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Emergence of *Aedes aegypti* in Madeira Island: origin, insecticide resistance and vector competence

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Seixas G, Jupille H, Yen PS, Viveiros B, Failloux AB, Sousa CA. Potential of *Aedes aegypti* populations in Madeira Island to transmit dengue and chikungunya viruses. Parasit Vectors. 2018:11; 509.

Seixas G, Grigoraki L, Weetman D, Vicente JL, Silva AC, Pinto J, Vontas J, Sousa CA. Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal). PLoS Negl Trop Dis. 2017:11(7), e005799.

Jupille H, Seixas G, Mousson L, Sousa CA, Failloux AB. Zika virus, a new threat for Europe? Plos Negl Trop Dis. 2016:10(8), e0004901.

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Seixas G, Jupille H, Mousson L, Yen P, Silva AC, Failloux AB, Sousa CA. Competência vetorial de *Aedes aegypti* da ilha da Madeira para a transmissão dos virús chikungunya, dengue e Zika. 2nd Vector-borne diseases workshop NovaSaúde, Lisbon, 2016. Oral presentation.

Seixas G, Grigoraki L, Weetman D, Silva AC, Pinto J, Vontas J, Sousa CA. Multiple mechanisms mediating insecticide resistance in *Aedes aegypti* from Madeira Island, Portugal. E-SOVE conference, Lisboa, 2016. Oral presentation.

Seixas G, Grigoraki L, Weetman D, Silva AC, Pinto J, Vontas J, Sousa CA. Insecticide resistance of the Zika vector, *Aedes aegypti*, from Madeira Island (Portugal). Ciência 2016 conference, Lisbon, 2016. Poster presentation.

Seixas G, Grigoraki L, Weetman D, Silva AC, Pinto J, Vontas J, Sousa CA. Molecular basis of insecticide resistance in the dengue and Zika vector, *Aedes aegypti*, from Madeira Island (Portugal). 2nd Genetics workshop NovaSaúde, Lisbon, 2016. Poster presentation.

Seixas G, Salgueiro P, Gordicho V, Vicente J, Silva A.C, Pinto J, Sousa CA. Resistência aos inseticidas do vetor de dengue *Aedes aegypti* da ilha da Madeira: implicações no controlo vetorial. V Jornadas Científicas do IHMT, Lisboa, 2014. Oral presentation.

Seixas G, Salgueiro P, Alves G, Silva A.C, Novo M.T, Pinto J, Sousa C.A. *Aedes aegypti* and the dengue outbreak in Madeira island: the burden of a recent colonisation. Abstract Book of Amazonian Conference on Emerging and Infectious Diseases (ACEID), p. 43. STRONGER Project, Cayenne, French Guiana, 2014. Oral presentation

To my parents

for "those who respect their mother are like those who lay up a treasure.

Those who honor their father will have joy in their children, and when they pray they will be heard"

Ecclesiasticus 3, 4-5

Wisdom is a shelter,

as money is a shelter,

but the advantage of knowledge is this:

wisdom preserves

those who have it.

Ecclesiastes 7:12

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Resumo

Aedes aegypti é altamente competente para a transmissão aos humanos de arbovírus, como chikungunya, dengue e Zika. A recente expansão desta espécie para áreas de onde foi eliminada e o seu estabelecimento em novos territórios, combinada com um crescimento urbano intensivo e aumento do movimento global de pessoas e mercadorias, desencadearam o dramático aumento da incidência de arboviroses nos últimos 40 anos. A vulnerabilidade da Europa aos arbovírus está a aumentar em áreas onde as populações de mosquitos vetores estão presentes. Um exemplo notável é o recente surto de dengue na ilha da Madeira em 2012. Apesar das medidas de controlo vetorial implementadas, a espécie *Ae. aegypti* encontra-se em contínua expansão desde o momento da sua introdução em 2005. Neste contexto, foram analisadas populações de *Ae. aegypti* da ilha da Madeira com o objetivo de i) caracterizar a sua suscetibilidade aos inseticidas e os potenciais mecanismos de resistência presentes, ii) determinar a origem geográfica de *Ae. aegypti* da região e a sua estrutura genética com recurso a marcadores genéticos, tais como microssatélites e ADN mitocondrial, e iii) avaliar a competência da espécie presente na ilha para a transmissão dos vírus chikungunya, dengue e Zika.

A espécie *Aedes aegypti* presente na Madeira foi considerada resistente a todos os inseticidas testados. Ensaios efetuados com sinergistas, que aumentaram significativamente as taxas de mortalidade, juntamente com os ensaios bioquímicos, que indicaram atividades enzimáticas aumentadas, sugerem a presença de resistência metabólica. A análise de *microarrays* revelou o aumento da expressão de genes associados à resistência aos inseticidas, principalmente proteínas cuticulares e citocromo oxidases P450. Destes, os genes mais expressos, *Cyp9J32* e *Cyp9J28*, são conhecidos metabolizadores de piretróides. A genotipagem de mutações *kdr* revelou a presença da mutação V1016I com uma frequência moderada enquanto a mutação F1534C encontra-se fixa.

As análises de genética populacional indicam pelo menos dois eventos de colonização de *Ae. aegypti* na Madeira, sendo a Venezuela o mais provável país de origem destas introduções. Estimativas de tamanho efetivo populacional são consistentes com a rápida expansão de *Ae. aegypti* na ilha, atingindo valores máximos em 2012, coincidente com o surto de dengue ocorrido neste ano. Mais ainda, os resultados sugerem que as medidas de controlo implementadas após o surto podem ter afetado o tamanho efetivo de *Ae. aegypti* no Funchal.

Finalmente, os estudos de competência vetorial revelaram a elevada suscetibilidade para a transmissão dos vírus chikungunya e dengue e moderada suscetibilidade para a transmissão do vírus Zika. Em conjunto, os resultados obtidos corroboram o potencial risco para a disseminação de arbovírus na população local de *Ae. aegypti*. Assim, é necessário que a Madeira mantenha, atualize e teste os planos de contigência para estas arboviroses, de modo a garantir a devida preparação para futuras epidemias. Mais ainda, este estudo contribuiu para uma melhor compreensão do estado de resistência aos inseticidas e da estrutura genética de populações de *Ae. aegypti* na ilha, conhecimentos que poderão ser usados no delineamento e implementação de novas estratégias de controlo que previnam novos surtos arbovíricos transmitido por esta espécie.

Palavras-chave: Resistência aos inseticidas, genética populacional, competência vetorial

Abstract

Aedes aegypti is highly competent to transmit arboviruses to humans, such as chikungunya, dengue and Zika. The recent expansion of this species into areas where it has been eliminated and its establishment in new territories, combined with intensive urban growth and increased global movement of people and goods, have triggered a dramatic increase in the incidence of arboviruses over the last 40 years. The vulnerability of Europe to arboviruses is increasing in areas where mosquito vector populations are present. A striking example is the recent dengue outbreak on Madeira Island in 2012. Despite the vector control measures implemented, *Ae. aegypti* has been in continuous expansion since its introduction in 2005. In this context, *Ae. aegypti* populations from Madeira Island were analysed with the objective of i) characterize their susceptibility to insecticides and potential resistance mechanisms present, ii) determine the geographical origin of *Ae. aegypti* in the region and its genetic structure using genetic markers such as microsatellites and mitochondrial DNA, and iii) evaluate the competence of the species present on the island for chikungunya, dengue and Zika viruses transmission.

The *Aedes aegypti* of Madeira was considered resistant to all insecticides tested. Bioassays performed with synergists, which significantly increased mortality rates, along with biochemical assays, which indicated increased enzymatic activities, suggest the presence of metabolic resistance. Microarray analysis revealed increased expression of genes associated with insecticide resistance, mainly cuticle proteins and cytochrome P450 oxidases. Of these, the most expressed genes, *Cyp9J32* and *Cyp9J28*, are known pyrethroid metabolizers. Genotyping of *kdr* mutations revealed the presence of the V1016I mutation at a moderate frequency while the F1534C mutation is fixed.

Population genetics analyses suggest at least two colonization events of *Ae. aegypti* in Madeira, with Venezuela being the most likely origin of these introductions. Effective population size estimates are consistent with a rapid expansion of *Ae. aegypti* on the island, reaching maximum values in 2012, coinciding with the dengue outbreak in this year. Moreover, results suggest that the control measures implemented after the outbreak may have affected the *Ae. aegypti* effective size in Funchal.

Finally, vector competence studies revealed high susceptibility for chikungunya and dengue viruses transmission and moderate susceptibility to Zika virus transmission. Altogether, the results obtained corroborate the potential risk for the dissemination of arboviruses in the local population of *Ae. aegypti*. Thus, it is necessary for Madeira to maintain, update and test contingency plans for these arboviruses in order to ensure proper preparation for future epidemics. Moreover, this study contributed to a better understanding of insecticide resistance status and the population genetic structure of *Ae. aegypti* populations on the island, knowledge that may be used in the design and implementation of new control strategies to prevent new outbreaks of arboviruses transmitted by this species.

Keywords: Insecticide resistance, population genetics, vector competence

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Abbreviations

Aaa	*Aedes aegypti aegypti*
Aaf	*Aedes aegypti formosus*
AChE	acetylcholinesterase
AR	allele richness
AS-PCR	allele-specific PCR
ATP	adenosine triphosphate
ATSB	attractive toxic sugar baits
Bti	*Bacillus thurigiensis israelensis*
CCE	carboxyl/choline esterases
CI	cytoplasmic incompatibility
CHIKV	chikungunya virus
CNV	copy number variation
COI	cytochrome c oxidase subunit I
CRISPR	clustered regularly interspaced short palindromic repeats
DAPC	discriminant analysis of principle components
DDT	dichlorodiphenyltrichloroethane
DE	Dissemination efficiency
DEM	diethyl maleate
DENV	dengue virus
DENV-1	dengue virus serotype 1
DENV-2	dengue virus serotype 2
DNA	deoxyribonucleic acid
Dpi	days post-infection
DR	dissemination rate
dsRNA	double stranded RNA
ECDC	European Centre for Disease Prevention and Control
EU	European Union
FBS	fetal bovine serum
FC	fold change

F_{is}	inbreeding coefficient
FFU	focus forming units
GABA	gama-aminobutyric acid
GHTM	Global Health and Tropical Medicine
GMO	genetically modified organisms
GST	Glutathione-S-transferase
Hd	haplotype diversity
H_e	expected Heterozygosity
i.e.	id est
IASAÚDE	Instituto de Administração da Saúde e Assuntos Sociais
IGR	insect growth regulator
IHMT	Instituto de Higiene e Medicina Tropical
IIT	incompatible insect technique
ITM	insecticide-treated material
IRS	indoor residual spraying
IR	infection rate
ISFVs	insect specific flaviviruses
Kdr	knockdown resistance mutations
LD	linkage disequilibrium
MCMC	markov chain monte carlo
MDSS	Madeira Dengue Surveillance System
MIB	midgut infection barrier
MEB	midgut escape barrier
mtDNA	mitochondrial deoxyribonucleic acid
N	sample size
N_e	effective population size
ND4	NADH dehydrogenase subunit 4
NGS	next generation sequencing
PAHO	Pan American Health Organization
P450s	cytochrome P450 monooxygenases
PBO	piperonyl butoxide

PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	Reverse transcription polymerase chain reaction quantitative real time
RAD	restriction site associated DNA markers
RAPD	random amplification of polymorphic DNA
RIDL	release of insects carrying a dominant lethal allele
RNA	ribonucleic acid
RNAi	RNA interference
SIT	sterile insect technique
SGB	salivary gland barrier
SMM	stepwise mutation model
SNP	single nucleotide polymorphism
TCID	tissue culture infective dose
TE	Transmission efficiency
TEs	transposable elements
TPM	two-phase model
TR	Transmission rate
USA	United States of America
Vgsc	voltage gated sodium channel
WHO	World Health Organization
YFV	yellow fever virus
ZIKV	Zika virus
ΔK	ad hoc approach to infer the most likely number of clusters in the sample by STRUCTURE software

Chapter 1.

General Introduction

1. Arboviruses and vector-borne diseases

Mosquitoes have a worldwide distribution and are a major health problem in most tropical and subtropical countries where they are vectors of parasitic and arboviral diseases (Figure 1).

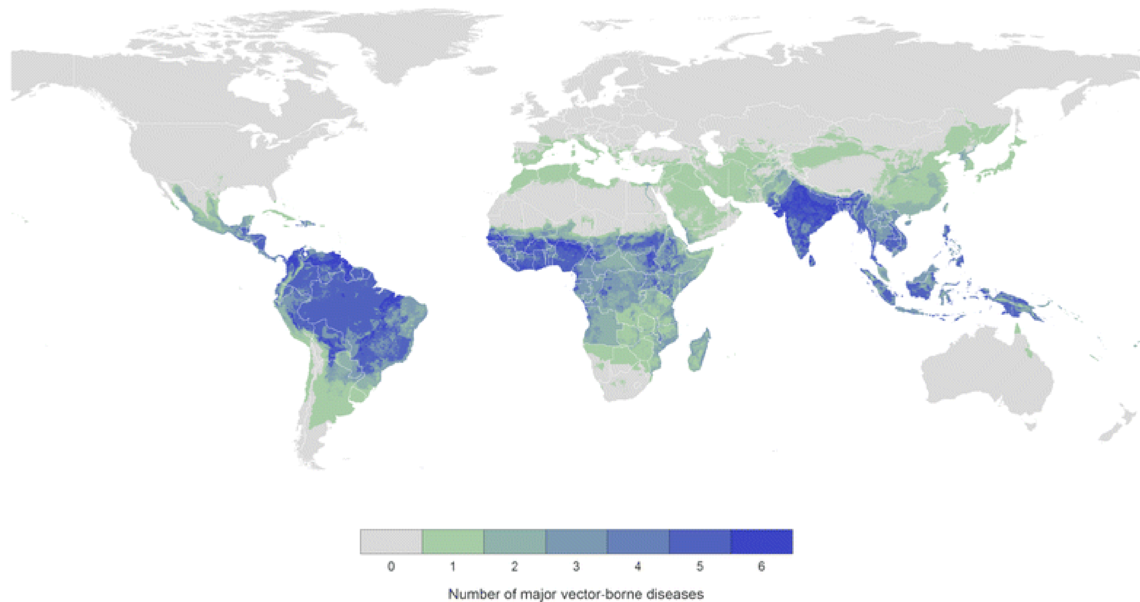

Figure 1. Combined global distribution of major vector-borne diseases. Retrieved from Golding et al. (2015).

Vector-borne diseases, especially those caused by arboviruses (arthropod-borne viruses), are among the leading causes of morbidity and mortality in humans and animals (Weaver and Reisen, 2010). Of these, more than 100 are transmitted by mosquitoes, proclaimed as the world's deadliest species in the world (Kamerow, 2014). *Aedes aegypti* (Linnaeus, 1762), also known as the yellow fever mosquito, is considered one of the mosquito species with major medical importance. This species is an important nuisance agent, causing allergic reactions due to its feeding behaviour and, most importantly, is a primary vector of arboviruses, such as dengue (Guzman et al. 2010), chikungunya (Leparc-Goffart et al. 2014) and Zika (Musso et al. 2015). The fact that *Ae. aegypti* females feeds primarily on humans (a process called anthropophily) is the main reason why this mosquito species is such an efficient arbovirus vector.

Mosquito-borne arboviruses are RNA viruses, comprising different viral families

(*Flaviviridae*, *Togaviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdodviridae*), and may be transmitted by different mechanisms: i) horizontal transmission - the most usual in which the transmission cycle involves a host and a hematophagous arthropod vector; ii) vertical transmission - involves the passage of the virus from an infected female to its progeny, and; iii) venereal transmission, in which virus is transmitted during mating (Weaver and Reisen, 2010). The parameters that shape the transmission potential of a given mosquito population to act as a disease vector are known and form the components of vectorial capacity (Figure 2) (Shaw and Catteruccia, 2018).

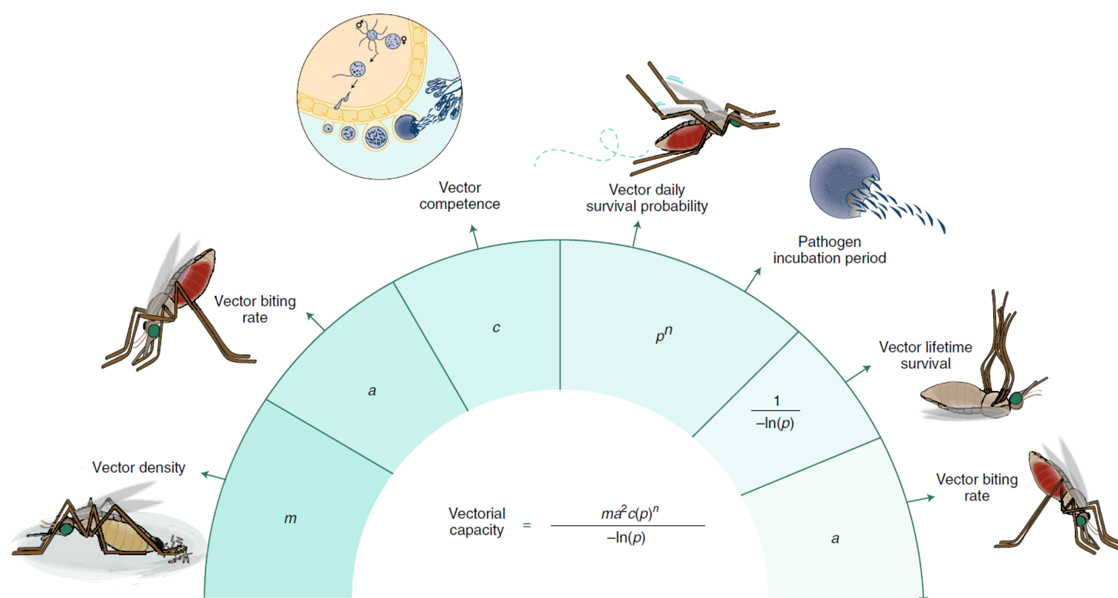

Figure 2. Components of mosquito vectorial capacity. Retrieved from Shaw and Catteruccia (2018).

Vector competence refers to the vector's capability to withstand infection, replication and transmission of a particular virus (Bennett et al. 2002). Before a mosquito-vector becomes infectious, the pathogens have to overcome several barriers in the invertebrate host. These include a midgut infection barrier (MIB), a midgut escape barrier (MEB) and a salivary gland barrier (SGB) (Bennett et al. 2002). These barriers are of extremely importance since a good vector must be easily infected by the pathogen and, subsequently, become infectious. The key features related to arboviral infection of mosquitoes comprises i) ingestion of viremic blood from an infected host, ii) infection of midgut cells, subsequent viral replication and midgut escape, iii) dissemination to the hemocoel and secondary

tissues, iv) infection of the salivary glands (Franz et al. 2015).

The genetic background of the vector and the pathogen greatly influences this important trait. In addition, vector competence may also be affected by environmental factors (Lambrechts et al. 2011) and by the mosquito microbiome and immune system (Hedge et al. 2015; Dennison et al. 2015; Tham et al. 2018).

Although described by Garrett-Jones and Grab (1964) as two individual parameters, currently some authors consider vector competence as part of vectorial capacity estimates.

1.1. Vector competence evaluation

To determine the vector competence of a mosquito-vector, the following parameters are used:

- Infection rate (IR), corresponding to the proportion of females presenting infected digestive tract among the analysed females;
- Dissemination rate (DR), corresponding to the proportion of females with infected wings/legs or head among females with infected digestive tract. This allows to assess the ability of the virus to cross the mosquito digestive tract barrier and to disseminate into the hemocele;
- Dissemination efficiency (DE), corresponding to the proportion of females with infected wings/legs or head among the females tested;
- Transmission rate (TR), corresponding to the proportion of females with infectious saliva among females with disseminated virus beyond the midgut barrier;
- Transmission efficiency (TE), corresponding to the proportion of females with infectious saliva among tested ones.

The rate of disseminated infection and its efficiency is essential to evaluate the ability of the virus to cross the MIB and MEB, with subsequent dissemination into the hemocele, while the rate and efficiency of transmission indicates that the virus has crossed the last barrier, the SGB, and is able to be released in the mosquito saliva. Therefore, vector competence parameters, such as TR and TE, are crucial for risk assessment of arbovirus transmission mediated by local mosquito populations.

Aedes aegypti displays high estimates of vector competence to several arboviruses (Weaver and Reisen, 2010). However, in order to understand the current worldwide distribution of *Ae. aegypti*-borne diseases, as well as the ability of this species to invade and thrive in new territories, it is necessary to analyse this mosquito ability to live in close association with humans, in the urban and domestic environment.

2. A successful invader

2.1. Bioecology

Ecological plasticity and desiccation-resistant eggs have been the major features associated with the success of establishment and subsequent expansion of *Ae. aegypti* worldwide. As for all mosquito species, *Aedes aegypti* life cycle involves two distinct stages: the aquatic phase, where the immature stages develop, and an aerial phase, also known as the adult stage (Figure 3).

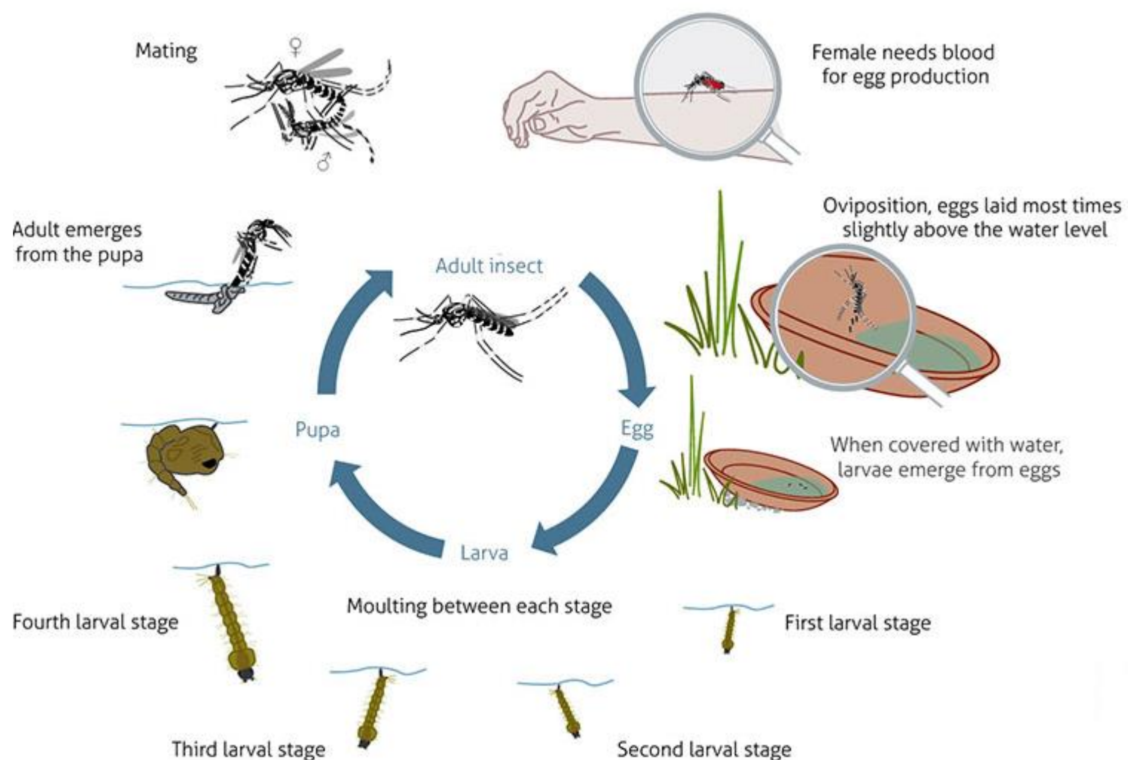

Figure 3. Schematic representation of *Aedes aegypti* life cycle. Retrieved from: https://us.biogents.com/aedes-aegypti-yellow-fever-mosquitoes/life-cycle-aedes-

aegypti-yellow-fever-mosquito-en/

The life cycle starts with egg laying at the water surface level, a process called oviposition. Females deposit around 100 eggs during a single gonotrophic cycle, i.e. the period encompassing the host-seeking behaviour to find a blood meal until the egg laying. Larval biotopes, or breeding sites, are typically small artificial containers such as ornamental flowerpots, tires or any water-holding container in or around the peridomestic or domestic area. In addition, although less frequently, *Ae. aegypti* may also lay eggs in natural breeding sites such as plant's armpits that accumulate water. After selecting a suitable breeding site, the female will place a few eggs above the water surface on the inner wall of the container and move to another location, repeating the process, until all eggs of the batch are layed. This dispersion of the egg batch through several breeding places, known as "skip oviposition", is a common behaviour among Aedine species and a strategy to increase the survival probability of the species (Reiter et al. 1995).

When submerged by water, eggs will hatch, and undergo four larval stages until reaching the pupa stage. During this phase, considered a quiescence stage, a deep tissue rearrangement takes place and, after 1-2 days, the adult will emerge. Newly emerged females will search for a blood meal and a male for mating. As for any anautogenus species, *Ae. aegypti* females need a blood meal in order to mature their eggs. This blood meal is often from a human but females also feed on other vertebrates. This species is a very aggressive day-biting mosquito with endophagic (mainly indoor) and endophilic (resting indoor) habits. Adult *Ae. aegypti* specimens rarely disperse from the place where they emerge (Harrington et al. 2005). The flight dispersal is short (around 200 metres), usually dependent upon the availability of food sources and suitable oviposition sites.

One particular characteristic is fundamental to understand the history of dispersal and expansion of *Ae. aegypti*: eggs are resistant to desiccation, remaining viable for up to one year. This characteristic together with its peridomestic behaviour, have contributed to the successful human-mediated worldwide dispersion of the species.

2.2. History of *Aedes aegypti*

Aedes aegypti is native from Africa, where an ancestral darker form, or subspecies, *Ae. aegypti formosus* (Aaf), considered zoophilic, can be found in sylvatic habitats, breeding in tree and rock holes (Powell and Tabachnick, 2013). Anthropogenic changes may have interfered in the behaviour of *Ae. aegypti formosus*. In particular, water holding containers in the domestic and peridomestic area may have given rise to suitable mosquito-breeding sites, especially during extended dry seasons (Powell, 2018). It is presumed that after some generations, the *Ae. aegypti* populations exploiting these new larval habitats may have evolved a preference for human blood feeding, eventually giving rise to a “domesticated” paler form, denoted *Ae. aegypti aegypti* (Aaa) (Figure 4).

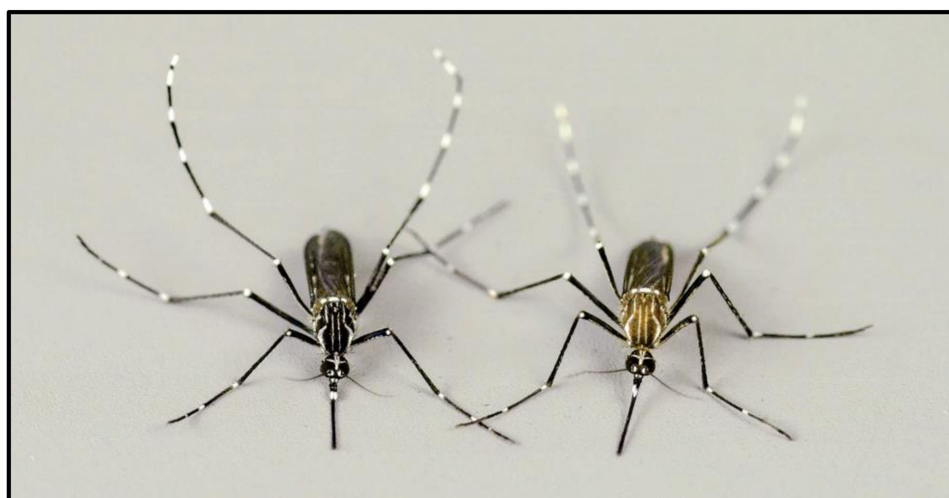

Figure 4. *Aedes aegypti* females of the African subspecies *formosus* (left) and the urban subspecies *aegypti* (right). Retrieved from Powell (2016).

Hybridization between forms is observed in the wild but its ecological or genetic consequences remains unclear (Powell, 2016). These subspecies differ in bioecological traits, vectorial capacity and competence, which render them different medical importance. Since *Ae. aegypti aegypti* is almost exclusively anthropophilic and it is the form that spread out of Africa becoming widely distributed across tropical and subtropical latitudes (Figure 5), it is considered the subspecies with major medical importance, responsible for the great majority of the arboviral outbreaks[1].

[1] For ease of communication, from here on we refer to *Ae. aegypti* as the subspecies *aegypti aegypti*, unless otherwise stated.

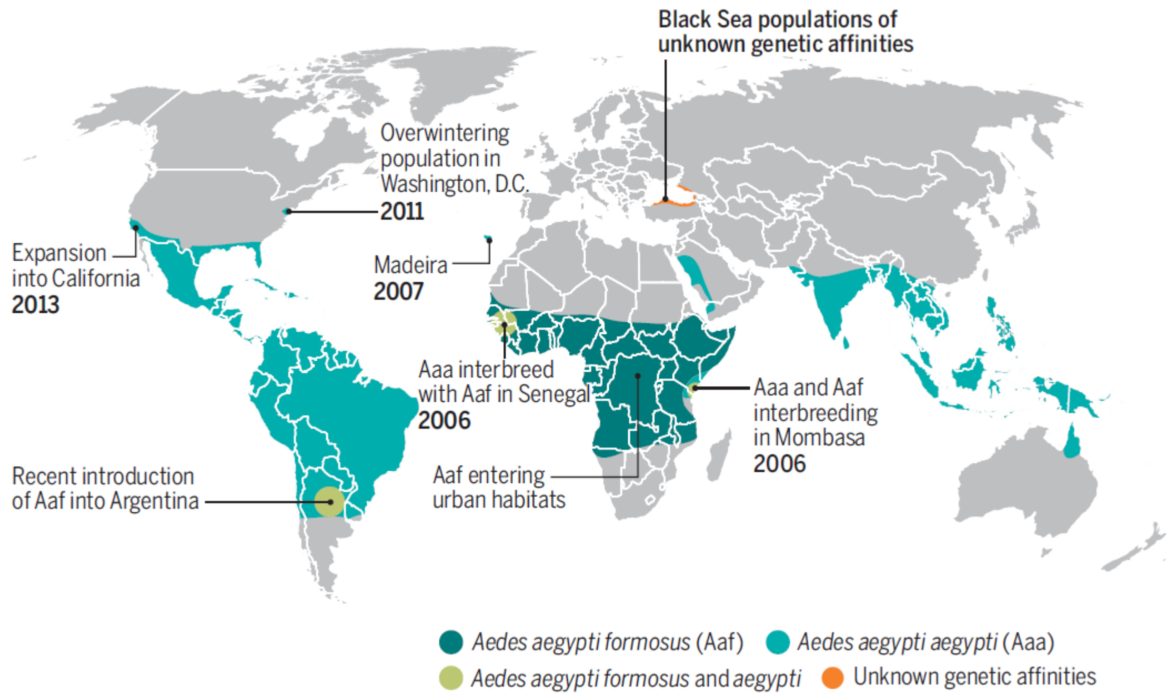

Figure 5. *Aedes aegypti* subspecies worldwide distribution. Year of introduction is given for locations newly infested with this mosquito species since 2006. Retrieved from Powell (2016).

