND FUTURE PERSPECTIVES

In the past 30 years, there was a remarkable resurgence of vector-borne viral diseases. Diseases such as caused by WNV, which have never been major public health problems, all of a sudden show up in places like Europe and North America to cause major outbreaks. As Reiter (2010[b]) points out, this is a sign of the times, with the changing demographics and societal behavior that allow more people being in contact with vectors. The increased movement of people, animals, and commodities via modern transportation into areas that do not have the public health infrastructures to deal with vector-borne diseases unquestionably increases the need to study and surveillance these re-emerging vector-borne diseases.

Mosquitoes are unquestionably the most medically important arthropod vectors of disease. The maintenance and transmission of viruses that may cause numeral infections and diseases in humans and animals are absolutely dependent on the availability of competent mosquito vectors. Arboviral diseases transmitted by mosquitoes are among the most important emergent/ resurgent infectious diseases that will have a major impact over the next 10–20 years. Climate change and human activities will cause the spread of mosquito vectors over the world and they will continue increasingly resistant to the pesticides available to deal with their abundance. The lack of preventive care in places previously free of mosquito-borne diseases will cause major epidemics that will not be resolved by administering vaccines. Only with an understanding of the biology of the mosquito will be possible to prevent and control vector-borne diseases.

The results presented in this thesis allowed a comprehensive revision on WNV in Portugal concerning its epidemiological determinants from the perspective of the mosquito vector until the bird reservoir and human host, including vector-host determinants:

1) The identification of mosquito species captured in different geographic regions of Portugal allowed an update on the composition and distribution of the culicidae fauna. The studies in vectors abundance and distribution presented on this work indicates *Ochlerotatus caspius*, *Culex theileri* and *Cx. pipiens* as the most abundant and widespread mosquitoes throughout mainland Portugal. This current abundance and distribution of mosquitoes is subject to dramatic future changes as a result of many factors cited above, related to the increase of human travelling and trade exchange as also climate change. Hence, surveillance of vectors and vector-borne diseases should be constantly performed to predict, detect and prevent on time any possible introduction of new mosquito species, namely the invasive species *Aedes aegypti* and *Ae. albopictus*, and to monitor abundance and distribution of the endemic vector species, particularly the biotypes of the *Cx. pipiens* complex. The European Centre for Disease Prevention and Control (ECDC) is reinforcing surveillance studies on mosquitoes and other vectors, since the incidence of vector-borne diseases in Europe is much

greater than generally recognized by physicians and health authorities (World Health Organization [WHO], 2004). The network of medical entomologists and public health experts (VBORNET) supports ECDC activities in the area of arthropod vector surveillance within the European Union to improve preparedness towards vector-borne diseases. The production of distribution maps of the major arthropod disease vectors and updated maps on invasive mosquitoes' species largely depend on the accuracy and awareness of the data gathered locally. Present challenges include the current presence of *Ae. aegypti* in Madeira Island and in the southern border of Russia to the Black Sea, and the presence of *Ae. albopictus* in most countries in the Mediterranean shore. Future studies on mosquitoes' abundance and distribution should analyse the relationship between insect populations and environmental parameters for a longer time. It would be interesting to better analyze the larval habitats in several locations and define the most important environmental factors determining the distribution of a particular species. This data should then be combined with defined parameters regarding globalization and to which extent trade exchange and human migration could affect vectors distribution.

2) The molecular identification of host blood meal in engorged females captured in nature, and 3) the genetic analyses of the primary vector of WNV - *Culex pipiens* - and its habitat determinants allowed several conclusions. The studies on blood feeding patterns showed *Cx. pipiens* feeding on birds and to a less extend on humans and other mammal hosts. First it demonstrated that *Cx. pipiens* might play a role both in the avian-to-avian enzootic WNV cycle but also in the avian-to-mammal transmission. Later, the performed molecular assays to identify the *Cx. pipiens* biotype revealed the sympatric presence of both biotypes and resulting hybrids forms in many study sites throughout Portugal. The combination of these data with the results of host feeding preference points to *Cx. pipiens f. pipiens* as primarily ornithophylic, *Cx. pipiens f. molestus* as anthropophilic and hybrids having opportunistic feeding behaviour. Concluding, the results from this study supported an innate host preference by *Cx. pipiens* different biotypes, with *pipiens* maintaining the enzootic cycle between birds and *molestus* and particularly hybrids forms ecologically competent to transmit the virus to humans. Under these circumstances it was considered that Portugal meets favourable ecological conditions for disease outbreaks of WNV. In future it would be interesting to deeply analyse the proportion and distribution of *pipiens*/ *molestus* by larvae sampling. This would increase sensitivity to the distribution analyses and also would better express the relationship between the biotype and the specific habitat. Another issue to address is how genetic determines feeding behaviour. Although much work has been done in