Due to its desiccation-resistant eggs, *Ae. aegypti* has been able to move out of Africa and rapidly disperse worldwide. Historical and epidemiological records allow us to understand the spread of *Ae. aegypti* and invasion timeline (Powell and Tabachnick, 2013). Altogether, these records suggest a West African origin of this mosquito species that has spread to the New World, 400-500 years ago, aboard slave ships. At the same time, it is believed that this species has invaded southern Europe, reaching Spain and Portugal also through maritime transport. Around 1960 this species was present in several European countries such as France, Italy, Bosnia, Macedonia and Russia (Christophers, 1960). Although present in the Iberian Peninsula until mid-20th century, no record of this species has been reported in the last six decades. Its supposed disappearance is often associated with the malaria eradication campaigns carried out in the 1950's (Powell and Tabachnick, 2013). It is believed that the Asian continent was colonized during 19th century by specimens with origin in the New World (Brown et al. 2014). In Europe, re-emergence of this species was recently reported the eastern Black Sea coast (southern

Russia, Georgia, eastern Turkey) (Kotsakiozi et al. 2018a), Netherlands (Ibanez-Justicia et al. 2018), Canary Islands (ECDC, 2018), and in the Portuguese island of Madeira (Figure 6).

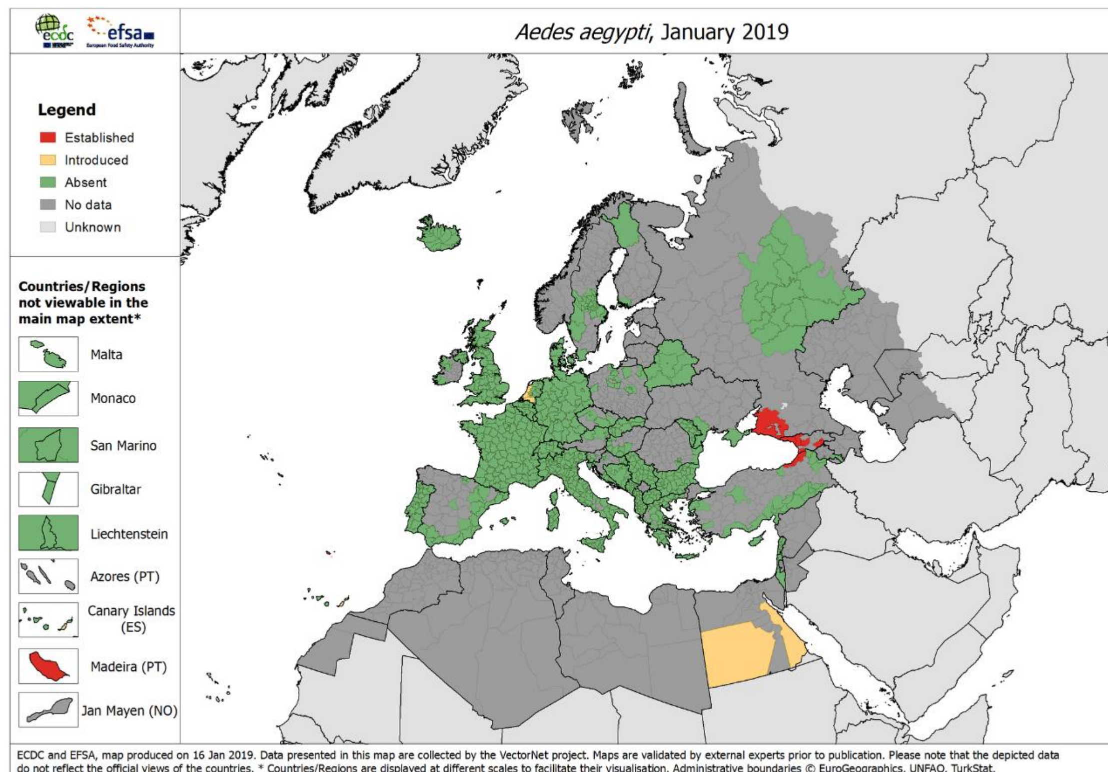

Figure 6. *Aedes aegypti* current distribution in Europe. Retrieved from https://ecdc.europa.eu/en/publications-data/aedes-aegypti-current-known-distribution-january-2019.

3. Population genetics as a tool against *Aedes aegypti*

Population genetics studies of *Ae. aegypti* have played an important role in tracing the colonization history of this species. It is presumed that the highly invasive capacity of this species is related to the high genetic variation of its genome (Mathews et al. 2018). For entomologists, population genetics has numerous applications. By determining the ongoing gene flow between populations we can more easily understand the role of arthropod vectors in disease epidemiology and design better vector control measures. For instance, population genetic studies helped unravelling the presence of the two subspecies of *Ae. aegypti* (Aaa and Aaf), with different epidemiological importances (Powell et al.

1980; Wallis et al. 1983; Brown et al. 2011; Brown et al. 2014; Kotsakiozi et al. 2018b). In addition, these studies also contributed to better elucidate the expansion and migration of *Ae. aegypti* between countries raising issues about the spread of insecticide resistance or other relevant genes, such as those involved in vector competence. The public health threat posed by a new species introduction may also be early assessed by determining the geographic origin of the invading individuals through population genetic studies (Failloux et al. 2002a).

The type of molecular markers used in population genetics studies has evolved overtime. Different genetic markers have been used for phylogenetic and population substructure analysis, ranging from isoenzymes (Failloux et al. 2002b), to random amplification of polymorphic DNA (RAPD) (Gorrochotegui-Escalante et al. 2000), mtDNA (Moore et al. 2013) or microsatellites (Slotman et al. 2007; Brown et al. 2011). Until recently, most of the population genetics studies with *Ae. aegypti* relied on mtDNA sequencing and microsatellite genotyping. However, the presence of mtDNA pseudogenes can make it difficult to obtain clear mtDNA sequences, resulting in misleading results (Hlaing et al. 2009; Behura et al. 2011). Nevertheless, mtDNA is still a useful marker to infer phylogenetic relationships between populations and colonization events in newly introductions of *Ae. aegypti* (Urdaneta-Marquez et al. 2008; Damal et al. 2013). Microsatellites were the genetic markers subsequently developed (Slotman et al. 2007; Lovin et al. 2009; Brown et al. 2011). Their relatively low cost, high levels of polymorphism and ease of scoring led to an increase on the number of studies using these markers (Putman and Carbone, 2014). Microssatellites are non-coding regions of simple repetitive DNA found throughout the eukaryote genome (Putman and Carbone, 2014). The length of a microsatellite sequence and the number of repeats may vary greatly from individual to individual due to the high rate of mutations that occur in these regions due to slippage during replication (Guichoux et al. 2011). Previous studies have used microsatellites to trace the evolutionary history (Brown et al. 2011; Brown et al. 2013; Gloria-Soria et al. 2016a), the population size (Saarman et al. 2017) and the temporal genetic stability of multiple Ae. *aegypti* populations (Gloria-Soria et al. 2016b).

More recently, the development of next generation sequencing technologies enabled the study of *Ae. aegypti* genetic background at a scale never observed before (Evans et al. 2015). Through genome annotation (Nene et al. 2007), these genomic technologies

enabled the design of high-throughput genotyping tools such as DNA arrays or chips (Strode et al. 2008; Evans et al 2015). These genomic tools are being used for the detection of detoxification genes overexpression associated with insecticide resistance (Strode et al. 2008), and for genotyping of thousands of single nucleotide polymorphisms (SNPs) that are being used to refine the evolutionary history of *Ae. aegypti* worldwide (Rašić et al. 2014; Rašić et al. 2015; Evans et al. 2015; Crawford et al. 2017; Sherpa et al. 2018; Kotsakiozi et al. 2018a). However, population genomics studies are still inaccessible to most laboratories due to its cost, need for specific equipment and bioinformatics expertise. Therefore, the cost-efficient microsatellite genotyping remains a widely used methodology for population genetics studies with *Ae. aegypti*. Futhermore, previous studies combining microsatellites and SNPs, showed comparable results in establishing patterns of genetic structure (Pless et al. 2017; Gloria-Soria et al. 2018; Kotsakiozi et al. 2018a). However, finer-resolution phylogenies are obtained when using SNPs markers when compared to microsatellites or mtDNA due to the higher number of polymorphic loci analysed (Pless et al. 2017; Gloria-Soria et al. 2018).

4. What else is driving *Aedes aegypti* expansion?

The ecological plasticity of *Ae. aegypti*, the ability to explore different larval breeding sites and the close association with humans, makes *Ae. aegypti* control a complex task. The most successful attempt to control and eradicate *Ae. aegypti* populations was made during the 1950-1960 period, when the Pan American Health Organization (PAHO) invested in a comprehensive, well-coordinated eradication campaign that resulted in the elimination of *Ae. aegypti* in almost all Central and South America territory (Hotez, 2016) (Figure 7). This eradication program was highly focused in breeding site reduction, implemented in collaboration with the military forces, and insecticide spraying with dichlorodiphenyltrichloroethane (DDT) (Hotez, 2016). However, after 1970, lack of financial support lead to the end of *Ae. aegypti* control activities. In consequence, regions free of *Ae.aegypti* were once more re-invaded (Figure 7).

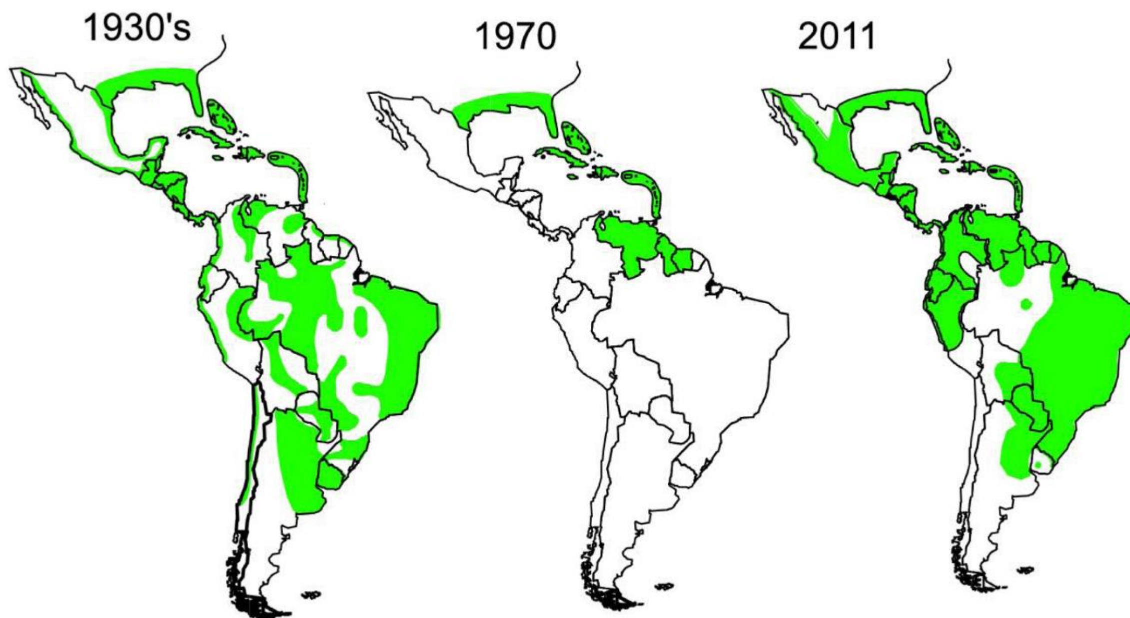

Figure 7. Distribution of *Ae. aegypti* in the Americas before and after the vector control program of PAHO. Retrieved from Gubler (2011).

4.1.Vector control methodologies

Since there are no vaccines or treatment against *aegypti*-transmitted arboviruses, vector control remains as the mainstay of arbovirus prevention by aiming to reduce human-mosquito contact (Simmons et al. 2012). Below, a selection of control methodologies, in use or under development, will be presented. These methodologies tools that derive from the understanding of *Ae. aegypti* bioecological features, in particular, the species breeding, resting and biting behaviours.

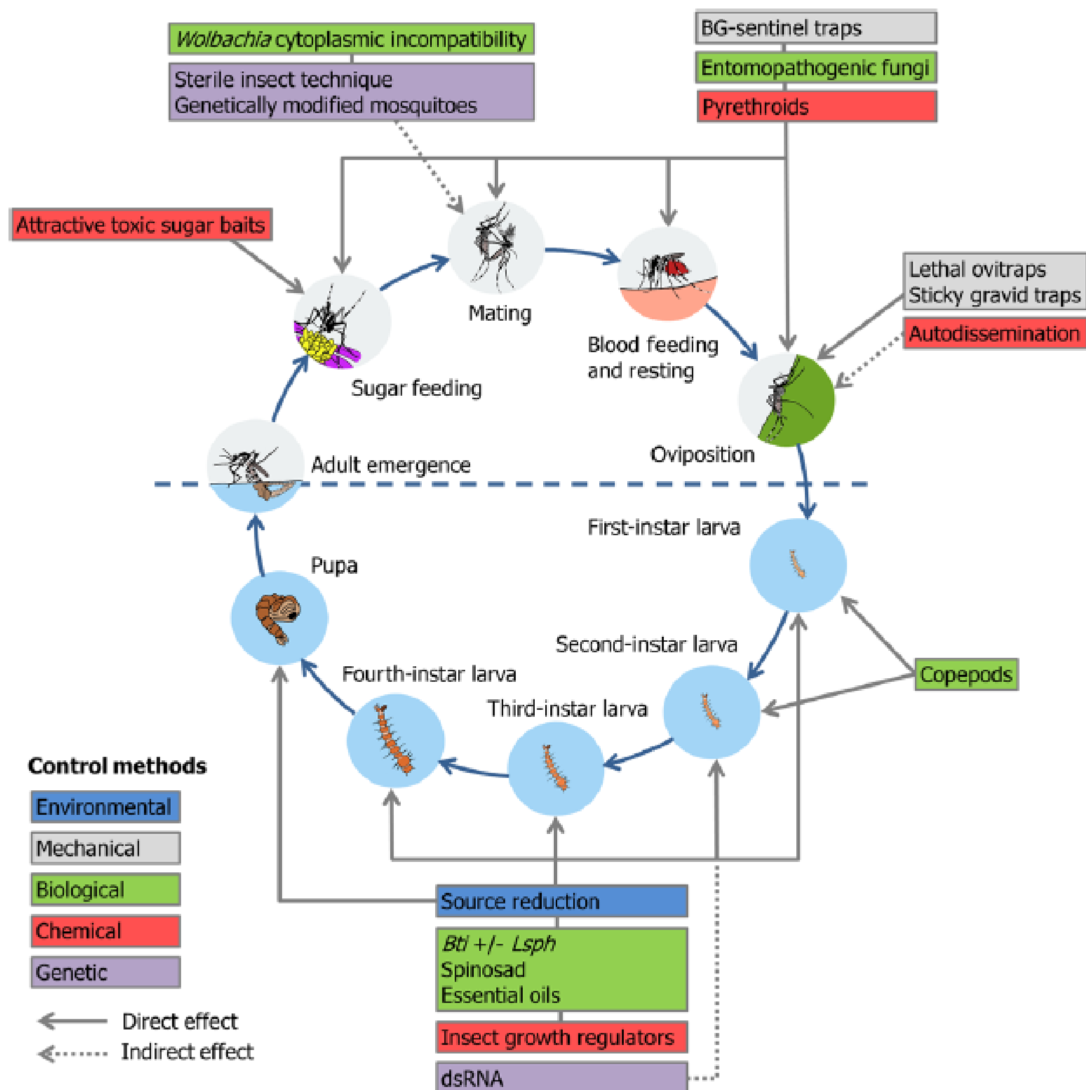

Figure 8. Control methodologies currently used or in development against *Ae. aegypti*. Retrieved from Baldacchino et al. (2015). Legend: dsRNA – double stranded RNA; *Bti* – *Bacillus thurigiensis israelensis*; *Lsph* – *Lysinibacillus sphaericus*.

Environmental management consists in the reduction of the mosquito population densities by reducing or eliminating present or potential *Ae. aegypti* breeding sites. This method is commonly known as source reduction and is a community-based strategy. This type of control approach is highly dependent on intersectorial collaboration with the participation of health authorities, education, public service and environmental entities in order to ensure community engagement. When community participation is significant, these strategies may play a major role in reducing mosquito densities since *Ae. aegypti* main

breeding sites are those inside or around the domestic and peridomestic area, frequently inaccessible to health or vector control workers (WHO, 2009).

Although mosquito traps are mainly used for monitoring of *Ae. aegypti* abundance, they can also be used as a control tool. Several traps have been developed to control and monitor immature or adult stages of *Ae. aegypti* populations. These include traps targeting gravid females (e.g. ovitraps or sticky traps) or host-seeking females (e.g. BG-Sentinel traps) in search for a blood meal (Figure 9) (Baldacchino et al. 2015).

Figure 9. Ovitrap (left image) and a BG-Sentinel trap (right image). Photos taken by the author.

Ovitraps are a simple and effective trap designed to be an inexpensive monitoring tool. They consist of a small black plastic bucket filled with water to two thirds and an oviposition support. Females will lay eggs in the support, enabling the detection of container-breeding mosquitoes and assessment of the adult population dynamics. However, when an insecticide or a sticky material is added to the ovitrap wall or water, entering females may be killed during oviposition. Different trap types and specifications are reviewed in Johnson et al. (2017).

The BG-Sentinel trap is nowadays the most commonly used adult monitoring tool to capture *Ae. aegypti* specimens (Krockel et al. 2006; Crepeau et al. 2013; Pombi et al. 2014). These traps can be used with a variety of mosquito attractants, which makes it a versatile tool for mosquito research and surveillance. In addition, *Ae. aegypti* specimens are kept alive inside the trap enabling virological or RNA-based arbovirus detection. The only difficulty using this trap is the constant need of electrical power. However, it is an eco-friendly tool since eliminates *Ae. aegypti* individuals from the total remaining

population without the use of insecticides (Englbrecht et al. 2015).

Most of the traps above mentioned are capable of a process called autodissemination, where contaminated mosquitoes are used as vehicles of insecticide dispersal (Devine et al. 2009). In this strategy, a mosquito is contaminated by an insecticidal compound in an autodissemination station and subsequently disperses the compound that will kill or interfere with mosquito development in subsequent contacts with untreated breeding sites or other mosquitoes. The compounds used in this strategy are insect growth regulators (IGRs) such as pyriproxyfen, and biocides such as the bacteria *Spinosad* and the fungus *Beauveria bassiana*, showing promising results in laboratory and field trials (Darriet et al. 2010; Jacups et al. 2014; Ocampo et al. 2014; Snetselaar et al. 2014).

Biological control is the reduction of a vector mosquito population by the introduction of natural predators, competitors, parasites and bacterial toxins of biological origin (Baldacchino et al. 2015). Biological agents are widely used in controlling mosquito larvae, including bacterial toxins (*e.g. Bacillus thurigiensis israelensis*, *Bti*), copepods, larvivorous fish and *Toxorhynchites* larvae that predate *Ae. aegypti* immatures. This method has some major advantages: i) no environmental contamination since it is chemical-free; ii) the organisms involved are predators and/or target-specific for mosquito species; iii) natural autodissemination may happen. However, larval control, either biological or chemical, may be logistically difficult to implement. The ability to detect, access and treat breeding sites is the greatest obstacle to *Ae. aegypti* larval control that often leads to a low area coverage in vector control programs.

Genetic control aims to induce genetic alterations in the target species that are disadvantageous to itself or the etiological agent that transmits. The first developed method was the Sterile Insect Technique (SIT) involving the release of sterile male insects to mate with the wild population present in the area. If mass releases of irradiated sterile males occur into an area, when these males mate with the local female population, non-viable offspring is produced. The continuous release of these sterile males will result in the reduction and subsequent elimination of a specific mosquito population (Alphey et al. 2010). However, this technique has not been widely used against mosquitoes due to the radiation effects on the male fitness, the need to produce large number of insects and the difficulty of separating males from females before field release. Nonetheless, this method

is being tested in Italy against invasive populations of *Aedes albopictus* as a tool to supress mosquito populations (Bellini et al. 2013). Sterile insect technique strategies may also be employed in combination with pyriproxyfen autodissemination, a strategy called "Boosted SIT" (Bouyer and Lefrançois, 2014), where pyriproxyfen-treated sterile males are released and will, subsequently, contaminate females and disseminate the compound throughout the target area.

Another genetic method with a potential role in the control of arboviral diseases involves *Wolbachia*-infected *Ae. aegypti* mosquitoes (Hoffmann et al. 2011). Although present in 65% of insect species (McGraw and O'Neill, 2013), *Wolbachia pipientis* is an endosymbiotic bacteria that is not naturally present in *Ae. aegypti* species. Recent studies revealed that *Wolbachia* can spread rapidly into the host population as a consequence of cytoplasmic incompatibility (CI) and due to maternal inheritance (Hoffmann et al. 2011; Hoffmann et al. 2014). There are two types of releases with *Wolbachia*-infected mosquitoes: one where *Wolbachia*-infected males mate with uninfected females generating unfertile eggs, called incompatible insect technique (IIT), meant to suppress the wild population; or the release of infected females that mate with uninfected and infected males and generate fertile offspring, facilitating the spread of *Wolbachia* in the target population (Figure 10). Previous laboratory and field experiments in Australia, Vietnam and Brazil revealed that *Wolbachia* spread quickly in the wild *Ae. aegypti* population (Hoffmann et al. 2011; Dutra et al. 2015;Nguyen et al. 2015). The importance of these results is related to the fact that *Wolbachia* interferes with the ability of *Ae. aegypti* to transmit several arboviruses including chikungunya, dengue and Zika (Aliota et al. 2016a; Aliota et al. 2016b). However, the long-term efficacy of this technology still needs to be proven.

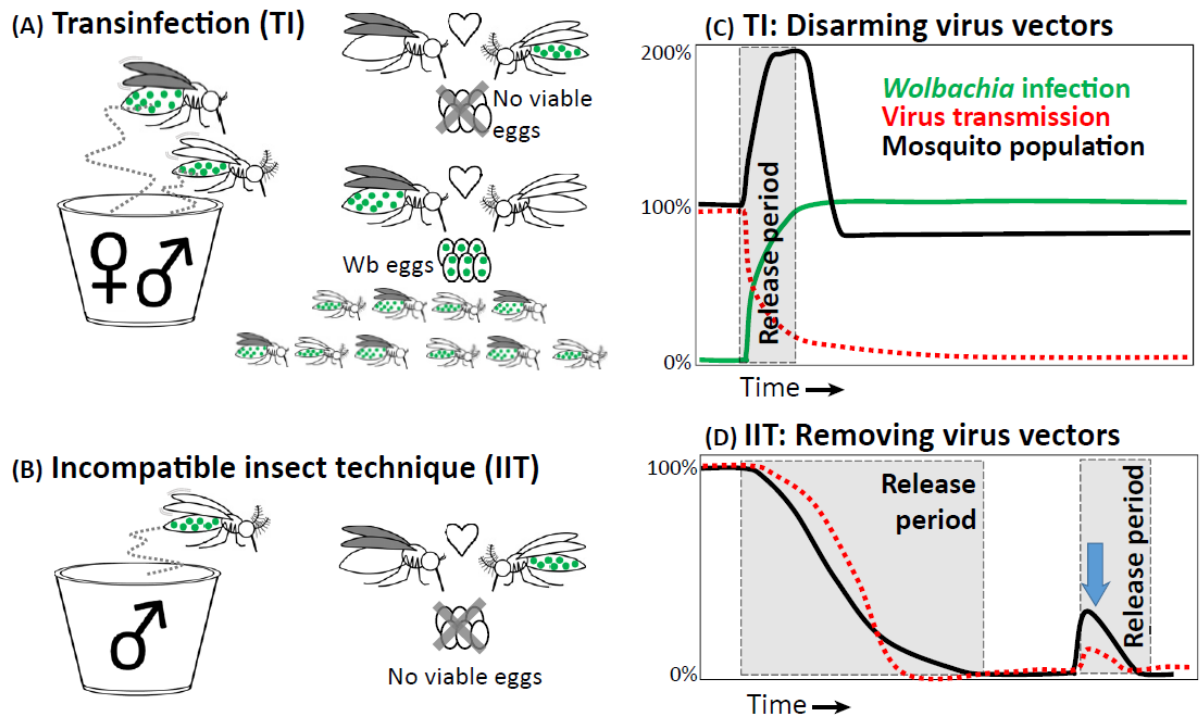

Figure 10. Different outcomes of *Wolbachia*-infected *Ae. aegypti* mosquitoes in the wild populations. Retrieved from Ritchie et al. (2018).

Another innovative approach that is being tested in field releases is known as release of insects carrying a dominant lethal allele (RIDL). This strategy replaces the need for harmful irradiation used in SIT by the use of genetically modified male mosquitoes carrying a late acting lethal gene that, when expressed, will cause the death of the larvae or pupae (Figure 11) (Alphey et al. 2010). This strategy has been field deployed in Cayman Islands (Harris et al. 2012), Malaysia (Lacroix et al. 2012), Brazil (Carvalho et al. 2015) and Panama (Gorman et al. 2016) and *Ae.aegypti* suppression was achieved with considerable results (80-95% of reduction in mosquito densities).

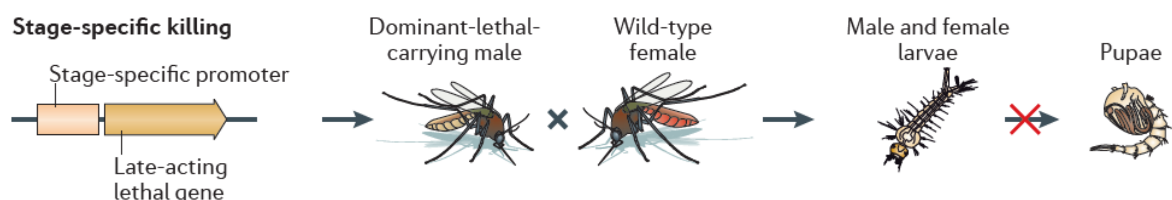

Figure 11. Release of insects carrying a dominant lethal allele (RIDL). Males carrying

the lethal transgene are released in the field and mate with wild type females. The resulting offspring die before reaching the pupal stage. Retrieved from McGraw and O'Neill (2013).

However, for genetic control technologies to succeed, public and community engagement is mandatory coupled with regulatory agencies requirements that are necessary to fulfil.

Chemical control is still a major tool for the control of *Ae. aegypti* (Vontas et al. 2012; Moyes et al. 2017; Fernandes et al. 2018). It is the primary strategy in an outbreak scenario when rapid reduction of adult mosquito densities is urgently needed. There are four major classes of insecticides approved for mosquito control: organophosphates, organochlorines, carbamates and pyrethroids. An alternative to these common insecticide classes are the IGRs in which increased interest is observed due to its different mode of action (Lau et al. 2015). Due to European directives, IGRs and pyrethroids are the most used chemical compounds in mosquito control strategies in European territory (Baldacchino et al. 2015). Therefore, special importance will be given to these chemical compounds.

Insect grown regulators, such as pyriproxyfen or methoprene, are chemical compounds that mimic the hormones responsible for insect development and growth (Devine et al. 2009). Hence, immature development is blocked and subsequent death is observed after few days. IGRs are environmentally safe, ideal for integration with other vector control tools and could provide high-coverage when using with the autodissemination technique (Devine et al. 2009). In addition, previous studies showed that, when in contact with IGRs, reduced female fertility and fecundity is observed (Ohba et al. 2013). Pyrethroids represent the latest synthetic insecticide class authorized by the European Union (EU) and the most used in vector control programs mostly due to their low toxicity for humans and mammals and because of their effectiveness and low cost (Baldacchino et al. 2015). Their target is the insect nervous system, where they act on the *Vgsc*, causing paralysis and subsequent mosquito death (Hemingway et al. 2004). Today, these compounds are used in insecticide-treated materials (ITMs), such as nets or curtains, in indoor residual spraying (IRS) (Paredes-Esquivel et al. 2016; Hladish et al. 2018) and as spatial repellents (Buhagiar et al. 2017; Bibbs et al. 2018).

Another approach is the development of attractive toxic sugar baits (ATSB) (Muller et al. 2010; Naranjo et al. 2013) involving the use of insecticides in a sugar meal solution that attract and kill sugar-seeking mosquitoes (Baldacchino et al. 2015). Since both males and females require sugar throughout mosquito's lifespan, the potential of these traps is considerable for vector control strategies. These bait stations have been successful against mosquito vectors (Stewart et al. 2013; Revay et al. 2014; Qualls et al. 2015a) and sand flies (Qualls et al. 2015b). In addition, viral surveillance in host-seeking mosquitoes is possible by using only the sugar-based solution and removing the toxic compound (van der Hurk et al. 2014; Girod et al. 2015). This approach allows tracking arbovirus circulation in wild mosquito populations and estimate the risk of transmission to humans (Flies et al. 2015).

In conclusion, chemical control has been the backbone of vector control programs against *Ae. aegypti*. Unfortunately, the outcome of these strategies have not been satisfactory and, instead, increased vector dispersal and the spread of *aegypti*-borne epidemics is alarming. Insecticide-based strategies are highly dependent on *Ae. aegypti* population susceptibility to the chemicals used. Therefore, the evolution and spread of insecticide resistance mechanisms in several mosquito populations have contributed for the inefficiency of these strategies (Maciel-de-Freitas et al. 2014).

5. Insecticide resistance

The continuous and disproportionate use of insecticides in agricultural practices and in public health has driven the evolution of insecticide resistance by selecting resistance mechanisms that allow a small proportion of the mosquito population to survive and thrive despite insecticide applications (Figure 12). Knowledge about the population susceptibility status and the underlying resistance mechanisms present in a new given mosquito vector population will be essential for future designing of efficient vector control programs.

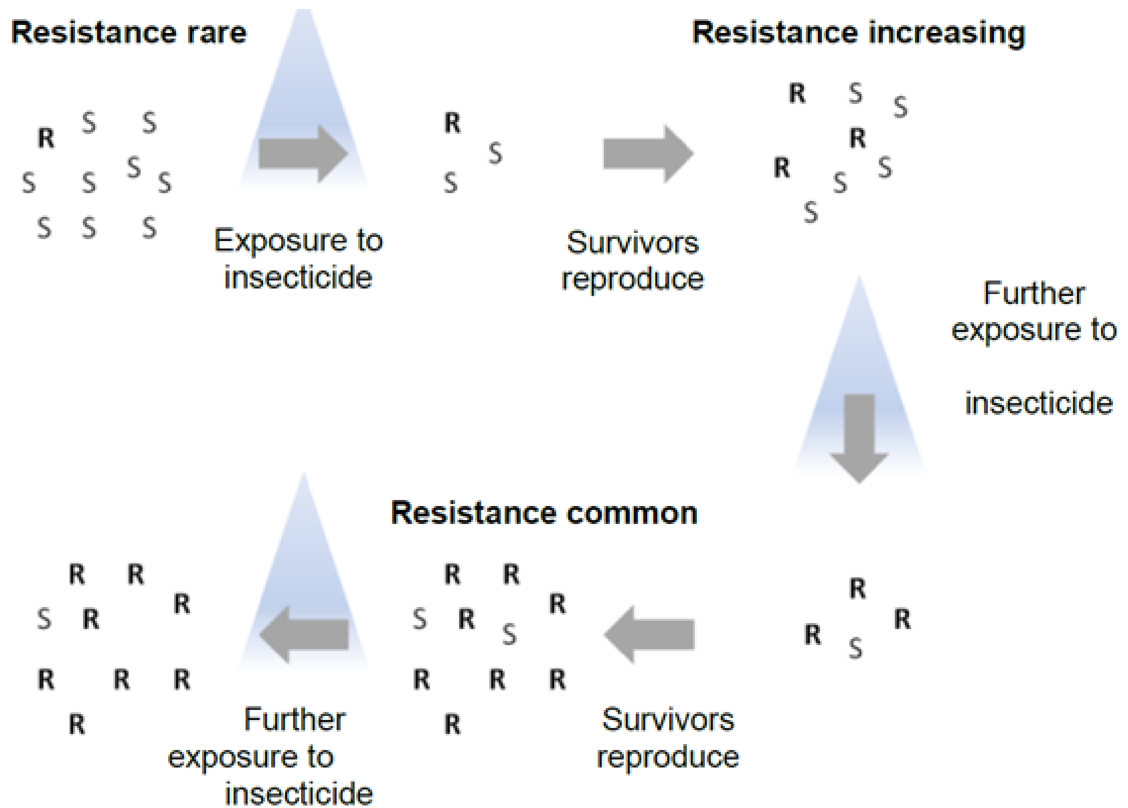

Figure 12. Evolution of insecticide resistance in mosquito populations. Adapted from IRAC (2010).

According to the WHO, insecticide resistance is "the ability of mosquitoes to survive exposure to a standard dose of insecticide; this ability may be the result of physiological or behavioural adaptation. The emergence of insecticide resistance in a vector population is an evolutionary phenomenon due to either behavioural avoidance (e.g. exophily instead of endophily) or physiological factors whereby the insecticide is metabolized, not potentiated, or absorbed less in resistant mosquitoes than by susceptible mosquitoes" (WHO, 2016).

Insecticide resistance is now widespread in several *Ae. aegypti* populations throughout the globe (Moyes et al. 2017), constituting a serious threat for vector control strategies. Therefore, it is essential to better elucidate these resistance mechanisms in order to prolong the effectiveness of current insecticides and ensure the sustainability of vector control programs.

5.1.Insecticide resistance mechanisms

Resistance to insecticides may occur due to several mechanisms: as an avoidance behaviour to the insecticidal compound (behavioural resistance), modification of the insecticide target by mutation or structural change (target-site resistance), a decrease in the insecticide penetration into the mosquito body (cuticular resistance), or sequestration within the body or degradation by detoxification enzymes (metabolic resistance) (Hemingway et al. 2004) (Figure 13).

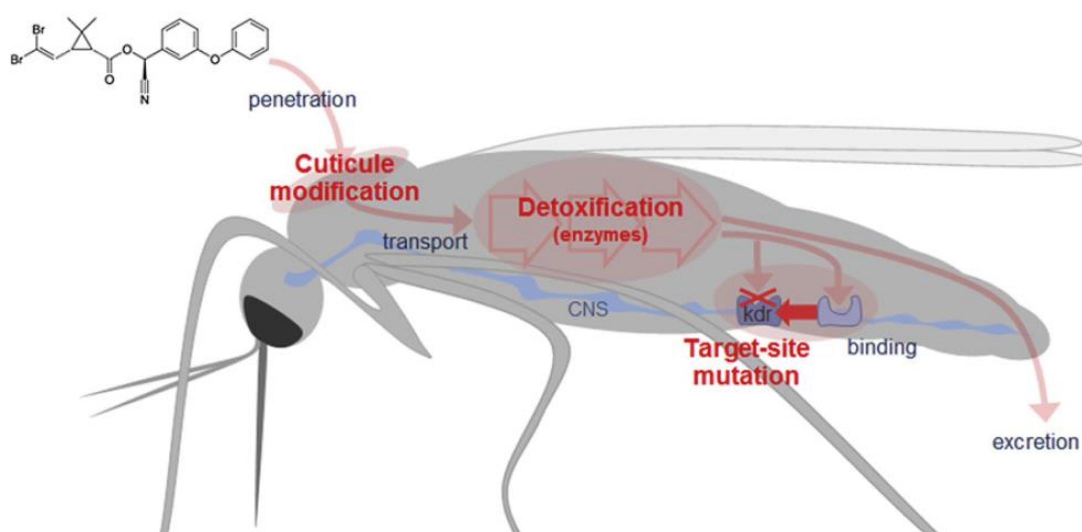

Figure 13. Main insecticide resistance mechanisms in mosquitoes. CNS: central nervous system. Retrieved from Nkya et al. (2013).

Multiple mechanisms may be present in a single insect providing cross-resistance to insecticides. Reduced penetration or cuticular resistance is caused by modifications in the cuticle that prevent or slow the absorption or penetration of insecticides into the mosquito (Balabanidou et al. 2018). However, further studies are required in order to identify the significance of this type of resistance in *Ae. aegypti* species (Moyes et al. 2017). Target-site resistance consists of an amino acid change in the target-site receptor that will reduce the binding to the insecticide compound. This type of resistance occurs in the acetylcholinestare (AChE), GABA and in the *Vgsc* receptors (Hemingway and Ranson, 2004). Of these, the presence of mutations on the *Vgsc*, also known as knockdown

resistance mutations (*kdr)*, have been the only target-site mutations widely detected in *Ae. aegypti* populations (reviewed in Moyes et al. 2017). Individuals carrying these mutations are less affected by the knockdown effect caused by the contact with pyrethroids or DDT, the insecticide classes that target the *Vgsc*. As more research about the molecular basis of insecticide resistance is made, additional *kdr* mutations associated with this resistance mechanism are detected (Haddi et al. 2017; Saavedra-Rodriguez et al. 2018). Metabolic resistance consists in the overexpression of several enzyme families that will degrade the insecticide before reaching its final target. This mechanism is conferred by the overexpression of detoxification enzymes and/or qualitative modifications of their protein sequence, improving their affinity and/or their catalytic activity against insecticides. The most important enzyme families involved in this mechanism are the cytochrome P450 monooxygenases (P450s), carboxyl/choline esterases (CCEs) and Glutathione-S-transferases (GSTs) (Figure 14) (Vontas et al. 2012; Smith et al. 2016; Moyes et al. 2017). These two important resistance mechanisms and their role in conferring resistance to each insecticide class are represented in Figure 14.

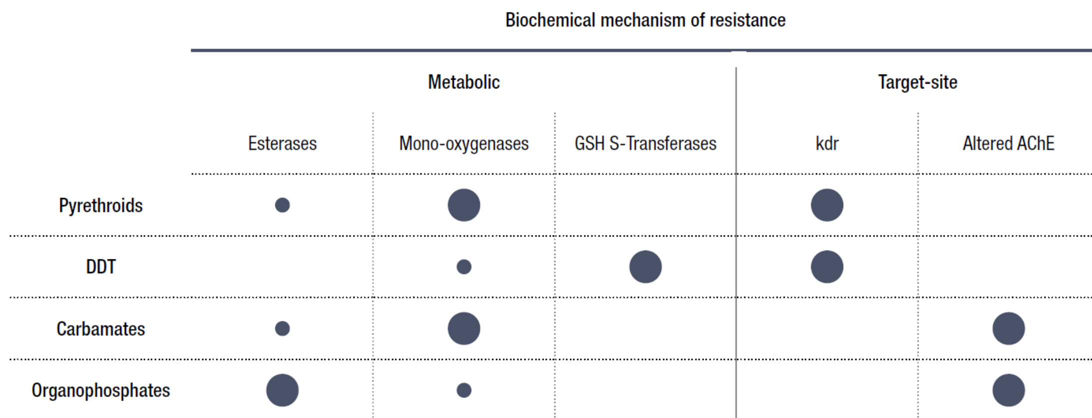

Figure 14. Cross resistance patterns of different classes of insecticides. Retrieved from **WHO (2012)**.

While there is no large-scale implementation of alternative vector control methods or vaccines against the pathogens transmitted by this vector, better knowledge about these resistance mechanisms is desirable for a better detection and resistance management of

Ae. aegypti field populations. Until recently, resistance mechanisms detection has been constrained by the available techniques, which consisted on polymerase chain reaction (PCR) for detection of target-site mutations and of synergist and biochemical assays for detection of increased enzymatic activity (Coleman and Hemingway, 2007). The annotation of *Ae. aegypti* genome (Nene et al. 2007) and the subsequent development of a microarray technique, named "*Aedes* detox chip", enabled the high-throughput analysis of overexpression of detoxification genes associated with metabolic resistance (Strode et al. 2008; Grisales et al. 2013). The recent discovery of copy number variations (CNVs) at genes coding for detoxification enzymes in multiple *Ae. aegypti* populations, using novel RNA-based sequencing techniques, suggests that this mechanism may play a major role in resistance phenotype (Faucon et al. 2015; Faucon et al. 2017). These studies also highlighted the presence of genetic polymorphisms in regulator/promoter gene regions affecting the final enzyme structure, which will allow the design of DNA-based markers for early-detection and monitoring of metabolic resistance in *Ae. aegypti.*

6. *Aedes aegypti* in Madeira Island

A paradigmatic example of an invasive and thriving *Ae. aegypti* population is the case of *Ae. aegypti* in Madeira Island. This mosquito was first detected in Madeira in October 2005 (Margarita et al. 2006). After complaints by the human population about aggressive mosquito bites causing severe allergic reactions, initial entomological studies revealed the presence of *Ae. aegypti* in several Funchal parishes (Margarita et al. 2006). Following the introduction and subsequent establishment, *Ae. aegypti* expanded to the neighbouring counties of Câmara de Lobos and Santa Cruz during 2006-2008 (Gonçalves et al. 2008). From 2008 to 2012, *Ae. aegypti* continued its successful expansion throughout the southern coast, reaching Calheta and Machico counties (Figure 15).

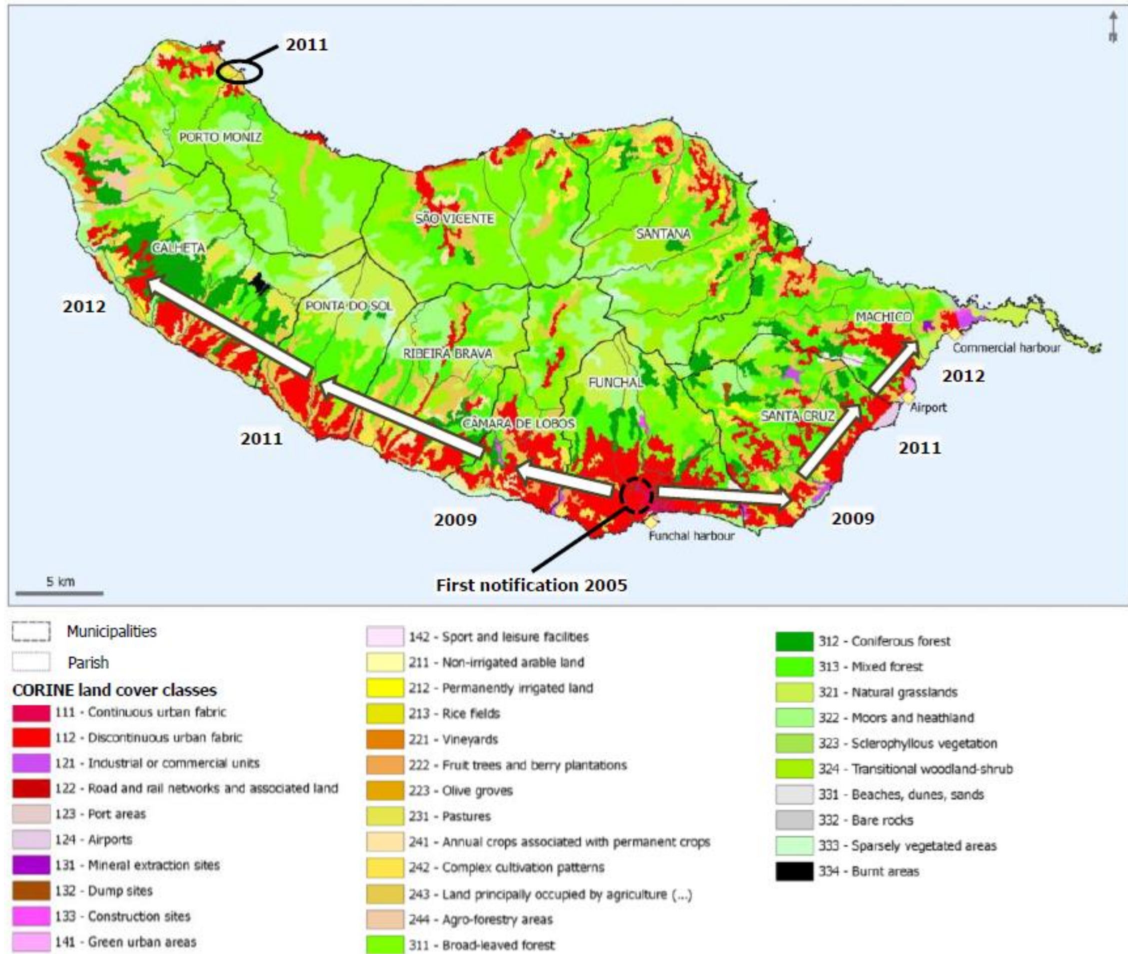

Figure 15. Expansion of *Ae. aegypti* and land cover categories in Madeira Island. Adapted from ECDC (2014).

The ecological plasticity of this mosquito species, together with the favourable climate and ecological conditions in Madeira, are major features associated with the success of establishment and subsequent expansion of *Ae. aegypti* in the island. In fact, the abundance of flowerpots, a famous tradition in Madeira, serves perfectly to *Ae. aegypti* as an optimal breeding site for larval stages (Almeida et al. 2007).

6.1. Surveillance and vector control implemented against *Aedes aegypti* in Madeira Island

After the introduction of *Ae. aegypti* in the island, Madeira Health Authorities promptly initiated vector control campaigns, during late 2005 to May 2008, in order to contain and,

if possible, eradicate the species from Funchal. These comprised educational campaigns for the community, about personal protection and source reduction, and chemical (pyrethroids and one organophosphate) and biological (*Bti*) insecticides to kill immatures and adult stages of *Ae. aegypti* (ECDC, 2014).

Unfortunately, *Ae. aegypti* in Madeira was able to withstand the control strategies implemented during this period. Trap monitoring made during 2006-2008, by the Natural History Museum of Funchal, revealed the expansion of *Ae. aegypti* inside and outside Funchal to near municipalities and counties (Gonçalves et al. 2008). After 2008, community-based strategies were intensified in order to provide knowledge and advice to the human population about reducing mosquito densities. In 2009, WHO bioassays were performed in order to estimate the susceptibility of *Ae. aegypti* from Funchal to several insecticides. Results revealed resistance to pyrethroids and DDT, and susceptibility to malathion (Seixas, 2012). Therefore, insecticide resistance may have contributed to the apparent failure in containing the expansion of *Ae. aegypti* in Madeira Island. However, these preliminary results should be considered with caution since assays were performed without a reference susceptible *Ae. aegypti* colony as a control.

In 2011, one year before the dengue outbreak, an island-wide survey with ovitraps was carried out in order to update the distribution of the mosquito in the island. Results showed the expansion *Ae. aegypti* west and eastwards, and even a sporadic introduction in Porto Moniz (North). However, the species was never again detected in this location, possibly due to the cooler climate conditions or to the low number of invading mosquitoes in this particular area. Based on the results of this survey, the ovitraps network was extended and complemented, with adult traps (BG-Sentinel trap) in Funchal area to monitor *Ae. aegypti* abundance and dispersal (Nazareth et al. 2014). In the same year, Madeira Health Authorities joined the "Rede de Vigilância de Vetores", an entomological surveillance network coordinated by the National Institute for Health.

The ovitrap and BG-Sentinel traps network (Figure 16) is still running nowadays and has been crucial in collecting weekly data for *Ae. aegypti's* surveillance in Madeira Island. During the dengue outrbreak of 2012, the network was critical in providing the Health Authorities with important data, pinpointing locations with the highest mosquito densities to deploy essential resources.

Figure 16. Ovitrap distribution in Madeira Island in the week 30 of 2018. Legend: green circle – negative ovitrap; red circle – positive ovitrap; white circle – ovitrap with no data. Retrieved from: http://iasaude.sras.gov-madeira.pt/naomosquito/.

Additional control efforts were made during the dengue outbreak in 2012. Educational campaigns were reinforced through television, radio communications and door-to-door visits in the hotspot mosquito areas, in order to show the residents how to remove *Ae. aegypti* breeding sites (ECDC, 2014). During the outbreak, storm drains were found to be highly infested with *Ae. aegypti* larvae. Therefore, the City Hall of Funchal implemented a widespread vector control campaign in Funchal, called "Plano Municipal de Combate ao Mosquito Vetor de Transmissão da Dengue", that relied on salt application in every positive storm drain for *Ae. aegypti* larvae. This program had the duration of two years, specifically during 2013-2014.

Nowadays, the ongoing vector control program in Madeira relies on community-based strategies and island-wide *Ae. aegypti* trap surveillance, especially in the International Airport and in the Funchal harbour where exportation/importation of *Ae. aegypti* or even new vectors may occur. In addition, the Madeira Dengue Surveillance System (MDSS), implemented since the dengue outbreak, is responsible for the detection of imported cases into the island. This notification system proved to be valuable for epidemiological surveillance of probable and confirmed cases of arboviral diseases in the region (ECDC,

2014).

6.2.Dengue outbreak in Madeira – a threat for Europe

Seven years after the introduction of *Ae. aegypti* in Madeira, a dengue outbreak occurred in the island. This outbreak was the first in European territory since the 1928 epidemics in Greece (Halstead and Papaevangelou, 1980), and the first ever in Portugal. Entomological surveillance and epidemiological studies found unusual high densities of *Ae. aegypti* and DENV-1, with phylogenetic relationships with South America, as the circulating virus in the region (Sousa et al. 2012; Alves et al. 2013). The magnitude of the outbreak and the fact that Madeira is an attractive tourist destination led to multiple exported cases to mainland Europe (Frank et al. 2013; Huhtamo et al. 2013). The majority of dengue cases were recorded in Funchal (77%) where more than half of the Madeira population lives (ECDC, 2014).

The risk for future *aegypti*-borne outbreaks in Madeira remains. The strong tourism-based economy and socioeconomic relationships with South America countries may facilitate the introduction of new *Ae. aegypti* populations, and, viremic travellers into Madeira. In addition, the recent crisis in Venezuela is causing the return of several Madeiran emigrants to the island, increasing the movement of people and goods coming from Caracas, the only direct flight from South America to Funchal city (Wilder-Smith et al. 2014). The high danger of importation of dengue as well as other arboviruses, such as chikungunya or Zika, highlight the need for continuous surveillance of mosquito populations. In this matter, the evaluation of vector competence is a crucial parameter for assessing the risk of arboviral transmission in Madeira. Competence for DENV-1 transmission is already presumed but no scientific data is available for other DENV serotypes or even for other arboviruses. Knowledge regarding this issue will allow adaptation of surveillance and control systems operating in the island, such as the MDSS implemented in 2012.

6.3.Genetics of *Aedes aegypti* in Madeira

The rapid expansion of *Ae. aegypti* in Madeira raised questions about the genetic

background of this recently introduced species. The first genetic study conducted in the region, with mosquito samples from 2009, revealed low mtDNA genetic variation levels (one mtDNA haplotype) and the presence of two common *kdr* mutations associated with insecticide resistance (Seixas et al. 2013). In addition, mtDNA results, together with *kdr* mutations and socioeconomic information, pointed to South America, namely Brazil and Venezuela, as the most likely source of *Ae. aegypti* in Madeira. However, this study did not provide information on vector population structure or the colonization history of this mosquito species in the island. More studies with highly polymorphic genetic markers, such as microsatellites, are required to refine the origin of this population and to provide information regarding the genetic structure and number of *Ae. aegypti* introductions into the island.

7. Objectives and thesis outline

The first objective of this thesis was to characterize insecticide susceptibility and resistance mechanisms in *Ae. aegypti* from Madeira Island in order to fill the knowledge gap about the resistance profile of this insular population. The second objective was to assess the origin, genetic structure and evolutionary history of this recently introduced population. These studies are of great interest, not only to associate with insecticide resistance data, but also to provide baseline information for eventual use of genetically modified mosquitoes in vector control strategies. Finally, the third objective was to perform vector competence studies to the major arboviruses transmitted by this vector, as these are mandatory to assess the risk of arboviral emergence in Madeira.

The studies conducted to accomplish the proposed objectives are described in the following chapters:

- **Chapter 2** describes the first study addressing the molecular basis of insecticide in *Ae. aegypti* from Madeira Island. The results obtained provide an update of insecticide resistance status of this species and possible vector control strategies are identified in order to reduce the probability of emergence and the impact of vector-borne diseases.
- **Chapter 3** is devoted to the population genetics of *Ae. aegypti* in Madeira. Microsatellite and mitochondrial DNA markers were used in order to detect single

or multiple introductions and assess the origin and genetic structure of mosquito populations in Madeira. This chapter also provided an opportunity to assess the outcome of previous and ongoing vector control programs in the region.

- **Chapters 4 and 5** describe the vector competence studies performed with *Ae. aegypti* populations from Madeira Island for dengue, chikungunya and Zika viruses.
- **Chapter 6** is dedicated to the concluding remarks of this thesis, as well as the perspetives for future research.

8. References

Aliota MT, Walker EC, Uribe Yepes A, Dario Velez I, Christensen BM, Osorio JE. The wMel Strain of *Wolbachia* reduces transmission of Chikungunya virus in *Aedes aegypti*. PLoS Negl Trop Dis. 2016a;10(4):1–13.

Aliota MT, Peinado SA, Velez ID, Osorio JE. The wMel strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*. Sci Rep; 2016b;6:1–7.

Almeida APG, Gonçalves YM, Novo MT, Sousa CA, Melim M, et al. Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Euro Surveill. 2007;12: E071115.6.

Alphey L, Benedict M, Bellini R, Clark GG, et al. Sterile-insect methods for control of mosquito-borne diseases: an analysis. Vector Borne Zoonotic Dis. 2010; 10(3), pp.295–311.

Alves MJ, Fernandes PL, Amaro F, Osório H, Luz T, Parreira P, et al. Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012. Euro Surveill. 2013 Jan;18(6):3–6.

Balabanidou V, Grigoraki L, Vontas J. Insect cuticle: a critical determinant of insecticide resistance. Curr Opin Insect Sci. 2018;27:68–74.

Baldacchino F, Caputo B, Chandre F, Drago A, della Torre A, Montarsi F, et al. Control methods against invasive *Aedes* mosquitoes in Europe: A review. Pest Manag Sci. 2015;71(11):1471–85.

Behura SK, Lobo NF, Haas B, deBruyn B, Lovin DD, Shumway MF, et al. Complete sequences of mitochondria genomes of *Aedes aegypti* and *Culex quinquefasciatus* and comparative analysis of mitochondrial DNA fragments inserted in the nuclear genomes. Insect Biochem Mol Biol. 2011;41(10):770–7.

Bellini R, Medici A, Puggioli A, Balestrino F, Carrieri M. Pilot field trials with *Aedes albopictus* irradiated sterile males in Italian urban areas. J Med Entomol. 2013;50(2):317–25.

Bennett KE, Olson KE, Muñoz MDL, Fernandez-Salas I, Farfan-Ale J a, Higgs S, et al. Variation in vector competence for dengue 2 virus among 24 collections of *Aedes aegypti* from Mexico and the United States. Am J Trop Med Hyg. 2002 Jul;67(1):85–92.

Bibbs CS, Hahn DA, Kaufman PE, Xue R. Sublethal effects of a vapour-active pyrethroid, transfluthrin, on *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae) fecundity and oviposition behaviour. Parasit Vectors. 2018;11(1):486.

Bouyer J, Lefrançois T. Boosting the sterile insect technique to control mosquitoes. Trends Parasitol. 2014;30(6):271–3.

Brown JE, McBride CS, Johnson P, Ritchie S, Paupy C, Bossin H, et al. Worldwide patterns of genetic differentiation imply multiple “domestications” of *Aedes aegypti*, a major vector of human diseases. Proc Biol Sci. 2011;278(1717):2446–54.

Brown JE, Evans BR, Zheng W, Obas V, Barrera-Martinez L, Egizi A, et al. Human impacts have shaped historical and recent evolution in Aedes aegypti, the dengue and yellow fever mosquito. Evolution. 2014;68(2):514–25.

Buhagiar TS, Devine GJ, Ritchie SA. Metofluthrin: investigations into the use of a volatile spatial pyrethroid in a global spread of dengue, chikungunya and Zika viruses. Parasit Vectors. 2017;10(1):1–11.

Carvalho DO, McKemey AR, Garziera L, Lacroix R, Donnelly CA, Alphey L, et al. Suppression of a field population of *Aedes aegypti* in Brazil by sustained release of transgenic male mosquitoes. PLoS Negl Trop Dis. 2015;9(7):1–15.

Christophers R. *Aedes aegypti* (L.) The Yellow Fever Mosquito: Its life history, bionomics and Structure. 1960;54.

Crawford JE, Alves JM, Palmer WJ, Day JP, Sylla M, Ramasamy R, et al. Population genomics reveals that an anthropophilic population of *Aedes aegypti* mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector. BMC Biol; 2017;15(1):1–16.

Crepeau TN, Healy SP, Bartlett-Healy K, Unlu I, Farajollahi A, Fonseca DM. Effects of Biogents sentinel trap field placement on capture rates of adult Asian tiger mosquitoes, *Aedes albopictus*. PLoS One. 2013;8(3):1–7.

Coleman M, Hemingway J. Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. J Pestic Sci. 2007;32(2):69–76.

Damal K, Murrell EG, Juliano SA, Conn JE, Loew SS. Phylogeography of *Aedes aegypti* (yellow fever mosquito) in South Florida: MtDNA evidence for human-aided dispersal. Am J Trop Med Hyg. 2013;89(3):482–8.

Darriet F, Marcombe S, Etienne M, Yébakima A, Agnew P, Yp-Tcha M-M, et al. Field evaluation of pyriproxyfen and spinosad mixture for the control of insecticide resistant *Aedes aegypti* in Martinique (French West Indies). Parasit Vectors. 2010;3:88.

Dennison NJ, Jupatanakul N, Dimopoulos G. The mosquito microbiota influences vector competence for human pathogens. Curr Opin Insect Sci. Elsevier Inc; 2014;3:6–13.

Devine GJ, Perea EZ, Killeen GF, Stancil JD, Clark SJ, Morrison AC. Using adult mosquitoes to transfer insecticides to *Aedes aegypti* larval habitats. Proc Natl Acad Sci. 2009;106(28):11530–4.

Dutra HLC, dos Santos LMB, Caragata EP, Silva JBL, Villela DAM, Maciel-de-Freitas R, et al. From bab to field: the influence of urban landscapes on the invasive potential of *Wolbachia* in Brazilian *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2015;9(4):1–22.

European Centre for Disease Prevention and Control. Dengue outbreak in Madeira, Portugal, March 2013. Stockholm: ECDC; 2014.

European Centre for Disease Prevention and Control, 2018. https://ecdc.europa.eu/en/news-events/new-settlements-aedes-aegypti-raising-concerns-continental-eu

Englbrecht C, Gordon S, Venturelli C, Rose A, Geier M. Evaluation of BG-Sentinel Trap as a management tool to reduce *Aedes albopictus* nuisance in an urban environment in Italy. J Am Mosq Control Assoc. 2015;31(1):16–25.

Evans BR, Gloria-Soria A, Hou L, McBride C, Bonizzoni M, Zhao H, et al. A Multipurpose high throughput SNP chip for the dengue and yellow fever mosquito, *Aedes aegypti*. G3; 2015;5:711–8.

Failloux A-B, Vazeille M, Rodhain F. Geographic genetic variation in populations of the dengue virus vector *Aedes aegypti*. J Mol Evol; 2002a;55(6):653–63.

Failloux A-B, Fouque F, Vazeille M, Rodhain F. Isoenzyme differentiation of *Aedes aegypti* populations in French Guiana. Med Vet Entomol. 2002b;16(4):456–60.

Faucon F, Dusfour I, Gaude T, Navratil V, Boyer F, et al. Unravelling genomic changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. Genome Res. 2015:1–13.

Faucon F, Gaude T, Dusfour I, Navratil V, Corbel V, Juntarajumnong W, et al. In the hunt for genomic markers of metabolic resistance to pyrethroids in the mosquito *Aedes aegypti*: An integrated next-generation sequencing approach. PLoS Negl Trop Dis. 2017;11(4):1–20.