the *Cx. pipiens* group, the systematic and evolution of this species complex remains challenging to research (Vinagradova, 2000).

Regarding the 4) studies on vector competence, *Cx. pipiens f. molestus* collected in Portugal was pointed out as a WNV competent vector for the first time, as no data on the transmission was available until this preliminary assay was conducted. Besides this species meets ecological criteria to transmit WNV by feeding behaviour, it displayed vector competence for WNV in laboratory that contributes to consider *Cx. pipiens* as primary vector. At this point, we may conclude that *Cx. pipiens* combines ecological and innate vector competence essential for local transmission.

Considering *Anopheles atroparvus*, Portugal was the only country where WNV was isolated from this species. The role of *An. atroparvus* in WNV epidemiology remains an issue to be addressed in the future. In this study, it was difficult to rear *An. atroparvus* in the laboratory, and the total number of specimens available for testing was low. This assay should be repeated in the future with more specimens in order to estimate accurately the rates of viral infection and dissemination. Vertical transmission of WNV, from infected females to progeny, is also an important issue to be addressed.

Concerning the 5) detection and identification of flaviviruses in mosquitoes caught in the wild, no positive pools for WNV were detected during the period of this work (2008-2012), suggesting that WNV may only be present in specific ecotypes being sporadically introduced or, otherwise, maintained in threshold levels that only sporadically rise high enough to enable transmission. However, the widespread presence of different mosquito only flaviviruses (MOFs) in Europe, related to other MOFs isolated worldwide, question the importance of these flaviviruses in mosquito physiology and the implications on transmission of pathogenic flaviviruses when co-infection occurs. Further studies to evaluate their potential to prevent or enhance the transmission of other pathogenic flaviviruses during co-infection are required. It would be interesting to infect *Cx. pipiens* with a MOF and WNV simultaneously and analyse the vector competence for WNV.

In relation to the 6) serological studies performed in birds, the detection of antibodies in a juvenile turtle-dove suggests that WNV infection occurred locally and supports viral circulation occurring in Portugal. In this study, the mallard and turtle-dove arise as potential reservoirs with an important role in WNV epidemiology. However, further studies directed to wild birds' reservoir identification are required to understand which birds operate as primary reservoirs importing WNV during migration events and maintaining it locally in circulation during the transmission season. Several important questions can be raised about the bird reservoirs, but susceptibility of species to infection and duration of viraemia are important

issues to be analysed in the Old World. The combination of these data with information on bird migration routes from Africa would help to understand the global scenario of WNV.

Finally, this work presented a WNV infection human case detected in 2010, which may represent the tip of the iceberg, with its largest submerged portion represented by the infected mosquitoes and birds. This is the second occurrence since 2004, when two linked cases were reported in the Algarve region. Since 2009, WNV activity throughout Europe has been increasing, with a number of outbreaks reported in countries around the Mediterranean basin, namely Italy and Greece. In conclusion, the increasing activity of the virus in Europe and the favorable epidemiological conditions recorded in Portugal, and addressed in this work, represent future challenges for Public Health and scientific research. In Europe, the distribution of WNV has been discontinuous, multifocal and unpredictable, but climate change together with globalization are changing this pattern, making it increasingly frequent, endemic in some countries and with a more continuous distribution.

It is hoped that the studies in this work have contributed significantly to the knowledge of WNV epidemiology in its various aspects, and have provided new lines for future research.

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