Fernandes JN, Moise IK, Maranto GL, Beier JC. Revamping Mosquito-borne disease control to tackle future threats. Trends Parasitol; 2018;xx:1–10.

Flies EJ, Toi C, Weinstein P, Doggett SL, Williams CR. Converting mosquito surveillance to arbovirus surveillance with honey-baited nucleic acid preservation cards. Vector-Borne Zoonotic Dis. 2015;15(7):397–403.

Frank C, Höhle M, Stark K, Lawrence J. More reasons to dread rain on vacation? Dengue fever in 42 German and United Kingdom Madeira tourists during autumn 2012. Euro Surveill. 2013;18(14):14–7.

Franz AWE, Kantor AM, Passarelli AL, Clem RJ. Tissue barriers to arbovirus infection in mosquitoes. Viruses. 2015;7(7):3741–67.

Garrett-Jones C, Grab B. the Assessment of insecticidal impact on the malaria mosquito's vectorial capacity, from data on the proportion of parous females. Bull World

Health Organ. 1964;31:71–86.

Girod R, Guidez A, Carinci R, Issaly J, Gaborit P, Ferrero E, et al. Detection of Chikungunya virus circulation using sugar-baited traps during a major outbreak in French Guiana. PLoS Negl Trop Dis. 2016;10(9):8–12.

Gloria-Soria A, Ayala D, Bheecarry A, Calderon-Arguedas O, Chadee DD, Chiappero M, et al. Global genetic diversity of *Aedes aegypti*. Mol Ecol. 2016a;25(21):5377–95.

Gloria-Soria A, Kellner DA, Brown JE, Gonzalez-Acosta C, Kamgang B, Lutwama J, et al. Temporal genetic stability of *Stegomyia aegypti* (= *Aedes aegypti*) populations. Med Vet Entomol. 2016b.

Gloria-Soria A, Lima A, Lovin DD, Cunningham JM, Severson DW, Powell JR. Origin of a high-latitude population of *Aedes aegypti* in Washington, DC. Am J Trop Med Hyg. 2018;98(2):445–52.

Golding N, Wilson AL, Moyes CL, Cano J, Pigott DM, Velayudhan R, et al. Integrating vector control across diseases. BMC Med. BMC Medicine; 2015;13(1):1–6.

Gonçalves Y, Silva J, Biscoito M. On the presence of *Aedes* (Stegomya) *aegypti* Linnaeus, 1762 (Insecta, Diptera, Culicidae) in the island of Madeira (Portugal). Bol do Mus Munic do Funchal. 2008;58(322):53–9.

Gorman K, Young J, Pineda L, Márquez R, Sosa N, Bernal D, et al. Short-term suppression of *Aedes aegypti* using genetic control does not facilitate *Aedes albopictus*. Pest Manag Sci. 2016;72(3):618–28.

Gorrochotegui-Escalante N, Munoz ML, Fernandez-Salas I, Beaty BJ, Black WC 4th. Genetic isolation by distance among *Aedes aegypti* populations along the northeastern coast of Mexico. Am J Trop Med Hyg. 2000;62(2):200–9.

Grisales N, Poupardin R, Gomez S, Fonseca-Gonzalez I, Ranson H, Lenhart A. Temephos resistance in *Aedes aegypti* in Colombia compromises dengue vector control. PLoS Negl Trop Dis. 2013;7(9):e2438.

Gubler DJ. Dengue, Urbanization and Globalization : The Unholy Trinity of the 21st Century. 201 ;39 :3-11.

Guichoux E, Lagache L, Wagner S, Chaumeil P, Léger P, Lepais O, et al. Current

trends in microsatellite genotyping. Mol Ecol Resour. 2011;11(4):591–611.

Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. Nat Rev Microbiol. 2010 Dec [];8(12 Suppl):S7-16.

Haddi K, Tomé HVV, Du Y, Valbon WR, Nomura Y, Martins GF, et al. Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of *Aedes aegypti*: A potential challenge for mosquito control. Sci Rep. 2017;7(April):1–9.

Halstead SB, Papaevangelou G. Transmission of dengue 1 and 2 viruses in Greece in 1928. Am J Trop Med Hyg. 1980;29(4):635-7.

Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, Clark GG, et al. Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. Am J Trop Med Hyg. 2005;72:209–20.

Harris AF, McKemey AR, Nimmo D, Curtis Z, Black I, Morgan SA, et al. Successful suppression of a field mosquito population by sustained release of engineered male mosquitoes. Nat Biotechnol. 2012;30(9):828–30.

Hegde S, Rasgon JL, Hughes GL. The microbiome modulates arbovirus transmission in mosquitoes. Curr Opin Virol. 2015;15:97–102.

Hladish TJ, Pearson CAB, Patricia Rojas D, Gomez-Dantes H, Halloran ME, Vazquez-Prokopec GM, et al. Forecasting the effectiveness of indoor residual spraying for reducing dengue burden. PLoS Negl Trop Dis. 2018;12(6):1–16.

Hlaing T, Tun-Lin W, Somboon P, Socheat D, Setha T, Min S, et al. Mitochondrial pseudogenes in the nuclear genome of *Aedes aegypti* mosquitoes: implications for past and future population genetic studies. BMC Genet. 2009;10:11.

Hemingway J, Hawkes NJ, McCarroll L, Ranson H. The molecular basis of insecticide resistance in mosquitoes. Insect Biochem Mol Biol. 2004;34:653–65.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, et al. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature; 2011;476(7361):454–9.

Hoffmann AA, Iturbe-Ormaetxe I, Callahan AG, Phillips BL, Billington K, Axford JK, et al. Stability of the wMel *Wolbachia* infection following invasion into *Aedes aegypti*

populations. PLoS Negl Trop Dis. 2014;8(9).

Huhtamo E, Korhonen EM, Vapalahti O. Imported dengue virus serotype 1 from Madeira to Finland 2012. Euro Surveill. 2013;18(8):1–4.

Ibañez-Justicia A, Gloria-Soria A, Den Hartog W, Dik M, Jacobs F, Stroo A. The first detected airline introductions of yellow fever mosquitoes (*Aedes aegypti*) to Europe, at Schiphol International airport, the Netherlands. Parasit Vectors; 2017;10(1):1–9.

IRAC: Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance In: Resistance Management for Sustainable Agriculture and Improved Public Health: Second Edition 2010 Insecticide Resistance Action Commitee; 2010:72pp.

Jacups SP, Paton CJ, Ritchie SA. Residual and pre-treatment application of starycide insect growth regulator (triflumuron) to control *Aedes aegypti* in containers. Pest Manag Sci. 2014;70(4):572–5.

Johnson BJ, Ritchie SA, Fonseca DM. The state of the art of lethal oviposition trap-based mass interventions for arboviral control. Insects. 2017;8(1).

Kamerow D. The world's deadliest animal. Bmj. 348;g3258.

Kotsakiozi P, Gloria-Soria A, Schaffner F, Robert V, Powell JR. *Aedes aegypti* in the Black Sea: recent introduction or ancient remnant? Parasit Vectors; 2018a;11(1):396.

Kotsakiozi P, Evans BR, Gloria-Soria A, Kamgang B, Mayanja M, Lutwama J, et al. Population structure of a vector of human diseases: *Aedes aegypti* in its ancestral range, Africa. Ecol Evol. 2018b:1–14.

Krockel U, Rose A, Eiras AE, Geier M. New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. J Am Mosq Control Assoc. 2006;22(2):229-238.

Lacroix R, McKemey AR, Raduan N, Kwee Wee L, Hong Ming W, Guat Ney T, et al. Open field release of genetically engineered sterile male *Aedes aegypti* in Malaysia. PLoS One. 2012;7(8):1–9.

Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD. Impact of daily temperature fluctuations on dengue virus transmission by *Aedes aegypti*. 2011;1–6.

Lau KW, Chen CD, Lee HL, Norma-Rashid Y, Sofian-Azirun M. Evaluation of insect

growth regulators against field-collected *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) from Malaysia. J Med Entomol. 2015;52(2):199–206.

Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014;383(9916):514.

Lovin DD, Washington KO, deBruyn B, Hemme RR, Mori A, Epstein SR, et al. Genome-based polymorphic microsatellite development and validation in the mosquito *Aedes aegypti* and application to population genetics in Haiti. BMC Genomics. 2009;10:590.

Maciel-de-Freitas R, Avendanho FC, Santos R, Sylvestre G, Araújo SC, Lima JBP, et al. Undesirable consequences of insecticide resistance following *Aedes aegypti* control activities due to a dengue outbreak. PLoS One. 2014;9(3).

Margarita Y, Santos Grácio AJ, Lencastre I, Silva AC, Novo T, Sousa C. First record of *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) (Diptera, Culicidae) in Madeira Island – Portugal (Portuguese, English abstract). Acta Parasitol Port. 2006; 13: 59-61.

Matthews BJ, Dudchenko O, Kingan S, Koren S, Antoshechkin I, Crawford JE, et al. Improved *Aedes aegypti* mosquito reference genome assembly enables biological discovery and vector control. bioRxiv. 2017;240747.

McGraw E a, O'Neill SL. Beyond insecticides: new thinking on an ancient problem. Nat Rev Microbiol; 2013;11(3):181–93.

Moore M, Sylla M, Goss L, Burugu MW, Sang R, Kamau LW, et al. Dual African origins of global *Aedes aegypti* s.l. populations revealed by mitochondrial DNA. PLoS Negl Trop Dis. 2013;7(4):e2175.

Moyes CL, Vontas J, Martins AJ, Ng LC, Koou SY, Dusfour I, et al. Contemporary status of insecticide resistance in the major *Aedes* vectors of arboviruses infecting humans. PLoS Negl Trop Dis. 2017;11(7):1–20.

Müller GC, Beier JC, Traore SF, Toure MB, Traore MM, Bah S, et al. Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the *Anopheles gambiae* complex in Mali, West Africa. Malar J. 2010;9(1):3–9.

Musso D, Cao-Lormeau VM, Gubler DJ. Zika virus: following the path of dengue and

chikungunya? Lancet. 2015; 386:243–4.

Naranjo DP, Qualls WA, Müller GC, Samson DM, Roque D, Alimi T, et al. Evaluation of boric acid sugar baits against *Aedes albopictus* (Diptera: Culicidae) in tropical environments. Parasitol Res. 2013;112(4):1583–7.

Nazareth T, Teodósio R, Porto G, Gonçalves L, Seixas G, Silva AC, et al. Strengthening the perception-assessment tools for dengue prevention: a cross-sectional survey in a temperate region (Madeira, Portugal). BMC Public Health. 2014 Jan;14(1):39.

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu Z, et al. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science. 2007;316(5832):1718–23.

Nguyen TH, Nguyen H Le, Nguyen TY, Vu SN, Tran ND, Le TN, et al. Field evaluation of the establishment potential of wMelpop *Wolbachia* in Australia and Vietnam for dengue control. Parasit Vectors. 2015;8(1):1–14.

Nkya TE, Akhouayri I, Kisinza W, David JP. Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects. Insect Biochem Mol Biol. 2012;43(4):407–16.

Ocampo CB, Mina NJ, Carabalí M, Alexander N, Osorio L. Reduction in dengue cases observed during mass control of *Aedes* (Stegomyia) in street catch basins in an endemic urban area in Colombia. Acta Trop. 2014;132(1):15–22.

Ohba S ya, Ohashi K, Pujiyati E, Higa Y, Kawada H, Mito N, et al. The effect of pyriproxyfen as a “population growth regulator” against *Aedes albopictus* under semi-field conditions. PLoS One. 2013;8(7):1–10.

Paredes-Esquivel C, Lenhart A, del Río R, Leza MM, Estrugo M, Chalco E, et al. The impact of indoor residual spraying of deltamethrin on dengue vector populations in the Peruvian Amazon. Acta Trop. 2016;154:139–44.

Pombi M, Jacobs F, Verhulst NO, Caputo B, Della Torre A, Takken W. Field evaluation of a novel synthetic odour blend and of the synergistic role of carbon dioxide for sampling host-seeking *Aedes albopictus* adults in Rome, Italy. Parasit Vectors. 2014;7(1):1–5.

Pless E, Gloria-Soria A, Evans BR, Kramer V, Bolling BG, Tabachnick WJ, et al.

Multiple introductions of the dengue vector, *Aedes aegypti*, into California. PLoS Negl Trop Dis. 2017;11(8):1–17.

Powell JR, Tabachnick WJ, Arnold J. Genetics and the origin of a new vector population: *Aedes aegypti*, a case study. Science. 1980;208:1385-1387.

Powell JR, Tabachnick WJ. History of domestication and spread of *Aedes aegypti* - A Review. Mem Inst Oswaldo Cruz. 2013;108 Suppl(August):11–7.

Powell JR. Mosquitoes on the move. 2016;354(6315):971-972.

Powell JR. Mosquito-Borne Human Viral Diseases: Why *Aedes aegypti*? Am J Trop Med Hyg. 2018;98(6):1563–5.

Putman AI, Carbone I. Challenges in analysis and interpretation of microsatellite data for population genetic studies. Ecol Evol. 2014;4(22):4399–428.

Qualls WA, Müller GC, Traore SF, Traore MM, Arheart KL, Doumbia S, et al. Indoor use of attractive toxic sugar bait (ATSB) to effectively control malaria vectors in Mali, West Africa. Malar J. 2015a;14(1):1–8. Qualls WA, Müller G, Khallaayoune K, Revay E, Zhioua E, Kravchenko V, Arheart K, Xue R, Schlein Y, Hausmann A, Kline D. and Beier J. Control of sand flies with attractive toxic sugar baits (ATSB) and potential impact on non-target organisms in Morocco. Parasit Vectors. 2015b:8(1), p.87.

Rašić G, Filipović I, Weeks AR, Hoffmann A. Genome-wide SNPs lead to strong signals of geographic structure and relatedness patterns in the major arbovirus vector, *Aedes aegypti*. BMC Genomics. 2014;15:275.

Rašić G, Endersby-Harshman N, Tantowijoyo W, Goundar A, White V, Yang Q, et al. *Aedes aegypti* has spatially structured and seasonally stable populations in Yogyakarta, Indonesia. Parasit Vectors. 2015;8:610.

Reiter P, Amador MA, Anderson RA, Clark GG. Short report: dispersal of *Aedes aegypti* in an urban area after blood feeding as demonstrated by rubidium-marked eggs. Am J Trop Med Hyg. 1995: 52(2);177-179.

Revay EE, Müller GC, Qualls WA, Kline DL, Naranjo DP, Arheart KL, et al. Control of *Aedes albopictus* with attractive toxic sugar baits (ATSB) and potential impact on non-target organisms in St. Augustine, Florida. Parasitol Res. 2014;113(1):73–9.

Ritchie SA, van den Hurk AF, Smout MJ, Staunton KM, Hoffmann AA. Mission Accomplished? We Need a Guide to the "Post Release" World of *Wolbachia* for *Aedes*-borne Disease Control. Trends Parasitol; 2018;xx:1–10.

Saarman NP, Gloria-Soria A, Anderson EC, Evans BR, Pless E, Cosme L V., et al. Effective population sizes of a major vector of human diseases, *Aedes aegypti*. Evol Appl. 2017;10(10):1031–9.

Saavedra-Rodriguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P, et al. Parallel evolution of Vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant *Aedes aegypti* from Mexico. Sci Rep. 2018;8(1):1–9.

Seixas, G. *Aedes* (Stegomyia) *aegypti* (Diptera: Culicidae) da ilha da Madeira: origem geográfica e resistência aos inseticidas. MsC Thesis, Instituto de Higiene e Medicina Tropical/Universidade Nova de Lisboa, 2012;123 pp.

Seixas G, Salgueiro P, Silva AC lara, Campos M, Spenassatto C, Reyes-Lugo M, et al. *Aedes aegypti* on Madeira Island (Portugal): genetic variation of a recently introduced dengue vector. Mem Inst Oswaldo Cruz. 2013;108(1998):3–10.

Shaw WR, Catteruccia F. Vector biology meets disease control : using basic research to fight vector-borne diseases. Nat Microbiol.

Sherpa S, Rioux D, Goindin D, Fouque F, François O, Després L. At the origin of a worldwide invasion: unraveling the genetic makeup of the Caribbean bridgehead populations of the dengue vector *Aedes aegypti*. Genome Biol Evol. 2018;10(1):56–71.

Simmons CP, Farrar JJ, Chau N, Wills B. Dengue. N Engl J Med. 2012;366:1423-32.

Slotman MA, Kelly NB, Harrington LC, Kitthawee S, Jones JW, Scott TW, et al. Polymorphic microsatellite markers for studies of *Aedes aegypti* (Diptera: Culicidae), the vector of dengue and yellow fever. Mol Ecol Notes. 2007;7(1):168–71.

Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. 2016.

Snetselaar J, Andriessen R, Suer RA, Osinga AJ, Knols BGJ, Farenhorst M. Development and evaluation of a novel contamination device that targets multiple life-stages of *Aedes aegypti*. Parasit Vectors. 2014;7:200.

Sousa CA, Clairouin M, Seixas G, Viveiros B, Novo MT, Silva AC, et al. Ongoing outbreak of dengue type 1 in the Autonomous Region of Madeira, Portugal: preliminary report. Euro Surveill. 2012 Jan;17(49):8–11.

Stewart ZP, Oxborough RM, Tungu PK, Kirby MJ, Rowland MW, Irish SR. Indoor application of attractive toxic sugar bait (ATSB) in combination with mosquito nets for control of pyrethroid-resistant mosquitoes. PLoS One. 2013;8(12):6–12.

Strode C, Wondji CS, David J, Hawkes NJ, Lumjuan N, Nelson DR, et al. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem Mol Biol. 2008;38:113-123.

Tham HW, Balasubramaniam V, Ooi MK, Chew MF. Viral determinants and vector competence of Zika Virus transmission. Front Microbiol. 2018;9:1–11.

Urdaneta-Marquez L, Bosio C, Herrera F, Rubio-Palis Y, Salasek M, Black IV WC. Genetic relationships among *Aedes aegypti* collections in Venezuela as determined by mitochondrial DNA variation and nuclear single nucleotide polymorphisms. Am J Trop Med Hyg. 2008;78(3):479–91.

van den Hurk AF, Hall-Mendelin S, Townsend M, Kurucz N, Edwards J, Ehlers G, et al. Applications of a sugar-based surveillance system to track arboviruses in wild mosquito populations. Vector-Borne Zoonotic Dis. 2014;14(1):66–73.

Vontas J, Kioulos E, Pavlidi N, Morou E, Torre A, Ranson H. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic Biochem Physiol; 2012;6–11.

Wallis GP, Tabachnick WJ, Powell JR. Macrogeographic genetic variation in a human commensal: *Aedes aegypti*, the yellow fever mosquito. Genet Res. 1983;41:241-258.

Weaver SC, Reisen WK. Present and future arboviral threats. Antiviral Res. 2010;85(2):328–45.

Wilder-Smith A, Quam M, Sessions O, Rocklov J, Liu-Helmersson J, Franco L, et al. The 2012 dengue outbreak in Madeira: exploring the origins. Euro Surveill. 2014;19(8):20718.

World Health Organization. Dengue: guidelines for diagnosis, treatment, prevention

and control – New edition. 2009. Geneve, Switzerland.

World Health Organization. Handbook for integrated vector management. 2012.

World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Second Edition. 2016.

Chapter 2.

Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal)

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Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal)

Abstract

Background

Aedes aegypti is a major mosquito vector of arboviruses, including dengue, chikungunya and zika. In 2005, *Ae. aegypti* was identified for the first time in Madeira Island. Despite an initial insecticide-based vector control program, the species expanded throughout the southern coast of the island, suggesting the presence of insecticide resistance. Here, we characterized the insecticide resistance status and the underlying mechanisms of two populations of *Ae. aegypti* from madeira island, Funchal and Paúl do mar.

Methodology/Principal findings

WHO susceptibility bioassays indicated resistance to cyfluthrin, permethrin, fenitrothion and bendiocarb. Use of synergists significantly increased mortality rates, and biochemical assays indicated elevated activities of detoxification enzymes, suggesting the importance of metabolic resistance. Microarray-based transcriptome analysis detected significant upregulation in both populations of nine cytochrome P450 oxidase genes (including four known pyrethroid metabolizing enzymes), the organophosphate metabolizer *CCEae3a*, Glutathione-S-transferases, and multiple putative cuticle proteins. Genotyping of knockdown resistance loci linked to pyrethroid resistance revealed fixation of the 1534C mutation, and presence with moderate frequencies of the V1016I mutation in each population.

Conclusions/Significance

Significant resistance to three major insecticide classes (pyrethroid, carbamate and organophosphate) is present in Ae. aegypti from Madeira Island, and appears to be mediated by multiple mechanisms. Implementation of appropriate resistance management strategies including rotation of insecticides with alternative modes of action, and methods other than chemical-based vector control are strongly advised to delay or reverse the spread of resistance and achieve efficient control.

Keywords: Insecticide resistance, Europe, *Aedes aegypti*, Madeira, Dengue

Author Summary

Aedes aegypti is the major mosquito vector of dengue, chikungunya and Zika worldwide. After its introduction in Madeira, it took a few years for the first dengue outbreak to occur in the region. Control strategies rely mostly on the use of insecticides but their efficiency is often being hampered by the ability of mosquitoes to resist to the compounds used. In fact, previous vector control programs using insecticides failed to eradicate, or even, to limit the spread of *Ae. aegypti* in Funchal, and now, the mosquito is widely distributed throughout the southern coast of the island. Bioassays to determine insecticide susceptibility profiles were carried-out in two populations of Madeira Island and the molecular mechanisms underlying the observed insecticide resistance phenotype were investigated. Transcription levels of detoxification genes were analysed, and screenings for *kdr* mutations, V1016I and F1534C, associated with pyrethroid resistance were performed. Our study showed the up-regulation of several detoxification genes of multiple enzyme families associated with metabolic resistance, and the presence of the two *kdr* mutations, with the F1534C being fixed. Another suggested mechanism probably involved in the resistance phenotype is cuticle thickening, as several cuticle genes were found overexpressed. This study reinforces the importance of alternative control strategies to suppress *Ae. aegypti* population and thus reduce the likelihood of arbovirus transmission in the region.

Introduction

Aedes aegypti (Linnaeus, 1762) is the most important vector of dengue, chikungunya and Zika viruses. Originally from Africa, this mosquito species invaded other territories and currently has a worldwide tropical distribution, probably aided by globalization [1]. Of major concern is that novel arboviral outbreaks have occurred following the establishment of *Ae. aegypti* in new areas [2,3]. In Europe, the most recent example of invasion and subsequent arboviral outbreak is the case of Madeira Island, a Portuguese territory in the Atlantic Ocean. In 2005, *Ae. aegypti* was first recorded in the Santa Luzia parish of Funchal (the capital city of Madeira) and soon the vector spread into other parishes of Funchal [4,5]. Control measures based on source reduction were soon implement together with insecticide spraying mainly with pyrethroid insecticides,

and application of *Bacillus thuringiensis israelensis* (*Bti*) for larval control [6,7]. This insecticide-based strategy was applied only in Santa Luzia municipality of Funchal, during the years 2006-2008. This strategy included outdoor ULV applications with alfa-cypermethrin and indoor sprayings with tetramethrin and d-fenothrin, in households, and pyrethrins, in warehouses and similar facilities. A liquid formulation of *Bti* was used to treat water fountains or other breeding sites that had standing water [7]. Despite the vector control measures, *Ae. aegypti* continued to expand throughout the entire south coast of the island [6].

In 2012, a dengue outbreak was declared by the Madeira Health Authorities with a total of 2,168 cases reported from September 2012 to March 2013 [7]. In this period, a total of 78 imported dengue cases were notified in 13 European countries, consisting of travelers that had visited the island during the outbreak [7]. This event was of particular public health concern given that competent dengue vector *Aedes albopictus* populations are established in southern Europe and have been implicated in autochthonous transmission of dengue and chikungunya viruses in Italy, France and Croatia [8-10].

Insecticide-based vector control efforts did not succeed in reducing the mosquito population, which thrived along the southern coast of the island. This is the most densely inhabited part of the island, resulting in a great availability of human hosts and breeding sites, which coupled with favourable climatic conditions may explain the rapid establishment of *Ae. aegypti*. Insecticide resistance might also have contributed to the apparently limited effectiveness of the implemented insecticide-based control measures, but investigation to date has been limited.

Reduced susceptibility to insecticides has been reported frequently in *Ae. aegypti* and is primarily associated with two major mechanisms of insecticide resistance, metabolic detoxification and target-site mutations. Over-expression of genes belonging to esterase, Glutathione-S-transferase (GST) and cytochrome P450 oxidase detoxification enzyme families has been reported in insecticide resistant *Ae. aegypti* populations from a broad range of geographic locations (reviewed in [11,12]). The role of several detoxification enzymes in insecticide resistance, including six P450s in pyrethroid resistance and the esterase *CCEae3a* in organophosphate resistance has been confirmed by *in vitro* and/or *in vivo* functional validation studies [13,14].

Multiple point mutations have been identified in the voltage-gated sodium channel gene (*Vgsc*) of pyrethroid-resistant *Ae. aegypti* populations worldwide. Of these, mutations at two codons are most commonly involved in resistance to pyrethroids in *Ae. aegypti*, V1016G or I and F1534C, which may act multiplicatively, especially in combination with an additional mutation, S989P (reviewed in [12]).

Cuticle thickening has also been implicated in insecticide resistance by interfering with the penetration of the insecticide and thus with the amount and rate of insecticide that reaches its target-site [15]. This type of resistance has been documented in several insect species including disease vectors [16-18], and genes encoding cuticle proteins have been found to be over-expressed in insecticide resistance strains of *Ae. aegypti* and *Ae. albopictus*, as well as *Anopheles stephensi*, and *Culex pipiens pallens* [19-22].

A previous study [23] detected the V1016I and F1534C point mutations in the *Vgsc* gene of *Ae. aegypti* from Madeira suggesting that target-site resistance is present on the island. However, no information on the prevalence of insecticide susceptibility, nor on other mechanisms of resistance in Madeira are available to date.

In order to clarify possible causes of inefficacy of insecticide-based control measures implemented on the island, and to assist health authorities in the planning of new interventions, we characterised resistance phenotypes and underlying mechanisms in *Ae. aegypti* from Madeira.

Methods

Study site and mosquito sampling

The archipelago of Madeira comprises seven islands and two islets situated in the north Atlantic *ca.* 685 km off the coast of Morocco, West Africa. Madeira is the largest island with 742 km^2 and a population of around 270,000 inhabitants. The island has a mountainous topography and most of the population lives along the coast, especially in the south, where the capital Funchal is located (32°39′4″N 16°54′35″W) and where nearly half of the population lives. This area is densely populated and domestic flower pots, which are the major breeding sites for *Ae. aegypti* in the island, are very abundant [24]. The subtropical climate, hilly landscape with exuberant Laurissilva forest and relative

proximity to the European continent make this insular territory a popular tourist destination [25].

Aedes aegypti were sampled by ovitrap collections carried out in Funchal and Paúl do Mar between September and November 2013.Ovitrap distribution in both localities is available in the citizen science online platform (http://iasaude.sras.gov-madeira.pt/naomosquito/). No specific permits were required for the described field collections. Immatures were reared to adulthood for subsequent use in insecticide susceptibility bioassays. Mosquito rearing and bioassays where performed in the facilities of Direção Regional da Agricultura, provided through a cooperation agreement with the Instituto de Administração da Saúde e Assuntos Sociais (IASAUDE). The *Ae. aegypti* Rockefeller strain was used as susceptible reference colony. Mosquitoes were reared in a controlled environment with stable temperature (26±2ºC), relative humidity (70±5%) and photoperiod (12h/12h light/dark). A subset of non-insecticide exposed females, which emerged from field collected immatures and from the Rockefeller reference susceptible strain were frozen in liquid nitrogen for subsequent use in biochemical assays. In addition, individuals from the Funchal and Paul do Mar populations, as well as individuals from the susceptible laboratory colonies Rockefeller and New Orleans were stored in RNALater (Invitrogen) to be used in the gene expression analysis.

Susceptibility bioassays

Bioassays were carried out with 3-5day old non-blood fed females using WHO insecticide susceptibility tests and protocols [26,27]. Filter papers impregnated with insecticide (bendiocarb 0.1%, cyfluthrin 0.15%, fenitrothion 1.0% and permethrin 0.75%) were provided by WHO-University of Sains Malaysia (Penang, Malaysia). Insecticides were chosen according to the three main classes of chemicals allowed to be used in vector control in the region, and based on previous knowledge regarding insecticide resistance status of the *Ae. aegypti* populations [7]. Females were exposed to the insecticide in groups of 20-25 per tube, for one hour. Four or five replicates per insecticide were used, depending in mosquito availability. After exposure, mosquitoes were transferred to a holding tube and supplied with a 10% sugar solution on a cotton pad. Mortality was scored 24 hours after exposure. The susceptibility status of each mosquito population was

assessed according to WHO recommendations, in which a mosquito population is deemed resistant to a given insecticide if mortality rates are below 90% [26,27] when testing a minimum of 100 specimens. The surviving mosquitoes (considered resistant) and dead (considered susceptible) were stored individually in 1.5ml tubes filled with silica gel desiccant for DNA-based analysis.

In addition, WHO susceptibility tests were carried out with pre-exposure to synergists to block the action of P450s, esterases and GSTs, in order to assess presence of metabolic resistance. Females were exposed to papers impregnated with 4% piperonyl butoxide (PBO) or 8% diethyl maleate (DEM) for one hour and then immediately exposed to each insecticide or to control papers as described above. Mortality rates were scored after 24 hours.

Biochemical assays

Biochemical assays were performed to quantify the enzymatic activity of the major detoxification families: esterases, Glutathione-S transferases (GST) and cytochrome P450 oxidases (MFO), following WHO protocols [28]. Forty 3-5 days- old individual females from Funchal, Paúl do Mar and an equal number of the Rockefeller reference strain were used in each assay. Comparisons of enzyme activity between field and reference mosquitoes were tested using a Mann-Whitney non-parametric analysis using Graphpad Prism v 6.03.

Microarray: RNA extraction, labeling and hybridization

Gene expression analysis was carried out at the Liverpool School of Tropical Medicine, UK. Three day-old females, non-blood fed and not exposed to insecticides, that were F1 progeny of mosquitoes collected in Funchal and Paúl do Mar were compared to three day-old non-blood fed, insecticide unexposed females from the susceptible reference colony Rockefeller. In addition, the Funchal population was compared to the second susceptible reference colony New Orleans, to further reduce the possibility that differences observed in expression levels could be related to differences in the genetic background of the laboratory strains unrelated to phenotype. Total RNA was extracted

from four replicate batches of five mosquitoes using the Arcturus PicoPure RNA isolation kit (Applied Biosystems). In all cases, RNA was treated with DNAse using the RNase-free DNase Set (Qiagen), according to the manufacturer's instructions. Quantity and quality of the RNA extracts were evaluated with a Nanodrop spectrophotometer (Nanodrop Technologies) and a 2100 Bioanalyzer (Agilent Technologies), respectively. The RNA pools were amplified and labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies). Quality and quantity of labeled cRNA was assessed as above before further use. Four hybridizations for each comparison (*i.e.* Funchal vs New Orleans, Funchal vs Rockefeller and Paul do Mar vs Rockefeller) were performed using the 15k Agilent "*Aedes* microarray" (ArrayExpress accession number A-MEXP-1966). After 17 hours of hybridization at 65ºC, the array was washed to remove non-specifically bound probes, using Agilent microarray washing buffers. Scanning was performed immediately after washing on an Agilent G2205B microarray scanner.

Microarray data analysis

Data processing was performed using the Agilent Feature extraction software and analysis of normalized data used Genespring v13. A strict filtering criterion was used for inclusion of probes where all had to be detectable (or marginal) in every array across each dataset, resulting in data from 9083 acceptable probes. Probability of differential expression was determined by a one-sample *t*-test (null hypothesis of a ratio of field/colony sample expression of 1) with the *P*-value threshold set at $P<0.05$. A fold change threshold of FC>2, or FC<-2 (for underexpressed probes) was also implemented. We employed a replication criterion for significance, such that a gene was considered differentially expressed if the probability and fold-change thresholds were met for each of the three comparisons with the susceptible reference strains. Although individually the use of a threshold of alpha=0.05 would lead to a high expected number of false positives (N≈450, ignoring the additional FC criterion), the use of a strict 3/3 replication criterion reduces this dramatically to N≈1 [29], again ignoring additional stringency from the FC criterion. Owing to this strict replication procedure we also identified probes as potentially significant if they exhibited P<0.05 in 2/3 analyses and an extreme level of expression (FC>20). For representation but not assessment of significance, fold changes

were averaged and P-values combined using Fisher's method for combining probabilities.

Microarray validation by qRT-PCR

The transcription level of candidate overexpressed genes was validated by qRT-PCR in the Funchal population. Two micrograms of DNAse-treated RNA from each sample (four biological replicates for each strain: Funchal, New Orleans and Rockefeller) were reverse-transcribed using oligo(dT)$_{20}$ (Invitrogen) and Superscript III (Invitrogen). Amplification reactions of 25μl final volume were performed in a MiniOpticon Two-Color Real-Time PCR Detection System (BioRad) using 2μl of 1/25 diluted cDNA,0.2μM primers (S1 Table) and Kapa SYBR FAST qPCR Master Mix (Kapa-Biosystems). For normalization of results, the ribosomal proteins L8_ AAEL000987 and S7 _AAEL009496 were used [30]. A fivefold dilution series of pooled cDNA was used to assess the efficiency of the qPCR reaction for each gene specific primer pair. A no template control (NTC) was included to detect contamination, and a melting curve analysis was done to check for the presence of a unique PCR product. The thermal profile of reactions was 95°C for 3min followed by 40 cycles of 95°C for 15sec, 58°C 30sec and 60°C for 30sec. Relative expression analysis was performed according to Pfaffl [31].

DNA isolation and *kdr* genotyping

A subsample of mosquitoes phenotyped as susceptible or resistant by WHO assays to pyrethroid insecticides (without pre-exposure to synergists) were genotyped for the presence of the two previously-detected mutations in the *Vgsc* gene [23]. Genomic DNA was extracted according to Collins et al [32]. Two allele-specific PCR assays (AS-PCR) were used to genotype *kdr* mutations V1016I and F1534C [23]. For the V1016I mutation, the protocol used was adapted from Saavedra-Rodriguez et al [33]. Amplifications were carried out in 25 μl of reaction mixture containing 1X buffer, 3 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.1 μM of primers Val1016f, Iso1016f and Iso1016r and 1U of *Taq* DNA polymerase. The PCR conditions were identical to those described in Saavedra-Rodriguez et al [33]. PCR products were separated by electrophoresis (90 minutes at 90V) in an ethidium bromide-stained 3% agarose gel and photographed under UV light.

The tetra-primer PCR assay described in Harris et al [34] was used to genotype

the F1534C mutation. Each reaction of 25 µl contained 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.25 µM of primers AaEx31P, AaEx31Q, AaEx31wt and AaEx31mut and 1 U of *Taq* DNA polymerase. The cycling conditions were the same used in Harris et al [34]. PCR products were size-fractioned by electrophoresis in ethidium bromide stained 2% agarose gels at 100V (45 minutes) and photographed under UV light.

All PCR assays contained negative controls (*i.e.* no DNA template) and positive controls, consisting of samples of known genotype confirmed by DNA sequencing [23].

Results

Susceptibility bioassays

Aedes aegypti from Funchal were found to be resistant to all insecticides tested (Fig 1A), with mortality rates ranging between 10.9% (after permethrin exposure) and 77.5% (after fenitrothion exposure). Mortality rates increased significantly when females were exposed to one or both of the synergists before the insecticide, suggesting involvement of metabolic resistance (Fig 1A). This was particularly evident for permethrin, after exposure to either PBO or DEM, and also for fenitrothion, for which complete restoration of susceptibility was attained with both synergists. For cyfluthrin and bendiocarb a significant increase in mortality was observed after exposure with PBO only.

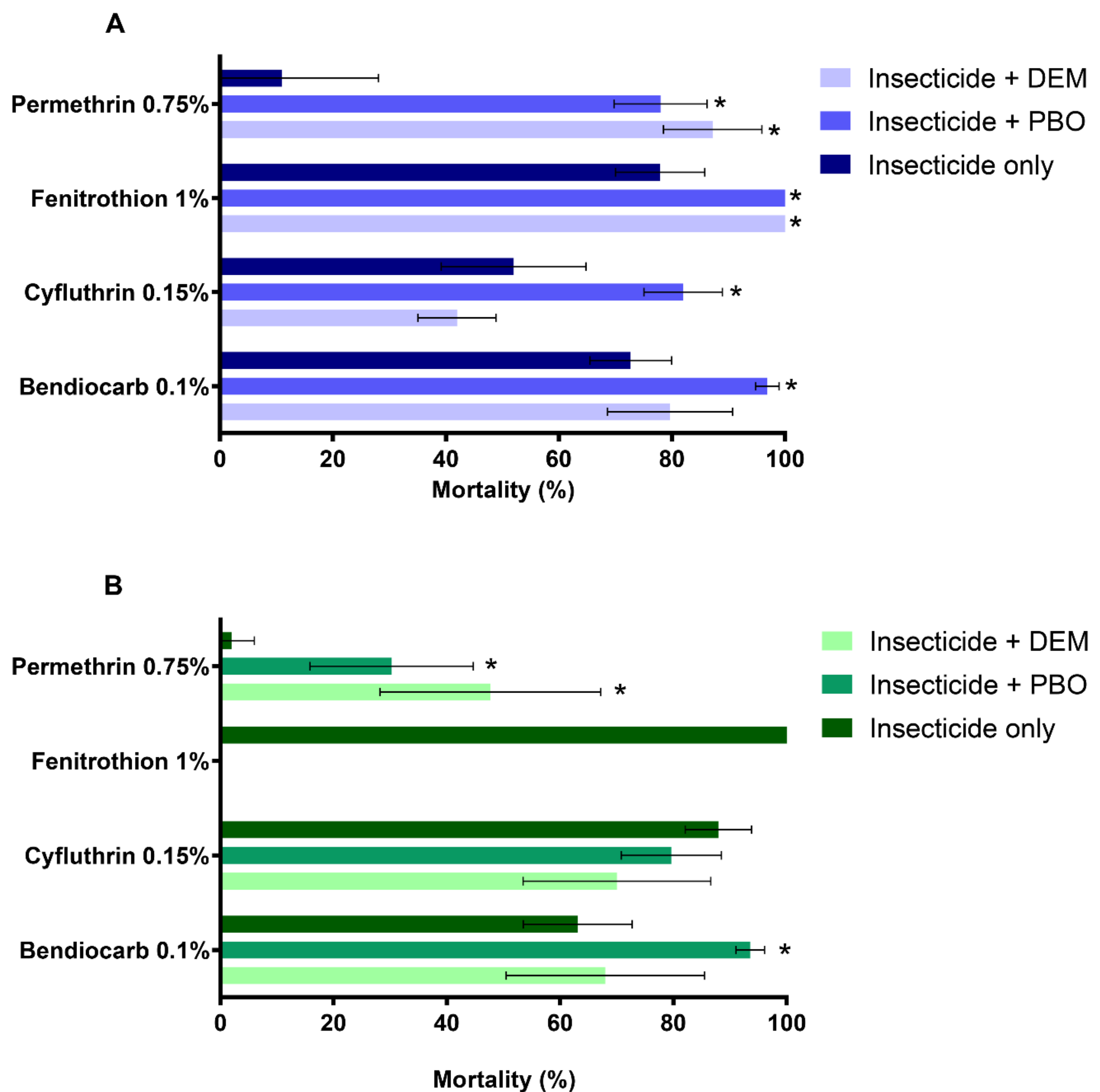

Fig 1. Susceptibility levels of *Ae. aegypti* from Funchal (A) and Paúl do Mar (B) to insecticides. * Significant differences in mortality rates between exposures with and without synergists (Fisher's exact test, *P*< 0.05). The error bars represent standard deviation.

Resistance to pyrethroids and carbamates was also observed in the Paúl do Mar population, with mortality rates between 2% (permethrin), 63.1% (bendiocarb) and 88% (cyfluthrin) (Fig 1B). In contrast, exposure to fenitrothion yielded 100% mortality indicating full susceptibility to this insecticide. As in the Funchal population, synergist assays suggest the presence of metabolic resistance (Fig 1B). Exposure to both synergists,

before insecticide contact, led to a significant increase in mortality rates with permethrin. In addition, near-full susceptibility to bendiocarb was achieved when previously exposing these mosquitoes to PBO. Synergist assays with cyfluthrin, in this *Ae. aegypti* population, did significantly alter the mortality rate. No mortality was observed in the control mosquitoes whether exposed to control papers alone or to synergists (with no insecticide).

Biochemical assays

A significantly higher enzymatic activity was detected for both α- and β-esterases in both populations when compared to the susceptible Rockefeller reference strain (Mann-Whitney tests, $P<0.05$), while no significant difference was observed in the enzymatic activity of GSTs. A statistically significant difference was seen in mixed function oxidases only in the Paúl do Mar population ($P = 0.01$). Funchal population did not show differences in the enzymatic activity of this enzyme family ($P = 0.7204$) (Fig 2).

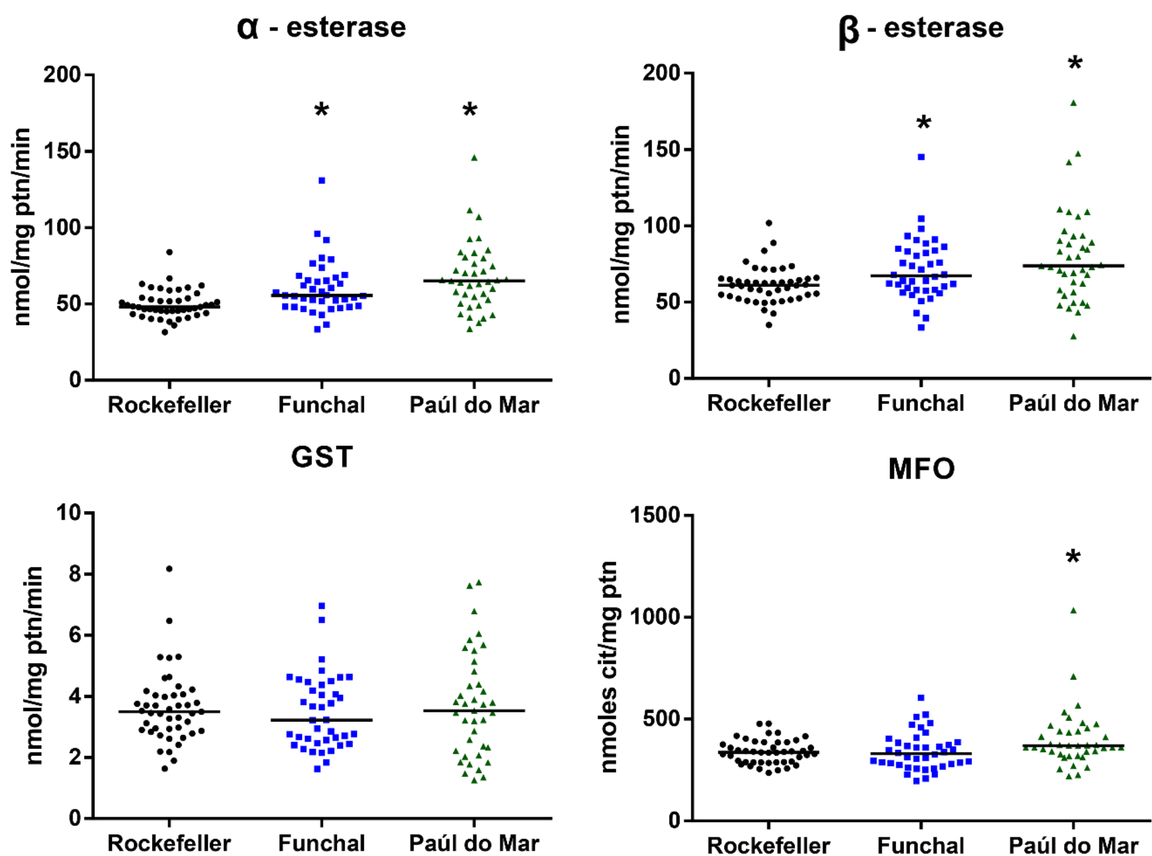

Fig 2. Activity profile of esterases, GST and mixed function oxidases (MFO) enzyme families of *Ae. aegypti* from Funchal and Paúl do Mar. * Significant differences in enzymatic activities between the wild population and the Rockefeller susceptible strain

(Mann-Whitney test, $P < 0.05$)

Microarray analysis of differential expression

From a total of 9083 probes passing quality control, 141 were differentially expressed (|Fold Change|>2, P<0.05, for three out of three comparisons to susceptible strains) across the Funchal and Paul do Mar populations (S1 Table). Among the 86 probes which were up-regulated (Fig 3), 11 genes were members of the three detoxification enzyme super-families (P450s, GSTs and Carboxyl/choline esterases) (Table 1). A further gene, *Cyp9J32* was also included in the table with the over-expressed genes as it showed extreme over-expression, and only marginally missed the threshold P-value in one (out of three) comparison. Thus we considered this as a false negative, resulting from our strict filtering procedure (Table 1). The P450 oxidases had the highest representation with nine genes, all from the CYP6 and CYP9 sub-families and included four known pyrethroid metabolizers, of which *Cyp9J32* and *Cyp9J28* were particularly strongly over-expressed (FC>20). Other detoxification genes found overexpressed in both wild populations were the *GSTd4, GSTd1* and *CCEae3a,* which metabolizes temephos-oxon (the toxic form of the larvicide temephos) and thus may be of relevance for resistance in the populations, although we did not evaluate temephos resistance in this study.

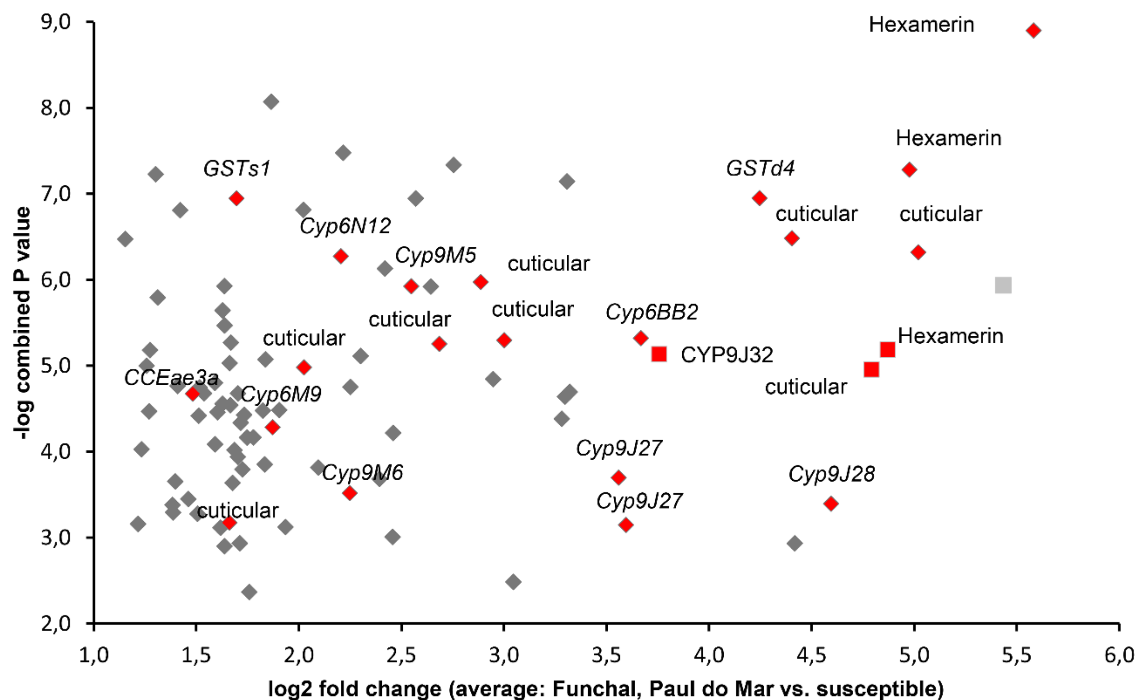

Fig 3. Commonly up-regulated transcripts in *Ae. aegypti* populations from Funchal and Paúl do Mar. A rhombus shape is used for transcripts meeting the criteria Fold Change >2 and P<0.05 in all three comparisons performed. Among these genes are the rer1 protein (possibly involved in the retrieval of endoplasmic reticulum proteins), a lyposomal aspartic protease precursor and many genes with unknown function. A square shape is used for potentially significant up-regulated transcripts, exhibiting a P<0.05 in 2/3 analyses and an extreme level of expression (FC>20). All transcripts falling into the categories of detoxification genes, hexamerins or genes encoding for cuticular proteins are shown with red color. *Cyp9J27* is present in two distinct locations in the genome and so is represented twice, though with different accession numbers (Table 1).

Beyond genes belonging to detoxification gene families the analysis revealed also the overexpression of eight transcripts encoding putative cuticle proteins, which have been implicated in resistance through lower insecticide penetration and also of three transcripts (AAEL011169, AAEL013759 and AAEL000765, which was highly and significantly, P<0.05, up-regulated in 2/3 comparisons) encoding hexamerins, which are involved in cellular trafficking and have previously been linked to insecticide resistance [35] (Fig 3, S1 Table).

Table 1. Commonly overexpressed transcripts in *Ae. aegypti* from Funchal and Paul do Mar belonging to detoxification gene families.

Class of detoxification gene	Gene accession number	Gene name	Funchal vs Rockefeller (FC)	*P*-value	Funchal vs New Orleans (FC)	P-value	Paul do Mar vs Rockefeller (FC)	P-value
P450s	AAEL008846	***Cyp9J32***	41	0.006	53	0.004	10	0.060
	AAEL014617	***Cyp9J28***	31	0.028	30	0.019	10	0.008
	AAEL014893	***Cyp6BB2***	15	0.005	19	$7*10^{-4}$	3.6	0.006
	AAEL014607	*Cyp9J27*	14	0.021	18	0.018	3.5	0.023
	AAEL014616	*Cyp9J27*	14	0.020	16	0.016	4.6	0.006
	AAEL001288	*Cyp9M5*	6.6	$6*10^{-4}$	8.5	0.009	2.3	0.001
	AAEL001312	***Cyp9M6***	5.4	0.015	4.7	0.006	4	0.031
	AAEL009124	*Cyp6N12*	5.1	$3*10^{-4}$	6.3	0.003	2.2	0.002
	AAEL017297	*Cyp6M9*	4.2	0.007	3.7	0.001	3	0.047
Esterases	AAEL005112	***CCEae3a***	2.7	0.009	3.4	0.012	2.1	0.001
GSTs	AAEL001054	*GSTd4*	24	$4*10^{-4}$	22	$2*10^{-4}$	9.8	0.004
	AAEL011741	*GSTs1*	4.9	$2*10^{-4}$	2.5	$7*10^{-5}$	2.2	0.032

FC represents the relative fold change in expression in the Funchal or Paul do Mar population compared to the respective susceptible colony. Bold type indicates a known insecticide metabolizer [13,14,40].

qRT-PCR validation

Quantitative real time PCR was used to validate the differential expression of five candidate genes detected as significantly up-regulated in the Funchal population (for which microarray data *vs.* both susceptible colonies was available) compared to the two susceptible colonies. We tested the highly overexpressed P450s *Cyp9J32* and *Cyp9J28*, the more highly overexpressed of the glutathione S transferases, *GSTd4*, the more highly overexpressed of the hexamerins, AAEL013757 and the more highly overexpressed member of genes encoding for putative cuticular proteins, AAEL002246 (S1 Table) (Table 2). Although the relative levels of overexpression estimated by qRT-PCR did not correspond closely with values obtained from the microarray experiment, the estimates from qRT-PCR confirmed up-regulation of the tested genes, thus providing validation of the significance indicated by microarrays.

Table 2. Validation of the transcriptional up-regulation of five candidate genes through qRT-PCR.

Transcript	Reference strain	**qRT-PCR** Fold change (95% CI)	**Microarray** Fold Change
Hexamerin	Rockefeller	63 (36-90)	57
(AAEL013757)	New Orleans	123 (54-191)	97
Cyp9J32	Rockefeller	17 (13-21)	41
(AAEL008846)	New Orleans	107 (81-132)	53
Cuticular	Rockefeller	42 (16-69)	36
(AAEL002246)	New Orleans	65 (25-105)	51
Cyp9J28	Rockefeller	13 (6-21)	31
(AAEL014617)	New Orleans	104 (63-144)	30
GSTd4	Rockefeller	169 (34-305)	24
(AAEL001054)	New Orleans	268 (148-387)	22

The relative expression ratio of five candidate genes in the Funchal population compared to two susceptible laboratory colonies (New Orleans and Rockefeller) is shown. Values

are estimated from four biological replicates and 95% confidence intervals are shown. Estimated values from the microarray experiment are given for comparison.

Kdr genotyping

Genotyping of the *kdr* locus was performed on a total of 91 Funchal and 80 Paúl do Mar specimens that had been previously exposed to cyfluthrin or permethrin (Table 3). The 1534C mutation was found in every specimen genotyped suggesting it may be fixed in both populations, while the V1016I mutation showed moderate and similar frequencies ranging from 17% in Funchal to 23% in Paúl do Mar (Fisher's exact tests, P = 0.263). Owing to ubiquitous occurrence of the 1534C mutation testing association with resistance was not possible. Although the V1016I frequency was slightly higher in resistant mosquitoes when compared to susceptible ones, there was no significant association between *kdr* genotypes and the resistance phenotype for either insecticide (Fisher's exact tests, Funchal - cyfluthrin: $P = 0.491$; permethrin: $P = 0.699$; Paúl do Mar - cyfluthrin: $P = 0.316$; permethrin: $P = 0.219$).

Table 3. Summary of *kdr* genotyping data in Funchal and Paúl do Mar *Ae. aegypti* populations.

				V1016I				**F1534C**		
Locality	**Insecticide**	**N**	**V/V**	**V/I**	**I/I**	**F.(I)**	**F/F**	**F/C**	**C/C**	**F.(C)**
Funchal	Cyfluthrin resistant	32	20	10	2	0.22	0	0	32	1.00
	Cyfluthrin susceptible	19	15	4	0	0.11	0	0	19	1.00
	Permethrin resistant	32	21	11	0	0.17	0	0	32	1.00
	Permethrin susceptible	8	6	2	0	0.13	0	0	8	1.00
	Total	**91**	**62**	**27**	**2**	**0.17**	**0**	**0**	**91**	**1.00**

Paúl do Mar	Cyfluthrin resistant	12	6	5	1	0.29	0	0	12	1.00
	Cyfluthrin susceptible	36	21	15	0	0.21	0	0	36	1.00
	Permethrin resistant	30	18	11	1	0.22	0	0	30	1.00
	Permethrin susceptible	2	0	2	0	0.5	0	0	2	1.00
	Total	**80**	**45**	**33**	**2**	**0.23**	**0**	**0**	**80**	**1.00**

N: sample size. Values correspond to absolute numbers for each genotype. F.(I) and F.(C) are the relative frequencies of the mutant allele for each mutation analyzed.

Discussion

The results of this study showed that *Ae. aegypti* from Madeira Island is resistant to insecticides of different chemical classes: carbamates (bendiocarb), organophosphates (fenitrothion) and both type I (permethrin) and type II (cyfluthrin) pyrethroids. Diagnostic exposures to these insecticides yielded mortality rates below the thresholds recommended by WHO to consider a mosquito population resistant [26,27], excepting the case of fenitrothion in Paúl do Mar. Combined bioassays with synergists and analysis of detoxification enzyme activities indicated the presence of enzyme-mediated metabolic resistance, and/or cuticular resistance. Pre-exposure to PBO, which inhibits P450s, some esterases and may also enhance cuticular penetration by the insecticide [36] resulted in a significant mortality increase for all insecticides tested. Synergist pre-exposures suggest involvement of the three major detoxification enzyme families in the resistance phenotype of *Ae. aegypti* from Madeira island. Biochemical assays only partially agreed with the results obtained by the bioassays with synergists, as significantly elevated enzymatic levels were detected for esterases only. These discrepancies were not completely unexpected as these assays cannot be considered reciprocal. While synergists act as inhibitors of enzymes suspected to be implicated in resistance, biochemical assays are a measure of enzyme activity without a direct link with the resistance phenotype. Furthermore, biochemical assays employ generic substrates which may not be recognized

by all variants of these large enzyme families, resulting in reduced sensitivity and specificity [36, 37, 38]. The microarray-based transcriptomic analysis showed overexpression of genes belonging to the three major detoxification enzyme families in agreement with the bioassays with synergists. The majority of overexpressed detoxification genes were cytochrome P450 oxidases, including *Cyp9J32*, *Cyp9J28*, *Cyp9J27*, *Cyp6BB2* and *Cyp9M6*, which have been found overexpressed in pyrethroid resistant *Ae. aegypti* populations from multiple countries [12,36,39]. In particular, *Cyp9J28* is an efficient pyrethroid metabolizer [13] that has also been shown to confer reduced susceptibility to deltamethrin when ectopically expressed in *Drosophila melanogaster* [40]. *Cyp9J32*, the most prominent pyrethroid metabolizer in *Ae. aegypti*, showing a very high catalytic efficiency against pyrethroids [13], was the most overexpressed P450 gene. The carboxyl-esterase *CCEae3A,* which has previously been associated with resistance to the organophosphate temephos-oxon [14] in both *Ae. aegypti* [30] and *Ae. albopictus* [41], and acts via sequestration and metabolism of temephos [14], was also among the overexpressed genes in Funchal and Paúl do Mar. This should be taken into account if temephos is considered for vector control in Madeira. The overexpression of two GSTs (*GSTd4* and *GSTs1*) was also revealed by the microarray analysis. Of these, *GSTd4* has been detected as highly overexpressed in the strongly permethrin resistant Singapore SP strain [42].

In addition to detoxification gene families, eight transcripts encoding putative cuticular proteins were up-regulated possibly indicating alteration of the cuticle as a mechanism of resistance. Resistance related to the cuticle by lowering the amount or rate of insecticide penetrating into the body has been reported in insects such as *Helicoverpa armigera* [43], *Drosophila melanogaster* [44], the *Trypanosoma cruzi* vector *Triatoma infestans* [16], and the *Plasmodium* vector *Anopheles funestus* [17]. Thickening of the whole cuticle, as well as the epicuticle layer, due to an increased number of cuticular hydrocarbons, has also been recently described in a multi-resistant strain of the malaria vector *Anopheles gambiae* [18]. Furthermore, there were also three transcripts (one marginally non-significant) encoding hexamerins among the most highly overexpressed genes. The link of hexamerins to insecticide resistance is poorly understood, but previous studies suggest a role for these storage proteins in cuticle formation [45,46]. It is also plausible that composition and thickening of the cuticle might reflect adaptive responses

to environmental challenges and/or seasonality, rather than insecticide resistance. Seasonal cuticular variations, mostly associated with adaptation to aridity (desiccation tolerance), have been previously observed in other insect species, including scorpions [47], crickets [48] and more recently in the malaria vector *A. coluzzii* [49]. There is evidence that *Ae. aegypti* of Madeira derives from a tropical south American source population [23], thus, adaptation of this mosquito to the more temperate climate of Madeira might have involved changes in cuticle composition and thickening.

Genotyping of the *kdr* locus confirmed not only the presence, but probable fixation of the pyrethroid resistance mutation F1534C, in line with previous studies [23]. However, the V1016I pyrethroid resistant mutation showed a significant frequency increase (8% in 2009 [23], to 17% in 2013, this study; Fisher's exact tests, $P = 0.019$). The role of V1016I in resistance to pyrethroids is currently unclear but frequencies of both mutations appear informative and should be routinely monitored [50]. Hu et al [51] found that the F1534C mutation is more effective in reducing sensitivity of the sodium channel to type I than to type II pyrethroids. This could explain the higher resistance level of *Ae. aegypti* from Funchal to permethrin when compared to cyfluthrin. However, reversal of resistance to permethrin with the synergist PBO was comparable to that obtained for cyfluthrin suggesting that resistance to both type I and type II pyrethroids may be primarily mediated by the metabolic activity of cytochrome P450 oxidases. These findings should be taken into consideration by the Health Authorities in Madeira when deciding between type I or type II pyrethroids for vector control. If this chemical class remains an option for chemical control of *Ae. aegypti* in Madeira island, the use of type II pyrethroids in combination with the synergist PBO appears to be a more effective option.

Conclusion

Insecticide resistance mediated by multiple mechanisms was identified in *Ae. aegypti* from two localities in Madeira. In addition to target-site (*kdr*) and metabolic resistance, a third mechanism consisting of cuticle thickening may also be involved, confirming that the insecticide resistance phenotype is multifactorial, and consequently is likely to be challenging to reverse. The recent presence of this species in the island and

the absence of a continuous, island-wide, insecticide-based control suggests that at least some, and maybe most, of the insecticide resistance mechanisms detected were already present in the colonizing specimens. Thus, the resistance status of these mosquitoes may have played some role in the establishment of this vector in the island, despite the 2006-2008 insecticide-based vector control campaign. With the current knowledge regarding insecticide resistance status and identification of underlying mechanisms, resistance management strategies including mode of action rotation (such as biocides and insect growth regulators), as well as alternative to chemical-based vector control interventions (ranging from environmental management to new paradigms and biotechnology-based approaches) is strongly advised, to control *Ae. aegypti* and thus decrease the probability of arbovirus transmission.

Supporting Information

S1 Table. List of probes analysed after the quality control and primers used in qPCR: https://doi.org/10.1371/journal.pntd.0005799.s001

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References

1. Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife. 2015;4: 1–18. doi:10.7554/eLife.08347
2. Beebe NW, Cooper RD, Mottram P, Sweeney AW. Australia’s dengue risk driven by human adaptation to climate change. PLoS Negl Trop Dis. 2009;3: e429. doi:10.1371/journal.pntd.0000429
3. Brown JE, Obas V, Morley V, Powell JR. Phylogeography and spatio-temporal genetic variation of *Aedes aegypti* (Diptera: Culicidae) populations

in the Florida Keys. J Med Entomol. 2013;50: 294–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/23540116

4. Margarita Y, Santos Grácio AJ, Lencastre I, Silva AC, Novo T, Sousa C. First record of *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) (Diptera, Culicidae) in Madeira Island – Portugal (Portuguese, English abstract). Acta Parasitol Port. 2006; 13: 59-61.
5. Almeida APG, Gonçalves YM, Novo MT, Sousa CA, Melim M, Gracio AJ. Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Euro Surveill. 2007; 12(46), pp.3311.
6. Gonçalves Y, Silva J, Biscoito M. On the presence of *Aedes* (Stegomyia) *aegypti* Linnaeus, 1762 (Insecta, Diptera, Culicidae) in the island of Madeira (Portugal). Bol Mus Mun Funchal. 2008;58: 53–59.
7. European Centre for Disease Prevention and Control. Dengue outbreak in Madeira, Portugal, March 2013. Stockholm: ECDC; 2014.
8. Gould EA, Gallian P, De Lamballerie X, Charrel RN. First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality! Clin Microbiol Infect. Blackwell Publishing Ltd; 2010;16: 1702–1704. doi:10.1111/j.1469-0691.2010.03386.x
9. Gjenero-Margan I, Aleraj B, Krajcar D, Lesnikar V, Klobučar a, Pem-Novosel I, et al. Autochthonous dengue fever in Croatia, August-September 2010. Euro Surveill. 2011;16. Available: http://www.ncbi.nlm.nih.gov/pubmed/21392489
10. Succo T, Leparc-Goffart I, Ferré J, Roiz D, Broche B, Maquart M, et al. Autochthonous dengue outbreak in Nimes, South of France, July to September 2015. Eurosurveillance. 2016;21: 1–7. doi:10.2807/1560-7917.ES.2016.21.21.30240
11. Vontas J, Kioulos E, Pavlidi N, Morou E, Torre A, Ranson H. Insecticide resistance in the major dengue vectors *Aedes albopictus* and *Aedes aegypti*. Pestic Biochem Physiol. Elsevier Inc.; 2012; 6–11. doi:10.1016/j.pestbp.2012.05.008

12. Smith LB, Kasai S, Scott JG. Pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus*: Important mosquito vectors of human diseases. Pestic Biochem Physiol. Elsevier B.V.; 2016; doi:10.1016/j.pestbp.2016.03.005
13. Stevenson BJ, Pignatelli P, Nikou D, Paine MJI. Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, *Aedes aegypti*: developing new tools to combat insecticide resistance. PLoS Negl Trop Dis. 2012;6: e1595. doi:10.1371/journal.pntd.0001595
14. Grigoraki L, Balabanidou V, Meristoudis C, Miridakis A, Ranson H, Swevers L, et al. Functional and immunohistochemical characterization of CCEae3a, a carboxylesterase associated with temephos resistance in the major arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Insect Biochem Mol Biol. 2016;74: 61–67. doi:10.1016/j.ibmb.2016.05.007
15. Nkya TE, Akhouayri I, Kisinza W, David JP. Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects. Insect Biochem Mol Biol. Elsevier Ltd; 2012;43: 407–416. doi:10.1016/j.ibmb.2012.10.006
16. Pedrini N, Mijailovsky SJ, Girotti JR, Stariolo R, Cardozo RM, Gentile A, et al. Control of pyrethroid-resistant chagas disease vectors with entomopathogenic fungi. PLoS Negl Trop Dis. 2009;3. doi:10.1371/journal.pntd.0000434
17. Wood OR, Hanrahan S, Coetzee M, Koekemoer LL, Brooke BD. Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus*. Parasit Vectors. 2010;3: 67. Available: http://www.parasitesandvectors.com/content/3/1/67
18. Balabanidou V, Kampouraki A, MacLean M, Blomquist GJ, Tittiger C, Juárez MP, et al. Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in *Anopheles gambiae*. Proc Natl Acad Sci. 2016;113: 201608295. doi:10.1073/pnas.1608295113
19. Vontas J, David JP, Nikou D, Hemingway J, Christophides GK, Louis C, et al. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. Insect Mol Biol. 2007;16: 315–324. doi:10.1111/j.1365-2583.2007.00728.x

20. David J-P, Coissac E, Melodelima C, Poupardin R, Riaz MA, Chandor-Proust A, et al. Transcriptome response to pollutants and insecticides in the dengue vector *Aedes aegypti* using next-generation sequencing technology. BMC Genomics. 2010;11: 216. doi:10.1186/1471-2164-11-216
21. Fang F, Wang W, Zhang D, Lv Y, Zhou D, Ma L, et al. The cuticle proteins: a putative role for deltamethrin resistance in *Culex pipiens pallens*. Parasitol Res. 2015;114: 4421–4429. doi:10.1007/s00436-015-4683-9
22. Ishak IH, Riveron JM, Ibrahim SS, Stott R, Longbottom J, Irving H, et al. The Cytochrome P450 gene CYP6P12 confers pyrethroid resistance in *kdr*-free Malaysian populations of the dengue vector *Aedes albopictus*. Sci Rep. Nature Publishing Group; 2016;6: 24707. doi:10.1038/srep24707
23. Seixas G, Salgueiro P, Silva AC, Campos M, Spenassatto C, Reyes-lugo M, et al. *Aedes aegypti* on Madeira Island (Portugal): genetic variation of a recently introduced dengue vector. 2013; 1–8. doi:10.1590/0074-0276130386
24. Nazareth T, Teodósio R, Porto G, Gonçalves L, Seixas G, Silva AC, et al. Strengthening the perception-assessment tools for dengue prevention: a cross-sectional survey in a temperate region (Madeira, Portugal). BMC Public Health. 2014;14: 39. doi:10.1186/1471-2458-14-39
25. Oliveira P, Pereira PT. Who values what in a tourism destination? The case of Madeira Island. Tour Econ. 2008;14: 155–168. doi:10.5367/000000008783554758
26. World Health Organization. Test procedures for insecticide resistance monitoring in malaria vector mosquitoes. Geneva: World Health Organization; 2013.
27. World Health Organization. Monitoring and managing insecticide resistance in Aedes mosquito populations Interim guidance for entomologists. Geneva: World Health Organization; 2016.
28. World Health Organization. Techniques to detect insecticide resistance mechanisms field and laboratory manual. Geneva: World Health Organization; 1998.

29. Moran MD. Arguments for rejecting the sequential Bonferroni in ecological studies. Oikos. 2003;100: 403–405. doi:10.1034/j.1600-0706.2003.12010.x

30. Poupardin R, Srisukontarat W, Yunta C, Ranson H. Identification of carboxylesterase genes implicated in temephos resistance in the dengue vector *Aedes aegypti*. PLoS Negl Trop Dis. 2014;8. doi:10.1371/journal.pntd.0002743

31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001 May 1; 29:e45

32. Collins FH, Mendez M a, Rasmussen MO, Mehaffey PC, Besansky NJ, Finnerty V. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. Am J Trop Med Hyg. 1987;37: 37–41. Available: http://www.ncbi.nlm.nih.gov/pubmed/2886070

33. Saavedra-Rodriguez K, Urdaneta-Marquez L, Rajatileka S, Moulton M, Flores a E, Fernandez-Salas I, et al. A mutation in the voltage-gated sodium channel gene associated with pyrethroid resistance in Latin American *Aedes aegypti*. Insect Mol Biol. 2007;16: 785–98. doi:10.1111/j.1365-2583.2007.00774.x

34. Harris AF, Rajatileka S, Ranson H. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. Am J Trop Med Hyg. 2010;83: 277–84. doi:10.4269/ajtmh.2010.09-0623

35. David J-P, Faucon F, Chandor-Proust A, Poupardin R, Riaz MA, Bonin A, et al. Comparative analysis of response to selection with three insecticides in the dengue mosquito *Aedes aegypti* using mRNA sequencing. BMC Genomics. 2014;15: 174. doi:10.1186/1471-2164-15-174

36. Bingham G, Strode C, Tran L, Khoa PT, Jamet HP. Can piperonyl butoxide enhance the efficacy of pyrethroids against pyrethroid-resistant *Aedes aegypti*? Trop Med Int Health. 2011;16: 492–500. doi:10.1111/j.1365-3156.2010.02717.x

37. Coleman M, Hemingway J. Insecticide resistance monitoring and evaluation in disease transmitting mosquitoes. J Pestic Sci. 2007;32: 69–76. doi:10.1584/jpestics.R07-01

38. Morou E, Dowd AJ, Rajatileka S, Steven A, Hemingway J, Ranson H, et al. A simple colorimetric assay for specific detection of glutathione-S transferase activity associated with DDT resistance in mosquitoes. PLoS Negl Trop Dis. 2010;4: 1–6. doi:10.1371/journal.pntd.0000808
39. Strode C, Wondji CS, David J, Hawkes NJ, Lumjuan N, Nelson DR, et al. Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem Mol Biol. 2008; 38: 113-123. doi:10.1016/j.ibmb.2007.09.007
40. Pavlidi N, Monastirioti M, Daborn P, Livadaras I, Van Leeuwen T, Vontas J. Transgenic expression of the *Aedes aegypti* CYP9J28 confers pyrethroid resistance in *Drosophila melanogaster*. Pestic Biochem Physiol. Elsevier Inc.; 2012;104: 132–135. doi:10.1016/j.pestbp.2012.07.003
41. Grigoraki L, Lagnel J, Kioulos I, Kampouraki A, Morou E, Labbé P, et al. Transcriptome profiling and genetic study reveal amplified carboxylesterase genes implicated in temephos resistance, in the asian tiger mosquito *Aedes albopictus*. PLoS Negl Trop Dis. 2015;9: e0003771. doi:10.1371/journal.pntd.0003771
42. Kasai S, Komagata O, Itokawa K, Shono T, Ng LC, Kobayashi M, et al. Mechanisms of pyrethroid resistance in the dengue mosquito vector, *Aedes aegypti*: target site Insensitivity, penetration, and metabolism. PLoS Negl Trop Dis. 2014;8. doi:10.1371/journal.pntd.0002948
43. Ahmad M, Denholm I, Bromilow RH. Delayed cuticular penetration and enhanced metabolism of deltamethrin in pyrethroid-resistant strains of *Helicoverpa armigera* from China and Pakistan. Pest manag Sci. 2006; 62: 805-810.
44. Strycharz JP, Lao A, Li H, Qiu X, Lee SH, Sun W, et al. Resistance in the highly DDT-resistant 91-R strain of *Drosophila melanogaster* involves decreased penetration, increased metabolism, and direct excretion. Pestic Biochem Physiol. Elsevier Inc.; 2013;107: 207–217. doi:10.1016/j.pestbp.2013.06.010
45. Burmester T. Evolution and function of the insect hexamerins. Eur J Entomol. 1999; 96: 213-225.

46. Chrysanthis G, Kaliafas AD, Mintzas AC. Biosynthesis and tissue distribution of four major larval serum proteins during development of *Ceratitis capitata* (Diptera). Insect Biochem Mol Biol. 1994;24: 811–818. doi:10.1016/0965-1748(94)90109-0
47. Gefen E, Talal S, Brendzel O, Dror A, Fishman A. Variation in quantity and composition of cuticular hydrocarbons in the scorpion *Buthus occitanus* (Buthidae) in response to acute exposure to desiccation stress. Comp Biochem Physiol -Part A Mol Integr Physiol. Elsevier Inc.; 2015;182: 58–63. doi:10.1016/j.cbpa.2014.12.004
48. Fedorka KM, Copeland EK, Winterhalter WE. Seasonality influences cuticle melanization and immune defense in a cricket: support for a temperature-dependent immune investment hypothesis in insects. J Exp Biol. 2013;216: 4005–10. doi:10.1242/jeb.091538
49. Arcaz AC, Huestis DL, Dao A, Yaro AS, Diallo M, Andersen J, et al. Desiccation tolerance in *Anopheles coluzzii*: the effects of spiracle size and cuticular hydrocarbons. J Exp Biol. 2016;219: 1675–1688. doi:10.1242/jeb.135665
50. Vera-Maloof FZ, Saavedra-Rodriguez K, Elizondo-Quiroga AE, Lozano-Fuentes S, Black IV WC. Coevolution of the Ile1,016 and Cys1,534 mutations in the voltage gated sodium channel gene of *Aedes aegypti* in Mexico. PLoS Negl Trop Dis. 2015;9: 1–22. doi:10.1371/journal.pntd.0004263
51. Hu Z, Du Y, Nomura Y, Dong K. A sodium channel mutation identified in *Aedes aegypti* selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. Insect Biochem Mol Biol. Elsevier Ltd; 2011;41: 9–13. doi:10.1016/j.ibmb.2010.09.005

Chapter 3.

Origin and expansion of the mosquito *Aedes aegypti* in Madeira Island (Portugal)

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Origin and expansion of the mosquito *Aedes aegypti* in Madeira Island (Portugal)

Abstract

Historically known as the yellow fever mosquito, *Aedes aegypti* invaded Madeira Island in 2005 and was the vector of the island's first dengue outbreak in 2012. We have studied genetic variation at 16 microsatellites and two mitochondrial DNA genes in temporal samples of Madeira Island, in order to assess the origin of the invasion and the population structure of this mosquito vector. Our results indicated at least two independent colonization events occurred on the island, both having a South American source population. In both scenarios, Venezuela was the most probable origin of these introductions, a result that is in accordance with the socioeconomic relations between this country and Madeira Island. Once introduced, *Ae. aegypti* has rapidly expanded along the southern coast of the island and reached a maximum effective population size (N_e) in 2012, coincident with the dengue epidemic. After the outbreak, there was a 10-fold reduction in N_e estimates, possibly reflecting the impact of community-based vector control measures implemented during the outbreak. These findings have implications for mosquito surveillance not only for Madeira Island, but also for other European regions where *Aedes* mosquitoes are expanding.

Introduction

Arbovirus transmission is becoming an increasing public health threat in Europe, mainly due to the establishment of invasive mosquito vectors and importation of arboviruses by viremic travelers[1]. The Asian tiger mosquito, *Aedes albopictus*, was first recorded in the European continent in Albania, in 1979[2]. Since then, this mosquito invaded most of central and western Europe and become established in 27 countries[3]. Coincidently, epidemics of chikungunya and dengue have been reported over the last 20 years, notably in Italy (2007, 2017)[4,5], France (2010, 2017)[6,7] and Croatia (2010)[8]. Another mosquito species responsible for arbovirus transmission is *Aedes aegypti*, previously present in Europe until mid-20th century and re-established in Madeira and in the Black Sea region[3].

In the Portuguese island of Madeira, *Ae. aegypti* was first reported in 2005, in the vicinity of Funchal city. Since then, this mosquito has subsequently expanded its distribution

throughout the southern coast of the island[9,10], being detected in Santa Cruz (East) in 2008 and in Paúl do Mar (West) in 2012 (Fig. 1).

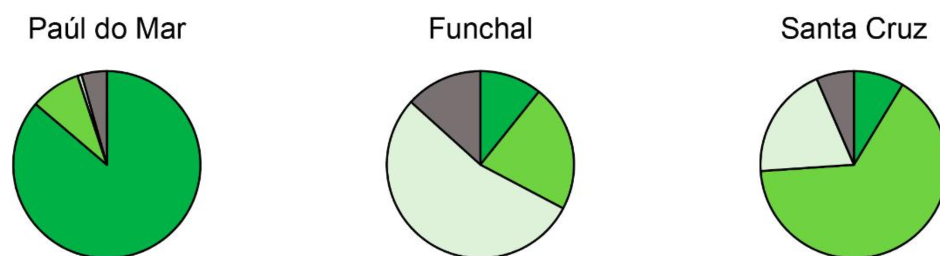

Figure 1. **Madeira Island map showing sampling sites**: Pául do Mar, a fishing village in the western point of *Ae. aegypti* distribution in the island; Funchal, the capital, where the main harbour is present; Santa Cruz, near the only Airport of the island. Below each locality name is the year of the first report of the introduction of the species. Pie charts indicate proportions of individuals assigned (Tq = 0.50) to each of the three genetic clusters determined by STRUCTURE (See text). Grey colour indicate admixed individuals with no cluster assignment. The map was produced using ArcGIS 10.2 (Esri, Redlands, CA).

The presence of this mosquito in the island, coupled with the introduction of DENV-1, led to an outbreak of dengue fever, with more than 2000 notified cases between October 2012 and March 2013[11]. The epidemic led to the reinforcement of vector control activities in the island for the subsequent months, particularly in the more densely populated area

of Funchal city[12]. Implemented anti-vector activities included larval control through massive salt application in the city's storm drains and community educational campaigns in order to remove flower dishes, the main *Ae. aegypti* breeding site in Madeira[13]. Targeted insecticide/biocide application was performed in health facilities and one school located in the most affected Funchal area, using pyrethroids and *Bacillus thurigiensis israelensis* (Bti)[12].

Madeira is a famous touristic destination, mostly for Europeans, with daily flights from/to several European countries and regular stops of cruise ships[12,14]. This scenario increases the risk of exportation of both *Ae. aegypti* and viremic individuals to Europe[12]. In fact, the dengue epidemic in Madeira was responsible for 78 imported cases that were notified in 13 different countries, the majority corresponding to tourists that had travelled to the island during the outbreak[15]. To mitigate the risk of importation/exportation of virus and vectors, local health authorities perform vector control activities at the International Airport and at the Funchal harbour, and coordinate an island-wide integrated Madeira Dengue Surveillance System (MDSS), responsible for the detection of imported cases.

Despite its insular condition, Madeira has a considerable risk of importing exotic vectors and pathogens from tropical regions. This is mainly due to the strong socio-economic relations that the island maintains with South American countries, such as Brazil and Venezuela[14]. Coincidently, phylogenetic analysis and an importation index based on the air-travel interconnectivity with dengue-endemic countries revealed Venezuela as the most likely country of origin for the circulating DENV-1 in the island[16]. In addition, previous studies showed that *Ae. aegypti* from Madeira is able to transmit dengue, chikungunya and Zika viruses[17,18], pinpointing the potential risk of local arbovirus transmission.

Previous genetic analyses involving different markers such as mitochondrial DNA, knockdown resistance associated genes[19], microsatellites[20], and Single Nucleotide Polymorphisms[21], provided evidence for a South American origin of the introduced *Ae. aegypti* population in Madeira. However, these analyses did not have sufficient resolution to precisely pinpoint the geographic origin and colonization dynamics of the *Ae. aegypti* Madeira population, mainly because i) a single sample from the island was used; ii) some of the most important putative source populations were not included. Moreover, these

studies did not provide information about the dynamics of the colonization of this mosquito in the island.

In this study, we analyzed microsatellites and mtDNA genes in *Ae. aegypti* samples from different localities of Madeira island collected at different time-points, as well as from additional sites in South America. In addition, genetic data were integrated with global genetic data available for this species in order to address the following questions:

1. What is the population genetic structure and demographic history of *Ae. aegypti* in Madeira island?
2. Are these populations the result of a single or multiple mosquito introductions?
3. What is the most likely country of origin of the source populations from which *Ae. aegypti* was introduced?

Results

Microsatellite genetic variation

Forty-eight out of 246 (19.5%) exact tests of Hardy-Weinberg proportions were significant (Supplementary Table S3). The majority of these departures were associated with positive F_{is} values, indicative of heterozygote deficits. Most heterozygote deficits were detected at a single locus, AC4, which accounted for 15 out of the 48 significant tests. Micro-checker results suggested that locus AC4 had a high probability of having null alleles in all but Fx05L and PM14A samples (Supplementary Table S3). The consistent heterozygote deficits and suspicion of null alleles lead us to remove locus AC4 from subsequent analyses of population structure. There were a total of 348 significant pairwise genotypic association tests out of 1851 performed. However, no pair of loci was consistently associated across samples, which suggests an absence of linkage disequilibrium among loci.

Microsatellite polymorphism in *Ae. aegypti* from Madeira was low to moderate, with mean over sample AR ranging from 1.7 (AC7) to 6.6 (88AT1) and mean *He* from 0.081 (AC7) to 0.789 (88AT) (Supplementary Table S4). Expected heterozygosity was significantly lower in the other two localities of Madeira when compared to the mean over-years of Funchal (Wilcoxon signed-rank tests, Paúl do Mar: $p = 0.001$; Santa Cruz: $p = 0.004$). In Paúl do Mar, no significant differences in *He* were found between 2013

and 2014 (Wilcoxon signed-rank test, $p = 0.168$). The proportion of unrelated individuals obtained by ML-RELATE was above 70% in all samples except for Santa Cruz. This sample had the highest frequency of related individuals (41%) with a high proportion of full sibs (15%) and backcrosses (20%).

The overall mean AR and *He* across temporal samples from Madeira was significantly different from those of the Brazilian samples (Wilcoxon signed-ranks tests, Santos: AR $p < 0.001$, *He* $p = 0.033$; São Sebastião: AR $p = 0.018$, *He* $p = 0.015$) but comparable to the sample of Caracas, Venezuela (Wilcoxon signed-ranks tests, Caracas: AR $p = 0.159$, *He* $p = 0.433$).

With the exception of Funchal in 2014, when the larval sample (Fx2014L) was less polymorphic, estimates of *He* and AR were largely similar between larval and adult samples collected in the same locality and year (Supplementary Table S3). The degree of relatedness among individuals was comparable between larval and adult samples, as shown in Supplementary Fig. S1. These results suggest that the genetic variation captured by both sampling methods is comparable. Therefore, larval and adult samples were pooled in subsequent temporal analyses to represent a single sample per collection year.

Effective population size and demographic stability

In Funchal, single-sample estimates of effective population size based on the linkage disequilibrium method (LD-N_e) increased overtime, from a minimum of 3.4 in 2005 to a maximum of 657.0 in 2012, the year of the dengue epidemic (Table 2). After 2012, there was ten-fold reduction of LD-N_e. This pattern of temporal variation was not evident in the two-sample estimates of N_e (F_s-N_e, Table 2). These estimates varied between 291.1 and 401.3 with no apparent trend for increase/decrease over years and with overlapping 95%CI.

Table 2. Estimates of effective population size and Mutation-Drift Equilibrium tests.

		Mutation-drift equilibrium tests			Effective population size		
Population	Year	SMM	TPM	Mode-Shift	LD-N_e	Year interval	F_s - N_e
Funchal	2005	10	11	Normal	3.4		
		(0.299)	(0.027)		[2.3-8.7]		
	2009	9	10	Normal	42.9	2005-2009	304.9
		(0.244	(0.009)		[28.0-75.0]		[185.1 – 454.3]
	2011	9	11	Normal	71.2	2009-2011	401.3
		(0.339)	(0.015)		[38.5 - 222.4]		[240.1 – 603.3]
	2012	9	12	Normal	657.0	2011-2012	291.1
		(0.165)	(**0.001**)		[166.9 - ∞]		[175.4 – 435.7]
	2013	10	12	Normal	54.9	2012-2013	383.1
		(0.103)	(<**0.001**)		[40.2 – 78.6]		[230.8 – 573.3]
	2014	11	12	Shifted	49.9	2013-2014	295.2
		(0.047)	(<**0.001**)		[36.5 – 71.1]		[176.6 – 443.8]
Paúl do Mar	2013	6	11	Normal	6.0		
		(0.227)	(<**0.001**)		[3.6 – 9.2]		
	2014	8	9	Normal	30.6	2013-2014	62.2

		(0.178)	(0.017)		[21.8 – 44.1]	[34.8 – 97.4]
Santa Cruz	2014	7	9	Normal	1.2	
		(0.596)	(0.312)		[1.0 – 1.4]	
Caracas	2013	10	10	Shifted	23.9	
		(0.116)	(0.021)		[11.1 – 68.8]	
Santos	2008	6	10	Normal	10.8	
		(0.939)	(0.138)		[8.7 – 13.2]	
São Sebastião	2008	9	12	Normal	12.1	
		(0.380)	(<**0.001**)		[8.5 – 17.2]	

MDE tests: 95% confidence intervals in square brackets. Upper values, number of loci (out of 15) in which $H_e > H_{eq}$; lower values are the *p-value* for the corresponding one-tailed Wilcoxon test; SMM: stepwise-mutation model; TPM (30%): two-phased model with 30% of indels greater than one repeat; In bold: significant tests after adjustment by the sequential Bonferroni procedure; Effective population size: generations sampled in the two-sample estimates were set at 0 and 10 based on the length of time between the two field collections.

For both methods, N_e estimates were consistently higher in Funchal, when compared to the other localities (Table 2). In Paúl do Mar, there was a 5-fold increase of LD-N_e between 2013 and 2014. The lowest LD-N_e estimate was obtained for the only sample available from Santa Cruz. The estimates of LD-N_e obtained for the three South American continental samples analyzed in this study were consistently lower than those obtained for Funchal and Paúl do Mar, except for the samples of 2005 and 2013, respectively (Table 2).

Significant departures from mutation-drift equilibrium were detected by heterozygote tests only under the TPM model (Table 2). There was a consistent surplus of loci with apparent heterozygote excess in all Madeiran samples, but these were significant only in 2012 and onwards. In Funchal, the sample of 2014 also showed a shifted allele frequency distribution, indicative of a recent bottleneck. In continental samples, heterozygosity tests suggest a recent bottleneck in the population of São Sebastião, Brazil. The sample of Caracas, Venezuela, presented a shifted allele frequency distribution but the corresponding heterozygosity test was only marginally significant ($p<0.05$).

Population structure and origin

A first STRUCTURE analysis was performed with the Madeira dataset only and the results for the three best K values (K=2 to K=4) are shown in Fig. 2. Graphical representations of Evanno's ΔK can be seen in Supplementary Fig. S2. The sample from Paúl do Mar consistently formed a homogenous distinct genetic cluster in the three population structure scenarios. In the K=3 clusters scenario, population partitioning corresponds to the geographic localities sampled, with distinct genetic clusters for Santa Cruz, Paúl do Mar and Funchal. The K=4 scenario maintains the geographic substructuring but separates the samples from Funchal into two different genetic backgrounds.

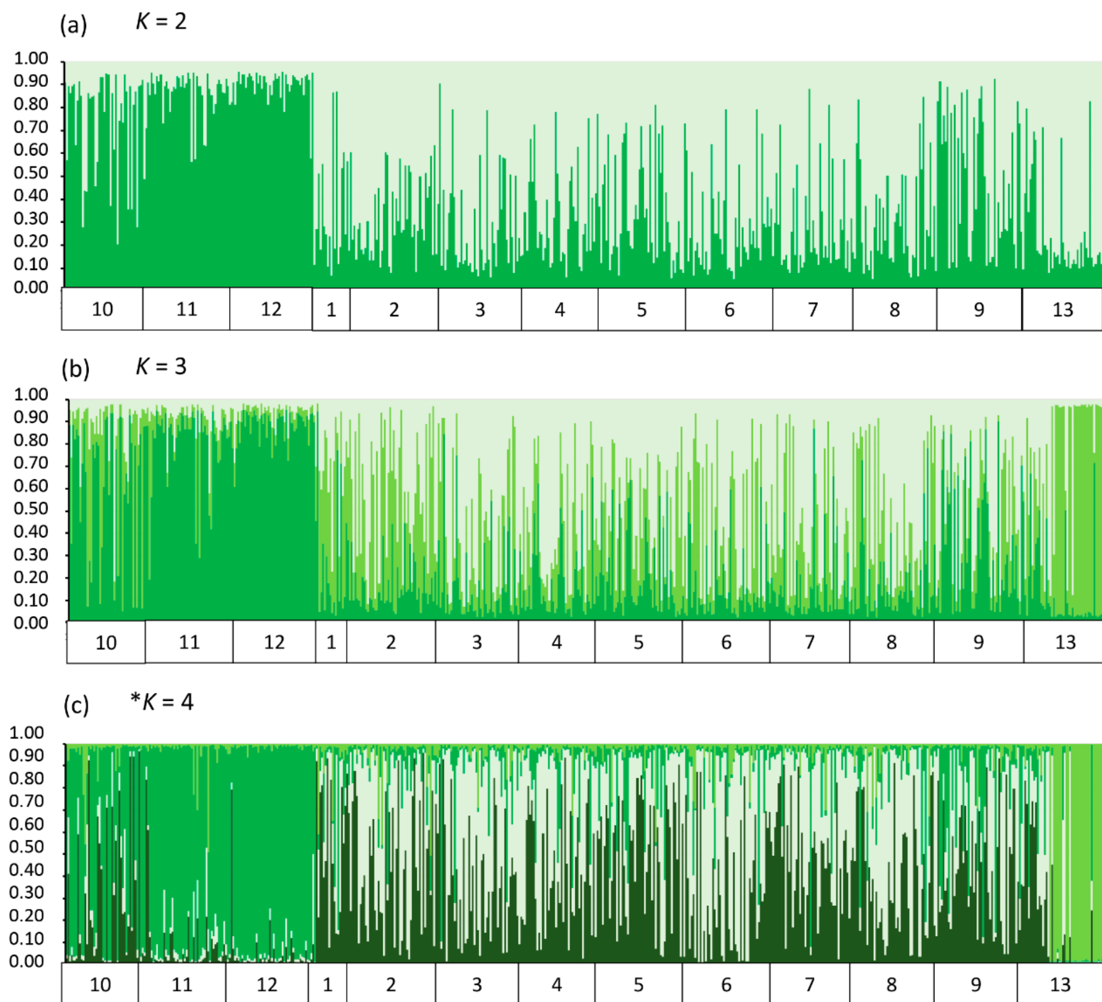

Figure 2. **Genetic structure of Madeira *Ae. aegypti* populations using 15 microsatellite markers**. Each bar represents an individual with the colour of the bar giving the probability of the individual belonging to a genetic population or cluster. (A, B, C) STRUCTURE plots of Madeira populations with *K* number of clusters as indicated. An asterisk indicates the plot representing the optimal *K* as determined by the delta *K* method. Legend: 1-9: Funchal populations; 10-12: Paúl do Mar populations; 13- Santa Cruz population. For population details, see Table 1.

This genetic partitioning within Funchal was not confirmed by the DAPC analysis (Fig. 3). Madeira samples were divided into two principal genetic clusters. The first discriminant function separates Paúl do Mar from Funchal and Santa Cruz while subdivision of these two localities in discriminant function two is less pronounced, judging from the respective eigenvalues.

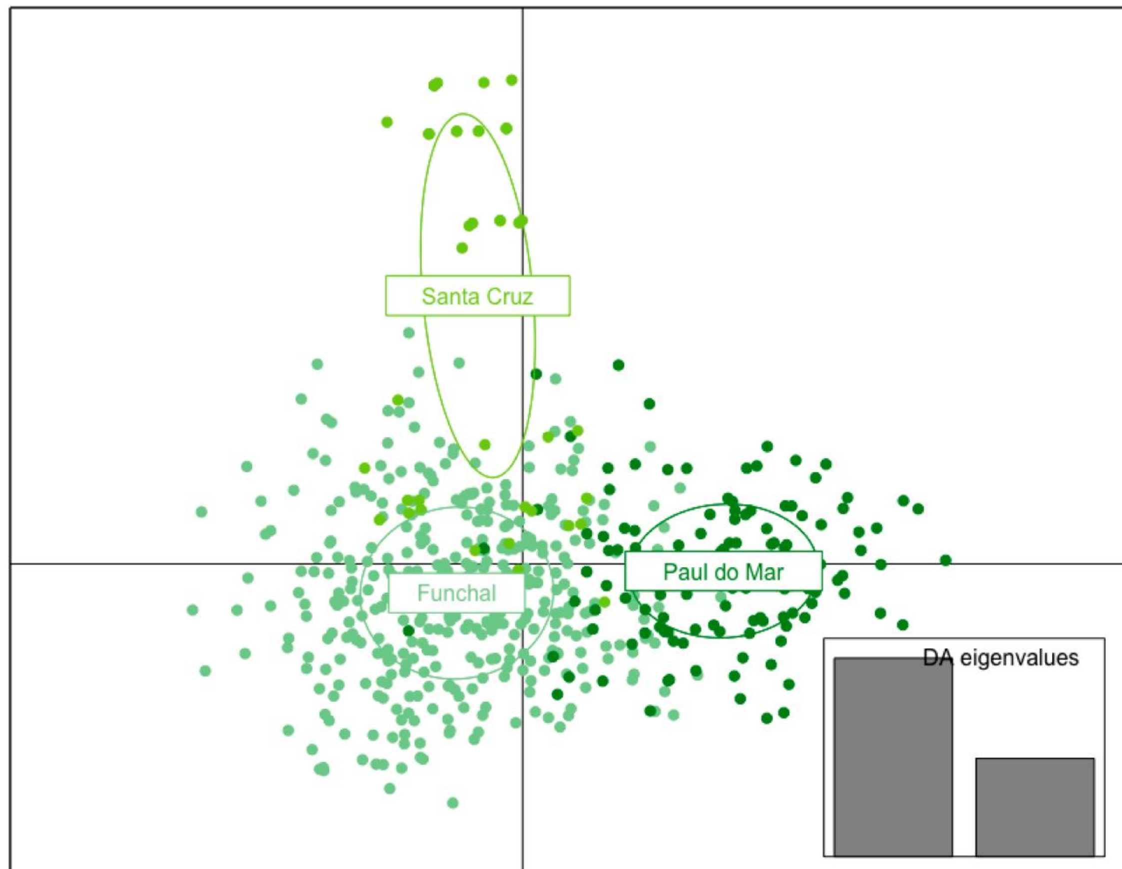

Figure 3. Discriminant analysis of principal components (DAPC) of *Ae. aegypti* populations in Madeira. Same populations depicted in the STRUCTURE plot shown in Figure 2.

A second STRUCTURE analysis was conducted with the complete dataset comprising the samples of Madeira and South America genotyped in this study, along with the dataset of Gloria-Soria *et al.* [20]. The best *K* obtained was *K=2*, reflecting the known segregation of the African *Aedes aegypti formosus* from out-of-Africa *Aedes aegypti aegypti* populations (Fig. 4a; see also Gloria-Soria *et al.*[20]).

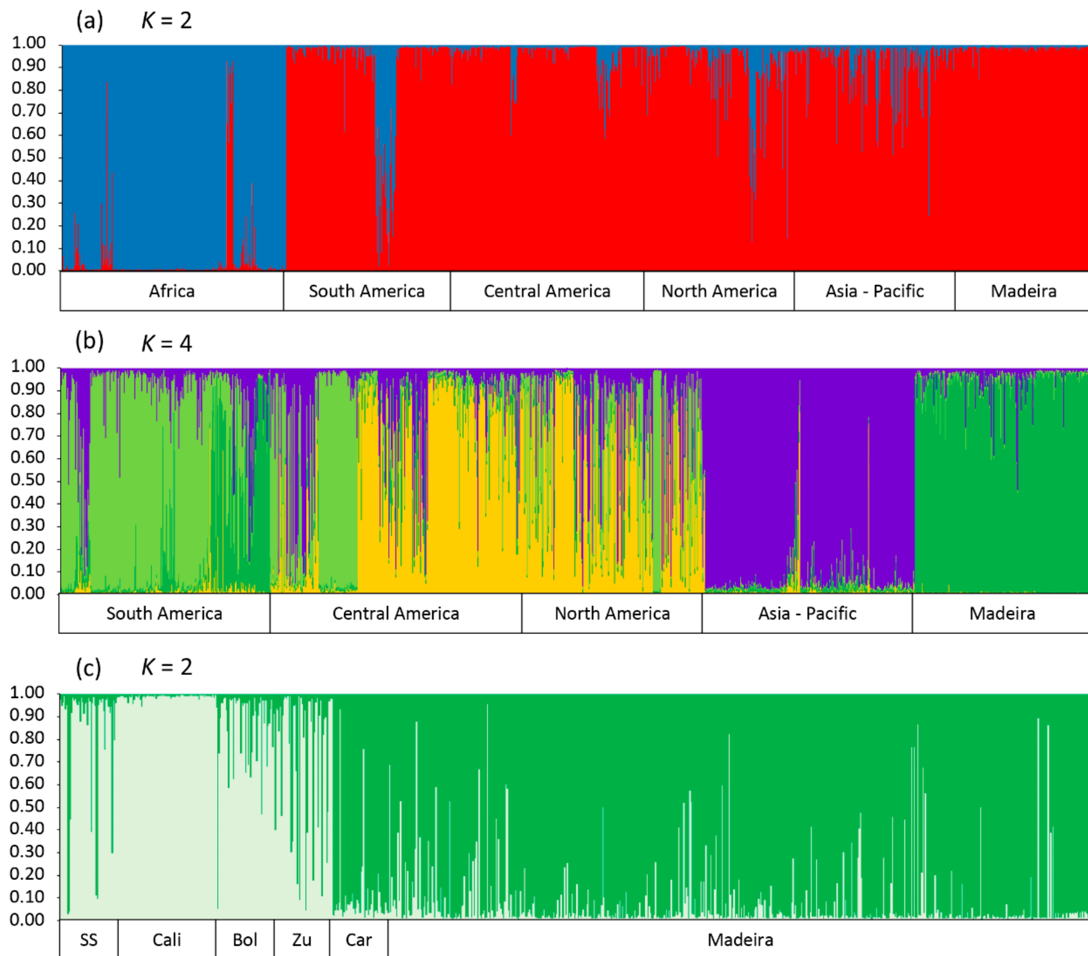

Figure 4. Analyses of *Ae. aegypti* from Madeira using 11 microsatellite markers. (A) STRUCTURE plot separating Aaa = *Ae. ae. aegypti*, red cluster; Aaf = *Ae. ae. formosus*, blue cluster. (B) Genetic structure of pantropical *Ae. aegypti* populations. (C) Genetic relationships between Madeira and South American populations. Colors of (A, B) are presented as in **Gloria-Soria et al. (2016).** Legend: SS – São Sebastião, Brazil; Cali – Cali, Colombia; Bol – Bolivar, Venezuela; Zu – Zulia, Venezuela; Car – Caracas, Venezuela.

All Madeiran individuals were homogenously assigned to the *Ae. aegypti aegypti* group. When the analysis was repeated without the African samples, the best value of *K* was equal to four (Fig. 4b). This partitioning reflected the previously shown three continental Asian/Pacific, North-Central American and South American clusters[20] along with one additional cluster that grouped all Madeira island samples with a subset of South-

American samples. A third STRUCTURE analysis was performed with samples of the fourth cluster only (Fig. 4c). This analysis gave a best K=2 and grouped the Madeiran samples mainly with Venezuelan samples from Caracas. A few individuals from São Sebastião, Brazil, were also assigned to this Madeira/Caracas cluster. The other cluster comprised individuals mainly from Brazil, Colombia and non-Caracas Venezuelan samples.

Results of a DAPC analysis conducted with the Madeira/South America subset confirmed a closer relationship between Madeira Island and the samples of Caracas, Venezuela, and São Sebastião, Brazil (Fig. 5).

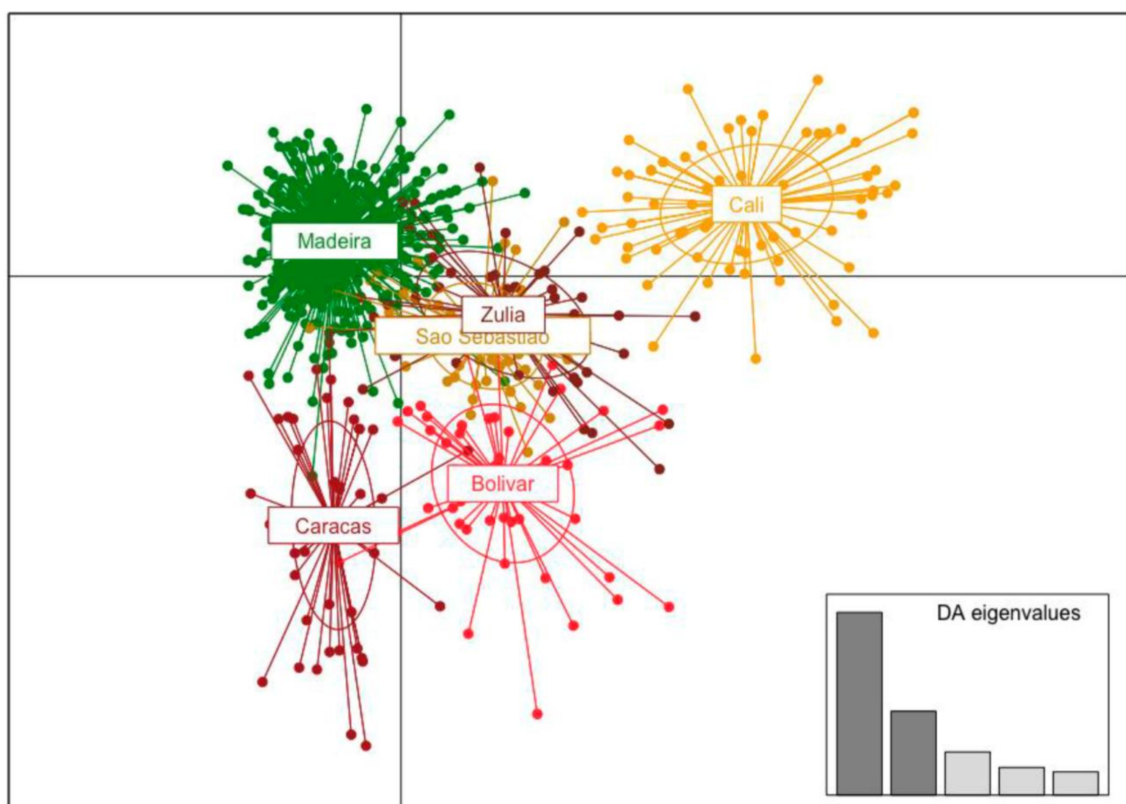

Figure 5. **Discriminant analysis of principal components (DAPC) of *Ae. aegypti* populations using a Madeira/South America subset** . Same populations depicted in the STRUCTURE plot shown in Figure 4C.

Mitochondrial DNA analysis

Summary statistics of genetic variation for each mtDNA gene in Madeira Island are shown in Supplementary Table S5. Partial COI sequences were obtained for 202

individuals (Supplementary Table S5). The 764 bp alignment revealed the presence of three distinct haplotypes and nucleotide diversity (π) of 0.00317. Partial ND4 sequences were analyzed from 191 mosquitoes (Supplementary Table S5). The 351 bp alignment revealed the presence of four haplotypes and π = 0.00545. Neutrality tests were non-significant for both genes.

A Median-Joining haplotype network for the concatenated COI/ND4 sequences is shown in Fig. 6.

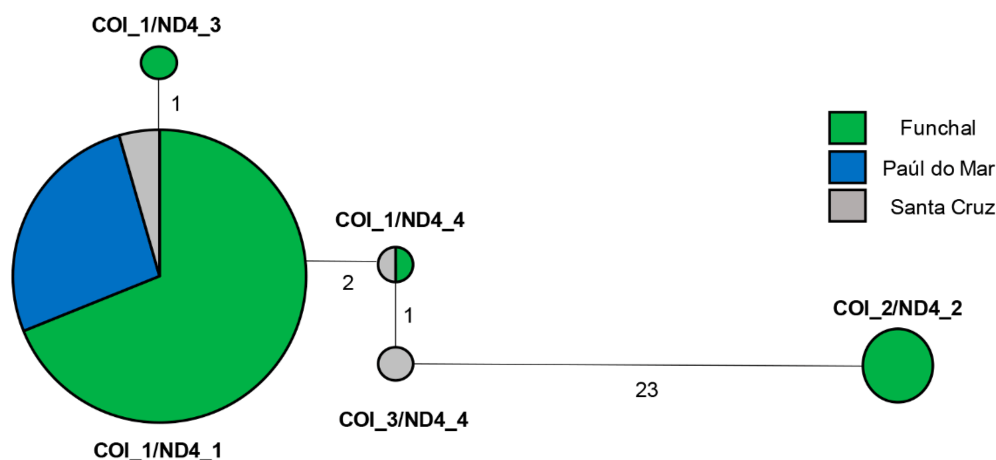

Figure 6. Median-joining network based on haplotypes obtained from the mtDNA concatenated COI and ND4 sequences as generated by Network version 5. The size of the nodes corresponds to the number of individuals with corresponding haplotypes. The number indicates the number of mutations between each haplotype.

Of the five different haplotypes identified, haplotype COI_1/ND4_1 was present in over 90% of all individuals in all localities and it was the only haplotype detected in Paúl do Mar in the two years sampled (Table 3). The second most frequent haplotype (COI_2/ND4_2) was separated by 23 mutational steps from the central COI_1/ND4_1 and it was only observed in Funchal. The two most frequent haplotypes were consistently detected in Funchal since the first collection in 2005. Three additional low frequency haplotypes derived from COI_1/ND4_1 by one or two mutational steps. One of these, COI_3/ND4_4 was unique to Santa Cruz only (2014) and haplotype COI_1/ND4_3 was unique to the 2005 collection of Funchal, in a single individual. Haplotype COI_1/ND4_4

was detected in 2014 simultaneously in Funchal and Santa Cruz. The 21 mtDNA sequences obtained from samples of Caracas, Venezuela, were all of the same haplotype, COI_1/ND4_1.

Table 3. Haplotype frequencies for concatenated COI/ND4 genes from *Ae. aegypti* populations in Madeira.

		N	COI_1/ND4_1	COI_1/ND4_3	COI_1/ND4_4	COI_2/ND4_2	COI_3/ND4_4
Funchal	2005	14	0.86	0.07	0.00	0.07	0.00
Funchal	2009	16	1.00	0.00	0.00	0.00	0.00
Funchal	2011	9	0.56	0.00	0.00	0.44	0.00
Funchal	2012	28	0.89	0.00	0.00	0.11	0.00
Funchal	2013	32	0.88	0.00	0.00	0.12	0.00
Funchal	2014	27	0.89	0.00	0.04	0.07	0.00
Paúl do Mar	2013	15	1.00	0.00	0.00	0.00	0.00
Paúl do Mar	2014	28	1.00	0.00	0.00	0.00	0.00
Santa Cruz	2014	9	0.78	0.00	0.11	0.00	0.11
	Total Madeira	178	0.90	0.006	0.01	0.08	0.006

We performed a phylogenetic analysis with concatenated COI-ND4 sequences to infer the phylogenetic relationships between Madeira and worldwide *Ae. aegypti* sequences[22]. The resulting phylogenetic tree revealed that *Ae. aegypti* in Madeira is represented by members of the two major mtDNA lineages known for this species (Fig. 7)[23]. The main COI_1/ND4_1 is included in the West African lineage and clusters with Venezuelan and USA haplotypes. Haplotypes COI_1/ND4_3, COI_1/ND4_4 and COI_3/ND4_4 are also included in this clade. Haplotype COI_2/ND4_2 is included in the East Africa lineage that contains sequences from Asia, Central America, Caribe and Brazil.

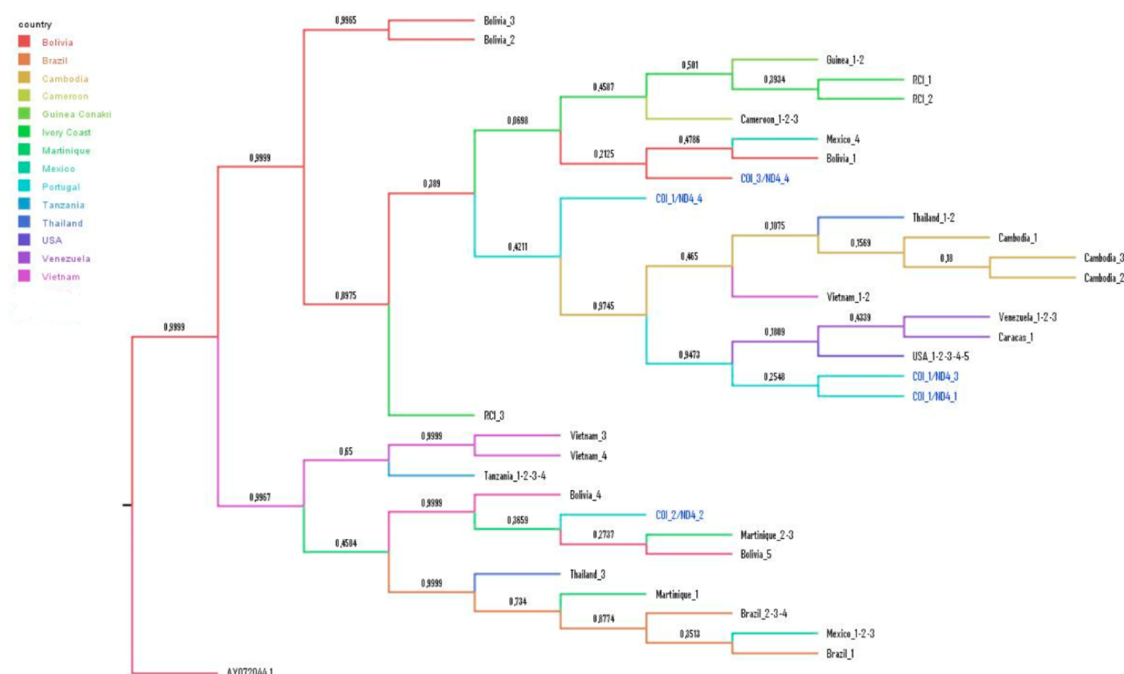

Figure 7. Phylogenetic tree obtained with a Bayesian inference of concatenated COI and ND4 sequences. Sequence number AY072044.1 is an outgroup *Aedes albopictus* specimen.

Discussion

The results of the present study indicate that the recently established *Ae. aegypti* population of Madeira has derived from at least two independent introductions, possibly occurring at different time-points. Venezuela is the most likely geographic origin but a Brazilian source population, at least for one of the introductions, cannot be fully excluded. After the initial colonization, *Ae. aegypti* has rapidly expanded throughout the southern

part of the island, reaching its maximum effective population size in 2012, which was coincident with the dengue outbreak that occurred in Madeira[12]. The reduction of N_e recorded after the outbreak may have been the result of the increased vector control implemented to halt virus transmission.

The lower mtDNA genetic diversity of *Ae. aegypti* in Madeira is consistent with a recently established island population. Values of haplotype diversity below 0.200 recorded in the island are lower than those recorded for continental populations of this mosquito species (e.g. Brazil[24]: Hd = 0.800; Florida, USA[25]: Hd = 0.886; Colombia[26]: Hd = 0.914). However, microsatellite genetic diversity of this 2005 sample (Fx05L) is comparable to other *Ae. aegypti* island[27] and mainland populations[28,29]. Furthermore, MDE tests did not provide evidence of a population contraction associated with a founder event mediated by only a few individuals. This may reflect a higher evolution rate of microsatellites, with mutation rates[30] around 10^{-4}-10^{-3}, or that the size of the founding population was still sufficient to maintain a representative gene pool of this species.

Both genetic and historical evidence support an initial introduction of *Ae. aegypti* in Funchal, possibly by maritime transportation. Funchal is the capital and the major urban centre of the island, where the international harbour is located. It was in Funchal that the first *Ae. aegypti* specimens were collected in October 2005[9]. In agreement, Funchal displays the highest genetic diversity for both mtDNA and microsatellites and the largest *Ne* estimates recorded in Madeira, suggesting a longer established population. After the introduction, *Ae. aegypti* rapidly expanded eastwards and westwards of Funchal, reaching Santa Cruz in 2008[10] and Paúl do Mar in 2012[12]. Again, genetic data supports an earlier colonization of Santa Cruz, judging from the higher haplotype diversity when compared to Paúl do Mar, with a single haplotype. The lower *Ne* estimates also agree with subsequent colonisations of those two localities after the initial introduction of *Ae. aegypti* in Funchal. This expansion was most probably human-mediated, through road transportation along the only highway that connects most of the southern part of the island. In fact, the pronounced island's topography is likely to act as a natural barrier to active dispersal of this mosquito and this may be the reason why *Ae. aegypti* has not yet established in the northern part of the island. The influence of human movement in shaping genetic diversity, structure and differentiation was also observed on other Ae. aegypti insular populations as in the Antilles islands[31] and in the Pacific region[32].

Concatenated mtDNA sequences revealed the presence of two highly divergent haplotypes separated by 23 mutation steps (COI_1/ND4_1 and COI_2/ND4_2). These two haplotypes were detected in the initial sample of Funchal 2005, providing evidence that the initial colonization was made by at least two different maternal lineages. However, the presence of a unique mtDNA haplotype (COI_3/ND4_4) in Santa Cruz may suggest one additional introduction on the island. Santa Cruz is a county where the International Airport of Madeira is located, providing an opportunity for airplane-mediated transportation of mosquitoes, an occurrence that has been previously detected[33]. While this haplotype was only detected in 2014, we cannot exclude an earlier introduction due to the lack of sample availability from previous years in this locality.

Both Bayesian clustering and DAPC analyses suggest a South American origin of *Ae. aegypti* in Madeira. All Madeira samples grouped in a distinct genetic cluster together with specimens from Venezuela, mainly from Caracas. Moreover, the most frequent mtDNA haplotype in Madeira (COI_1/ND4_1) is the same haplotype found in all Caracas specimens sequenced in this study. A Venezuelan origin of *Ae. aegypti* in Madeira is not surprising given that Madeira has an important emigrant community living in Caracas[34]. During summer, there is extensive movement between Caracas and Madeira because of holidaying by the migrant community. Coincidently, the only direct flight connecting Madeira and South America is between Funchal and Caracas[16].

A Venezuelan origin of *Ae. aegypti* also agrees with the insecticide susceptibility profile of *Ae. aegypti* in Madeira. This population was found to be resistant to three different insecticide classes and resistance was associated with knockdown resistance (*kdr*) mutations F1534C and V1016I and elevated expression of detoxification enzymes[35]. Similarly, insecticide resistance studies in *Ae. aegypti* from Venezuela revealed high frequencies of F1534C and V1016I *kdr* mutations and increased activity of glutathione-S transferases, esterases and mixed-function oxidases[36,37], the same profile as that observed in Madeiran *Ae. aegypti*.

In addition to a Venezuelan origin, at least one introduction may have derived from Brazil. There were 7 individuals from São Sebastião, Brazil, which grouped in the Madeira/Caracas genetic cluster and 21 individuals from Madeira with genetic ancestry closest to the Brazilian/Colombian cluster. Moreover, the phylogenetic tree indicates that

haplotype COI_2/ND4_2 groups in a clade in sequences from Brazil but not from Venezuela. However, this may simply reflect that the number of specimens sequenced from Venezuela was too low to capture all the haplotype diversity. Therefore, we cannot exclude the possibility of haplotype COI_2/ND4_2 also being present in Venezuela but not sampled.

Estimates of LD-*Ne* in Funchal consistently increased overtime from the first sample time-point in 2005 until reaching its highest value in 2012. Coincidently, 2012 was the year of the dengue epidemic on the island[36]. Such an increase in *Ne* agrees with the rapid expansion of this mosquito vector on the island. The precise ecological conditions driving this expansion are not fully understood but the mild temperate climate suitable for sustaining a mosquito population throughout the year coupled with an extensive availability of breeding sites (flower pots)[13] in urban and rural areas may have facilitated adaptation and subsequent population expansion on the island[14].

The utility of genetic markers in assessing the impact of vector control on the mosquito population has been tested previously, with varying degrees of success[39,40,41]. Interestingly, estimates of F_s-*Ne* did not show any trend of temporal variation. While two-sample estimates are sensitive to population fluctuations, these methods are influenced by the initial (T_0) genetic variation of the population, since N_e is retrieved from an unbiased estimate of allelic variance[42,43]. Therefore, the initial low microsatellite polymorphism of the Madeiran *Ae. aegypti* population may have affected the sensitivity of F_s-*Ne* in detecting population contractions. Coincidently, F_s-*Ne* samples of Funchal in the order of the hundreds are within the average values obtained in a previous study analyzing a global dataset for *Ae. aegypti*[44].

After the 2012 outbreak, estimates of *Ne* significantly decreased in 2013 and 2014. Heterozygosity tests and mode-shift allele detected a recent bottleneck during this period. These results suggest that the vector control measures implemented after the dengue outbreak were effective in reducing *Ae. aegypti* densities. It should be emphasized that vector control during the dengue outbreak of Madeira was predominantly based on community-based larval source reduction, enforced by a strong communication campaign led by the local health authorities[12]. Given the high insecticide resistance of *Ae. aegypti* on the island, alternative non-insecticidal methods are essential to contain the mosquito

population and, subsequently, prevent arbovirus transmission. In this context, larval source reduction is a valid option for Madeira. This method is also recommended by the World Health Organization as a primary vector control tool for *Ae. aegypti*[45].

To conclude, *Ae. aegypti* has recently arrived in Madeira and rapidly expanded its population size to levels able to sustain transmission of an arbovirus epidemic. The Venezuelan origin is coherent with the socioeconomic relations of this insular territory with that country and highlights the importance of monitoring mosquito populations at points of entry such as international harbours and airports. This study also provided evidence for the effectiveness of non-insecticidal vector control methods. The relatively small effective size of this island vector population may also be regarded as advantageous for the implementation of vector control tools that rely on genetically modified mosquitoes[46].

Methods

Mosquito samples

Mosquito collections were performed in three localities of Madeira: Funchal, the capital city; Santa Cruz and Paúl do Mar, representing the eastern and western distribution limits of *Ae. aegypti* in the island (Fig. 1). Collections were made at six time points in Funchal (2005, 2009, 2011, 2012, 2013 and 2014) and two (2013 and 2014) in Paúl do Mar. In addition, mosquito samples from two localities in Brazil (Santos and São Sebastião) and one in Venezuela (Caracas) were also analysed. The two Brazilian localities represent coastal cities with major international harbours. Caracas is the capital of Venezuela and located ca. 30 km away from the major harbour city La Guaira. Sample details, including the sampling method, collection year and sample sizes, are available in Supplementary Table S1.

Both immature and adult mosquitoes were sampled. Collected immatures (eggs and larvae) were reared to adults under insectary conditions. Adults were identified to species using morphological keys[47] and stored individually in silica-gel at -20ºC until DNA extraction.

DNA extraction

Genomic DNA was extracted using the NZY tissue gDNA isolation kit (NZYtech Portugal) for the 2005 Madeira sample, and with the protocol of Collins *et al*.[48] for the other Madeira samples. DNA of the individuals from Brazil and Venezuela were extracted using a Chelex100® Molecular Biology Grade resin (Bio-rad Laboratories) protocol according to the manufacturer's protocols.

Microsatellite genotyping

A total of 16 microsatellites were genotyped by fragment size analysis of polymerase chain reaction amplified products. Primer sequences and PCR conditions followed previously published protocols[49,50,51] and are described in Supplementary Table S2. Fragment size analysis was performed by capillary electrophoresis on an ABI3130xl genetic analyser (Applied Biosystems), at the DNA Analysis Facility at Science Hill, Yale University. Microsatellite alleles were scored using GENEMARKER software (SoftGenetics, PA, USA).

The genotypes obtained were integrated into the microsatellite genotypic database of Gloria-Soria *et al*.[20], available in VectorBase (Project ID: VBP0000138). This database has genotypes for 12 of the 16 microsatellites genotyped in a total of 3,566 individuals from 78 countries representing Asian, African and American *Ae. aegypti* populations. In order to calibrate inter-lab allele scoring, 20 individuals from Gloria-Soria *et al*.[20], kindly provided by the Jeffrey Powell laboratory at Yale University, were analysed at GHTM and the genotypes were compared with the original scorings.

The genotypes obtained for the samples here analysed are available in VectorBase (Project ID: VBP0000303).

Microsatellite data analysis

Expected heterozygosity (H_e) and the inbreeding coefficient (F_{is}) were estimated using GENEPOP[52]. The same software was used to perform exact tests of departure from Hardy-Weinberg proportions and of linkage disequilibrium (LD) among pairs of loci. Estimates of allele richness (AR) were obtained for each population by the statistical

rarefaction approach implemented in HP-RARE[53]. The software Micro-checker 2.2.3[54] was used to test for the presence of null alleles (99% confidence interval) at each locus/sample.

Two estimates of current effective population size (N_e) were made: single-sample estimates based on the linkage disequilibrium method[55]; and two-sample temporal estimates, based on the F-statistic of Jorde & Ryman[56]. Calculations were performed using NeEstimator v2[57]. Because rare alleles may bias linkage disequilibrium N_e estimates, alleles with frequency below 0.05 at each locus were removed from the analysis.

Evidence of recent population perturbations was assessed by heterozygosity tests as implemented in BOTTLENECK version 1.2.02[58]. Expected heterozygosity estimates assuming mutation drift equilibrium (MDE) were calculated from the number of alleles and sample size under two mutation models, considered more suitable for microsatellites: the stepwise mutation model (SMM) and a two-phased model (TPM) with 30% multistep mutations (variance = 30%). Although the SMM has been considered as better suited for the type of mutation process most frequent in microsatellites (i.e. DNA slippage[59]), there is evidence that intermediate mutation models such as the TPM with increasing proportions of multistep mutations are less prone to detect false positives[60]. Wilcoxon tests were used to assess significance between observed and MDE-expected heterozygosities, as recommended for analysis with less than 20 loci[61]. In addition, the allele frequency distribution method was also used[60]. Under MDE, an L-shaped allele frequency distribution is expected, whereas a shifted distribution due to loss of low-frequency alleles is consistent with a recent bottleneck.

In order to assess the degree of relatedness among individuals, the maximum-likelihood method implemented in ML-RELATE was used[63]. For each pair of individuals, log-likelihood estimates are calculated for four pedigree classes: unrelated, half-siblings, full-siblings and parent-offspring.

Bayesian clustering analysis was performed using the software STRUCTURE version 2.3.4.[64], in order to assess within-island population subdivision and to determine the most likely source populations of *Ae. aegypti* in Madeira. In a first analysis, only Madeira island samples were used. Subsequently, the Madeira island genotypes were analysed with the

continental sample dataset of Gloria-Soria *et al.*[20]. Twenty independent runs were made for each value of *K*, which varied from one to 10 for within island analysis, including source population determination, and from one to five at the subspecies/species level. Each run was conducted with a burn-in of 100,000 iterations and 500,000 replicates, assuming an admixture model with correlated allele frequencies. The optimal number of clusters was determined following the guidelines of Pritchard *et al.*[64] and the delta *K* statistic of Evanno *et al.*[65], using STRUCTURE HARVESTER version 0.6.94[66]. The information from the outputs of each *K* was aligned by the Greedy method implemented in CLUMPP[67].

Discriminant Analysis of Principal Components (DAPC), as implemented by ADEGENET[68], was used to visualize patterns of genetic differentiation among individual mosquitoes belonging to different genetic clusters in a two-dimensional plot. This analysis was performed with the samples from Madeira and a subset of candidate source populations selected from the Bayesian clustering analysis.

Whenever multiple tests were performed, the nominal significance level ($\alpha = 0.05$) was adjusted by the sequential Bonferroni procedure[69].

Mitochondrial DNA sequencing

The mitochondrial genes COI and ND4 were analysed by direct sequencing from amplified products, corresponding to 764 bp and 351 bp, respectively, using previously published primers[22,24] and protocols[19]. In addition to Madeira individuals, mtDNA sequences for 21 individuals from Caracas, Venezuela, were also obtained to compensate for the scarcity of sequences available in Genbank for this country. Sequences were aligned and manually corrected using BioEdit v7.0.5[70]. For each gene, haplotype diversity (*Hd*), nucleotide diversity (π) and the Tajima and Fu and Li neutrality tests were computed by DNAsp v5.10[71].

In order to infer the relationships between haplotypes in Madeira, haplotype networks were constructed for concatenated COI-ND4 sequences using a median-joining algorithm as implemented in the NETWORK software[72].

The BEAST v1.8.4 software[73] was used to generate a phylogenetic tree based on the COI-

ND4 concatenated haplotypes, obtained from sequences produced in this study and others retrieved from GenBank (Supplementary Table S2). The analysis was run in three separate independent runs with 500 million generations, sampled every 100 000 runs for the concatenated genes. A Bayesian skyline population growth model was used. The substitution model HKY[74] with gamma and invariant sites and three partitions into codon positions was selected. MCMC analysis was run long enough for convergence to be obtained. To analyze convergence and stability, we used Tracer v1.6 software[75]. TreeAnnotator was used to estimate the final Maximum Clade Credibility Tree, summarizing the posterior probability of each clade of the trees, as well as the average and confidence interval for the evolutionary rate of each branch of the tree. The obtained Bayesian trees were visualized and edited with FIGTREE 1.4.3. (http://tree.bio.ed.ac.uk/software/figtree/).

References

1. Fernandez-Garcia, M. D. *et al.* Chikungunya virus infections among travellers returning to Spain, 2008 to 2014. *Euro Surveill* **21,** 1–9 (2016).
2. Adhami, J. & Murati, N. The presence of the mosquito *Aedes albopictus* in Albania. *Rev Mjekësore* **1**, 13–16 (1987).
3. Medlock, J. M. *et al.* A Review of the Invasive Mosquitoes in Europe: Ecology, Public Health Risks, and Control Options. *Vector-Borne Zoonotic Dis* **12,** 435–447 (2012).
4. Rezza, G. *et al.* Infection with chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* **370,** 1840–1846 (2007).
5. Venturi, G. *et al.* Detection of a chikungunya outbreak in Central Italy Detection of a chikungunya outbreak in Central. *Euro Surveill* **22,** 1–4 (2017).
6. Gould, E. A., Gallian, P., De Lamballerie, X. & Charrel, R. N. First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality! *Clin Microbiol Infect* **16,** 1702–1704 (2010).
7. Calba, C. *et al.* Preliminary report of an autochthonous chikungunya outbreak in France, July to September 2017. *Euro Surveill* **22,** 5–10 (2017).
8. Gjenero-Margan, I. *et al.* Autochthonous dengue fever in Croatia, August-September 2010. *Euro Surveill* **16,** (2011).

9. Margarita, Y. *et al.* First record of *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) (Diptera, Culicidae) in Madeira Island – Portugal (Portuguese, English abstract). *Acta Parasitol Port* **13**: 59-61. (2006).
10. Gonçalves, Y. *et al.* On the presence of *Aedes* (Stegomyia) *aegypti* Linnaeus, 1762 (Insecta, Diptera, Culicidae) in the island of Madeira (Portugal). *Bol Mus Mun Funchal* **58**, 53–59 (2008).
11. Alves, M. J. *et al.* Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012. *Euro Surveill* **18,** 3–6 (2013).
12. European Centre for Disease Prevention and Control. Dengue outbreak in Madeira, Portugal, March 2013. Stockholm: ECDC; (2014).
13. Nazareth, T. *et al.* Strengthening the perception-assessment tools for dengue prevention: a cross-sectional survey in a temperate region (Madeira, Portugal). *BMC Public Health* **14,** 39 (2014).
14. Lourenço, J. & Recker, M. The 2012 Madeira dengue outbreak: epidemiological determinants and future epidemic potential. *PLoS Negl Trop Dis* **8,** (2014).
15. Frank, C., Höhle, M., Stark, K. & Lawrence, J. More reasons to dread rain on vacation? Dengue fever in 42 German and United Kingdom Madeira tourists during autumn 2012. *Euro Surveill* **18,** 14–17 (2013).
16. Wilder-Smith, A. *et al.* The 2012 dengue outbreak in Madeira: exploring the origins. *Euro Surveill* **19,** 20718 (2014).
17. Seixas, G. *et al.* Potential of Aedes aegypti populations in Madeira Island to transmit dengue and chikungunya viruses. *Parasit. Vectors* **11,** 509 (2018).
18. Jupille, H., Seixas, G., Mousson, L., Sousa, C. A. & Failloux, A. B. Zika Virus, a New Threat for Europe? *PLoS Negl. Trop. Dis.* **10,** 1–8 (2016).
19. Seixas, G. *et al. Aedes aegypti* on Madeira Island (Portugal): genetic variation of a recently introduced dengue vector. *Mem Inst Oswaldo Cruz* 1–8 (2013). doi:10.1590/0074-0276130386
20. Gloria-Soria, A. *et al.* Global genetic diversity of *Aedes aegypti*. *Mol Ecol* **25,** 5377–5395 (2016).
21. Gloria-Soria, A. *et al.* Origin of a high-latitude population of *Aedes aegypti* in Washington, DC. *Am J Trop Med Hyg* **98,** 445–452 (2018).

22. Paupy, C. *et al.* Genetic structure and phylogeography of *Aedes aegypti*, the dengue and yellow-fever mosquito vector in Bolivia. *Infect Genet Evol* doi:10.1016/j.meegid.2012.04.012
23. Bracco, J. E., Capurro, M. L., Lourenço-de-Oliveira, R. & Sallum, M. A. M. Genetic variability of *Aedes aegypti* in the Americas using a mitochondrial gene: evidence of multiple introductions. *Mem. Inst. Oswaldo Cruz* **102,** 573–80 (2007).
24. Paduan, K. D. S. & Ribolla, P. E. M. Mitochondrial DNA polymorphism and heteroplasmy in populations of *Aedes aegypti* in Brazil. *J Med Entomol* **45,** 59–67 (2008).
25. Damal, K., Murrell, E. G., Juliano, S. A., Conn, J. E. & Loew, S. S. Phylogeography of *Aedes aegypti* (yellow fever mosquito) in South Florida: MtDNA evidence for human-aided dispersal. *Am J Trop Med Hyg* **89,** 482–488 (2013).
26. Jaimes-Dueñez, J., Arboleda, S., Triana-Chávez, O. & Gómez-Palacio, A. Spatio-Temporal distribution of *Aedes aegypti* (Diptera: Culicidae) mitochondrial lineages in cities with distinct dengue incidence rates suggests complex population dynamics of the dengue vector in Colombia. *PLoS Negl Trop Dis* **9,** 1–21 (2015).
27. Gloria-Soria, A. *et al.* Temporal genetic stability of *Stegomyia aegypti* (= *Aedes aegypti*) populations. *Med Vet Entomol* (2016). doi:10.1111/mve.12153
28. Monteiro, F. A. *et al.* Genetic diversity of Brazilian *Aedes aegypti*: patterns following an eradication program. *PLoS Negl Trop Dis* **8,** 1–10 (2014).
29. Kotsakiozi, P. *et al.* Tracking the return of *Aedes aegypti* to Brazil, the major vector of the dengue, chikungunya and Zika viruses. *PLoS Negl Trop Dis* **11,** 1–20 (2017).
30. Ellegren, H. Microsatellite mutations in the germline: Implications for evolutionary inference. *Trends Genet* **16,** 551–558 (2000).
31. Sherpa, S. *et al.* At the origin of a worldwide invasion: unraveling the genetic makeup of the Caribbean bridgehead populations of the dengue vector *Aedes aegypti*. *Genome Biol. Evol.* **10,** 56–71 (2018).
32. Calvez, E. *et al.* Genetic diversity and phylogeny of *Aedes aegypti*, the main arbovirus vector in the Pacific. *PLoS Negl. Trop. Dis.* **10,** e0004374 (2016).

33. Ibañez-Justicia, A. *et al.* The first detected airline introductions of yellow fever mosquitoes (*Aedes aegypti*) to Europe, at Schiphol International airport, the Netherlands. *Parasit. Vectors* **10,** 1–9 (2017).
34. Dinneen, M. Portuguese immigrants in Caracas: social networks and transnational connectivity. *Port J Soc Sci* **10,** 169 (2011).
35. Seixas, G. *et al.* Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal). *PLoS Negl Trop Dis* **11,** 1–16 (2017).
36. Alvarez, L. C., Ponce, G., Oviedo, M., Lopez, B. & Flores, A. E. Resistance to malathion and deltamethrin in *Aedes aegypti* (Diptera: Culicidae) from western Venezuela. *J Med Entomol* **50,** 1031–9 (2013).
37. Alvarez, L. C., Ponce, G., Saavedra-Rodriguez, K., Lopez, B. & Flores, A. E. Frequency of V1016I and F1534C mutations in the voltage-gated sodium channel gene in *Aedes aegypti* in Venezuela. *Pest Manag Sci* **71,** 863–869 (2015).
38. Sousa, C. A. *et al.* Ongoing outbreak of dengue type 1 in the Autonomous Region of Madeira, Portugal: preliminary report. *Euro Surveill* **17,** 8–11 (2012).
39. Pinto, J. *et al.* Genetic structure of *Anopheles gambiae* (Diptera: Culicidae) in São Tomé and Príncipe (West Africa): implications for malaria control. *Mol Ecol* **11,** 2183–7 (2002).
40. Wondji, C. *et al.* Impact of insecticide-treated bed nets implementation on the genetic structure of *Anopheles arabiensis* in an area of irrigated rice fields in the Sahelian region of Cameroon. *Mol Ecol* **14,** 3683–93 (2005).
41. Hodges, T. K. *et al.* Large fluctuations in the effective population size of the malaria mosquito *Anopheles gambiae* s.s. during vector control cycle. *Evol Appl* **6,** 1171–1183 (2013).
42. Luikart, G., Ryman, N., Tallmon, D. A., Schwartz, M. K. & Allendorf, F. W. Estimation of census and effective population sizes: The increasing usefulness of DNA-based approaches. *Conserv Genet* **11,** 355–373 (2010).
43. Wang, J. A comparison of single-sample estimators of effective population sizes from genetic marker data. *Mol Ecol* **25,** 4692–4711 (2016).
44. Saarman, N. P. *et al.* Effective population sizes of a major vector of human diseases, *Aedes aegypti*. *Evol Appl* **10,** 1031–1039 (2017).

45. WHO. Global strategy for dengue prevention and control 2012-2020, Geneve, Switzerland: World Health Organization (2012).
46. Marsden, C. D. *et al.* An analysis of two island groups as potential sites for trials of transgenic mosquitoes for malaria control. *Evol Appl* **6,** 706–20 (2013).
47. Ribeiro, H. & Ramos, H.C. Identification keys of the mosquitoes (Diptera: Culicidae) of Continental Portugal, Açores and Madeira. *European Mosquito Bulletin* **3**, pp.1-11 (1999).
48. Collins, F.H. *et al*. A ribosomal RNA gene probe differentiates member species of the *Anopheles gambiae* complex. *Am J Trop Med Hyg* **37**: 37–41 (1987).
49. Slotman, M. A. *et al.* Polymorphic microsatellite markers for studies of *Aedes aegypti* (Diptera: Culicidae), the vector of dengue and yellow fever. *Mol Ecol Notes* **7,** 168–171 (2007).
50. Lovin, D. D. *et al.* Genome-based polymorphic microsatellite development and validation in the mosquito *Aedes aegypti* and application to population genetics in Haiti. *BMC Genomics* **10,** 590 (2009).
51. Brown, J. E. *et al.* Worldwide patterns of genetic differentiation imply multiple 'domestications' of *Aedes aegypti*, a major vector of human diseases. *Proc Biol Sci* **278,** 2446–54 (2011).
52. Rousset, F. Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. *Mol Ecol Resour* **8**: 103-106 (2008).
53. Kalinowski, S.T. Counting alleles with rarefaction: private alleles and hierarchical sampling designs. *Conserv Genet* **5**, 539-543 (2004).
54. Van Oosterhout, C., Hutchinson, W. F., Wills, D. P. M. & Shipley, P. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* **4**, 535–538 (2004).
55. Waples, R. S. & Do, C. LDNE: A program for estimating effective population size from data on linkage disequilibrium. *Mol Ecol Resour* **8**, 753–756 (2008).
56. Jorde, P. E. & Ryman, N. Unbiased estimator for genetic drift and effective population size. *Genetics* **177**, 927–935 (2007).
57. Do, C. et al. NeEstimator v2: Re-implementation of software for the estimation of contemporary effective population size (Ne) from genetic data. *Mol Ecol Resour* **14**, 209–214 (2014).

58. Piry, S,. Luikart, G., Cornuet, J.M. Computer note. BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. *J Hered* **90**:502–3 (1999).
59. Slatkin, M. Hitchhiking and associative overdominance at a microsatellite locus. *Mol Biol Evol* **12,** 473–480 (1995).
60. Peery, M. Z. *et al.* More precisely biased: Increasing the number of markers is not a silver bullet in genetic bottleneck testing. *Mol Ecol* **22,** 3451–3457 (2013).
61. Cornuet, J. M. & Luikart, G. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144,** 2001–2014 (1996).
62. Luikart, G., Allendorf, F. W., Cornuet, J.M. & Sherwin, W.B. Distortion of allele frequency distributions provides a test for recent population bottlenecks. *J Hered* **89,** 238–247 (1998).
63. Kalinowski, S.T, Wagner, A.P, Taper, ML. ML-RELATE: a computer program for maximum likelihood estimation of relatedness and relationship. *Mol Ecol Notes* **6** (2006). doi:10.1111/j.1471-8286.2006.01256.x
64. Pritchard, J. K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).
65. Evanno, G., Regnaut, S. & Goudet, J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* **14**, 2611–2620 (2005).
66. Earl, D. A. & vonHoldt, B. M. Structure Harvester: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour* **4**, 359–361 (2012).
67. Jakobsson, M. & Rosenberg, N. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* **23**, 1801–1806 (2007).
68. Jombart, T., Devillard, S. & Balloux, F. Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. *BMC Genet* **11,** 94 (2010).
69. Holm, S. A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65-70 (1979).

70. Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41,** 95–98 (1999).
71. Librado, P. & Rozas, J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451-1452 (2009).
72. Bandelt, H.J., Forster, P., Röhl, A. Median-Joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* **16**, 37-48 (1999).
73. Drummond, A.J., Suchard, M.A., Xie, D., Rambaut A. Bayesian Phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* **29**, 1969–1973 (2012). doi:10.1093/molbev/mss075
74. Hasegawa, M., Kishino, H., Yano TA. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174 (1985).
75. Rambaut, A., Drummond, A.J., Xie D., Baele, G. & Suchard, M.A. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*. **syy032** (2018). doi:10.1093/sysbio/syy032

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Table 1. ***Aedes aegypti* samples included in this study.**

Sample code[a]	Sample name	Country	Locality	GPS coordinates	Collection method	Collection month/year	Sample size
1	Fx05L	Portugal	Funchal	32.64134,-16.916687	Larval	November/2005	22
2	Fx09L	Portugal	Funchal		Larval	October/2009	48
3	Fx11A	Portugal	Funchal		BG-traps	October/ 2011	45
4	Fx12A	Portugal	Funchal		BG-traps	October/2012	46
5	Fx12L	Portugal	Funchal		Ovitrap	October/2012	46
6	Fx13A	Portugal	Funchal		BG-traps	October/2013	46
7	Fx13L	Portugal	Funchal		Ovitrap	October/2013	46
8	Fx14A	Portugal	Funchal		BG-traps	October/2014	46
9	Fx14L	Portugal	Funchal		Ovitrap	October/2014	46
10	PM13L	Portugal	Paúl do Mar	32.75907, -17.230439	Ovitrap	October/2013	46
11	PM14A	Portugal	Paúl do Mar		BG-traps	October/2014	46
12	PM14L	Portugal	Paúl do Mar		Ovitrap	October/2014	46
13	SC14L	Portugal	Santa Cruz	32.689282, -16.79074	Ovitrap	October/2014	46
	Car	Venezuela	Caracas	10.480594, -66.903606	Ovitrap	April/2013	46
	ST	Brazil	Santos	-23.967882, -46.328887	Ovitrap	NA/2008	47
	SS	Brazil	São Sebastião	-23.806347, -45.401653	Ovitrap	NA/2008	47
	Gloria-Soria *et al*. (2016)	NA	NA	NA	NA	NA	3566

NA, not applicable; [a] sample codes used in Figure 2.

Supplementary Table S1 - Description of the 16 microsatellite markers used.

Locus		Primer Sequence	Fluorescent Primer	Source	Analysis
AC1	For	TCCGGTGGGTTAAGGATAGA	M13-FAM	Slotman et al. (2007)	Within Madeira and worldwide analysis
	Rev	ACTTCACGCTCCAGCAATCT			
AC2	For	AATACAACGCGATCGACTCC	M13-FAM	Slotman et al. (2007)	
	Rev	AACGATTAGCTGCTCCGAAA			
AC4	For	GCGAATCGGTTCCCATAGTA	M13-FAM	Slotman et al. (2007)	
	Rev	CTTTATCGATCGACGCCATT			
AC5	For	TGGATTGTTCTTAACAAACACGAT	M13-FAM	Slotman et al. (2007)	
	Rev	CGATCTCACTACGGGTTTCG			
AG1	For	AATCCCCACACAAACACACC	M13-HEX	Slotman et al. (2007)	
	Rev	GGCCGTGGTGTTACTCTCTC			
AG2	For	TCCCCTTTCAAACCTAATGG	M13-HEX	Slotman et al. (2007)	
	Rev	TTTGCCCTCGTATGCTCTCT			
AG5	For	TGATCTTGAGAAGGCATCCA	M13-HEX	Slotman et al. (2007)	
	Rev	CGTTATCCTTTCATCACTTGTTTG			
CT2	For	CGCAGTAGGCGATATTCGTT	M13-HEX	Slotman et al. (2007)	
	Rev	ACCACCACCAACACCATTCT			
A1	For	GACGTAAACCGAGTGGGAGA	M13-FAM	Brown et al. (2011)	
	Rev	GCATTTAACCGCGCTAGAAC			
A9	For	GCAGCATGCACTTCACATTT	M13-FAM	Brown et al. (2011)	
	Rev	CGAATGGCATCTGATTCAAG			

B2	For	GGAAACACTTGCAGGGACAT	M13-HEX	Brown et al. (2011)	
	Rev	GCAGATGGTGGCAGTAGTGA			
B3	For	GCAAGTTGCAAAGTGCTCAA	M13-HEX	Brown et al. (2011)	
	Rev	ACCCACCGTTTGCTTTGTAG			
AG4	For	AAAACCTGCGCAACAATCAT	M13-FAM	Slotman et al. (2007)	Within Madeira analysis
	Rev	AAGGACTCCGTATAATCGCAAC			
AC7	For	TCGGCAAATTACCACAAACA	M13-FAM	Slotman et al. (2007)	
	Rev	CATTGGACTCGCTATAACACACA			
88AT1	For	CGTCGACGTTATCTCCTTGTT	M13-HEX	Lovin et al. (2009)	
	Rev	CCAACGCAAGATGCAAGATA			
201AAT1	For	GATCGTTCGACAGCATCTGA	M13-HEX	Lovin et al. (2009)	
	Rev	GGAAAGCTCATCGCCTACTG			

*All forward primers were designed with a short M13 tail at the start (TCCCAGTCACGACGT)

Supplementary Table S2 - Geographic origin and GenBank accession number of sequences used in the phylogenetic analyses.

COI	ND4	Name
JQ926682	JQ926708	Bolivia_1
JQ926682	JQ926707	Bolivia_2
JQ926683	JQ926707	Bolivia_3
JQ926681	JQ926705	Bolivia_4
JQ926676	JQ926705	Bolivia_5
JQ926703	JQ926718	Brazil_1
JQ926703	JQ926719	Brazil_2-3-4
JQ926698	JQ926713	Mexico_1-2-3
JQ926699	JQ926714	Mexico_4
JQ926696	JQ926711	Martinique_1
JQ926697	JQ926712	Martinique_2-3
JQ926701	JQ926726	Venezuela_1-2-3
JQ926684	JQ926725	USA_1-2-3-4-5
JQ926691	JQ926720	Thailand_1-2
JQ926692	JQ926721	Thailand_3
JQ926685	JQ926723	Vietnam_1-2
JQ926686	JQ926724	Vietnam_3
JQ926687	JQ926724	Vietnam_4
JQ926688	JQ926722	Cambodia_1
JQ926689	JQ926722	Cambodia_2
JQ926690	JQ926722	Cambodia_3
JQ926704	JQ926715	Tanzania_1-2-3-4
JQ926693	JQ926709	RCI_1
JQ926694	JQ926709	RCI_2
JQ926695	JQ926710	RCI_3
JQ926700	JQ926717	Guinea_1-2
JQ926702	JQ926716	Cameroon_1-2-3

Supplementary Table S3. Microsatellite genetic variation and null alleles frequency. https://1drv.ms/x/s!Agj5UMQqVs9xg8UPmQWlu6Y2oAqhvg

Supplementary Table S4. **Summary statistics for mtDNA genes in *Ae. aegypti* from Madeira.**

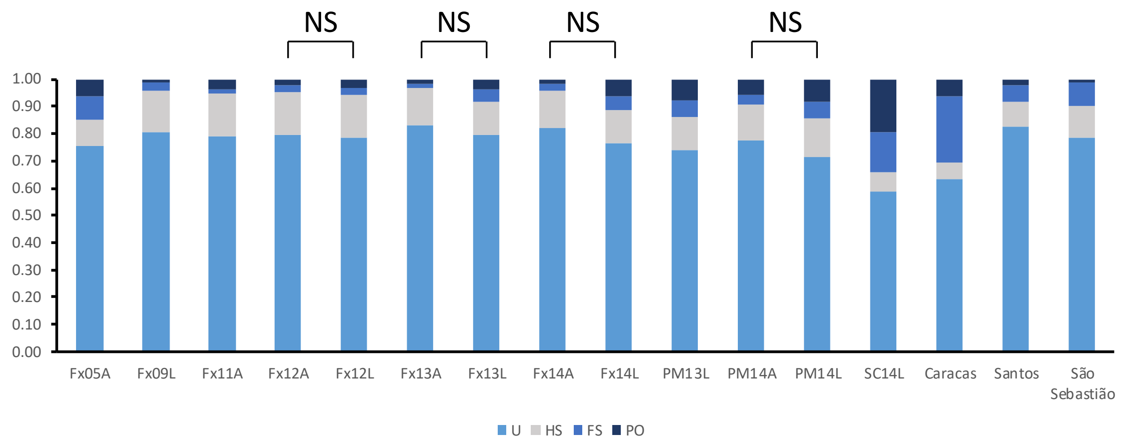
	N	**S**	**H**	***Hd***	**π**	**D (Tajima)**	**D***	**F***	**Gene size**
COI	202	14	3	0.198	0.00317	0.044	1.525	1.158	764 bp
ND4	191	13	4	0.192	0.00545	-0.353	0.822	0.458	351 bp
COI/ND4	178	27	5	0.177	0.00345	-0.5077	1.524	0.839	1115 bp

N, sample size; S, number of segregating sites; H, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; D, Tajima's D statistic; D* and F*, Fu and Li's statistics.

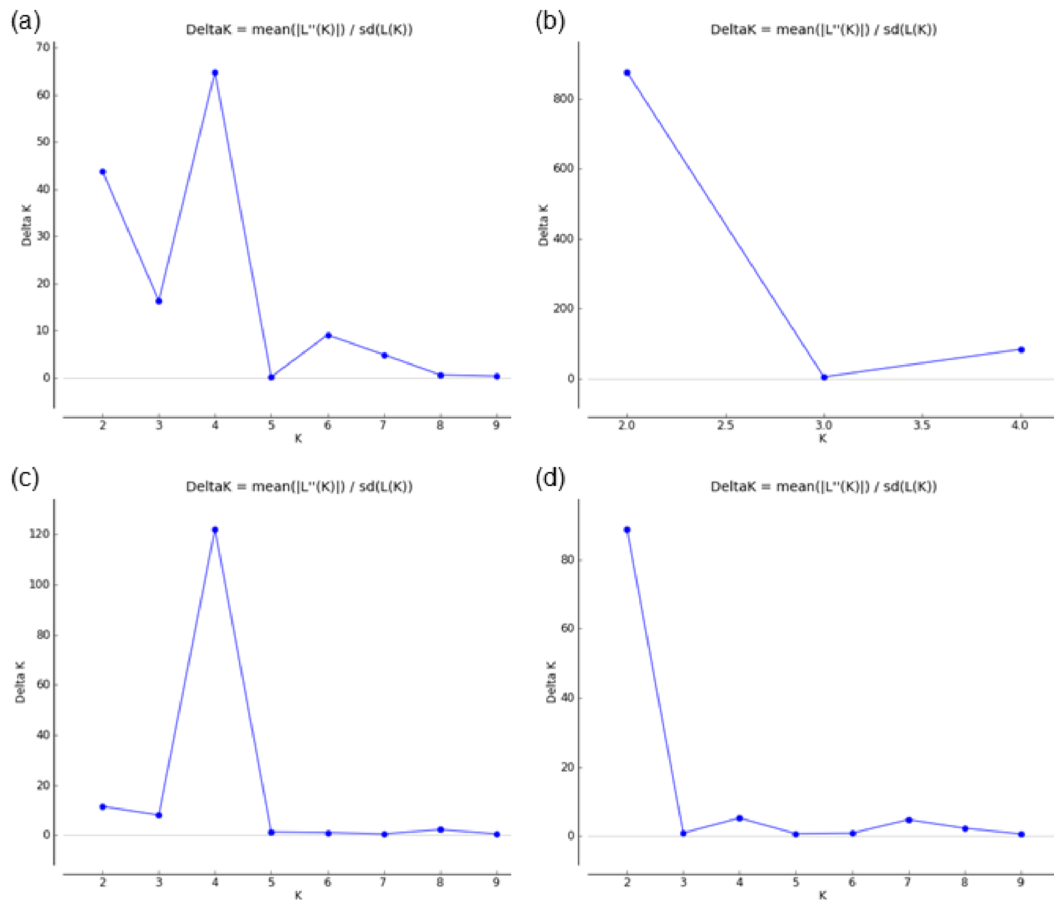
Supplementary Table S5. MtDNA haplotype sequences for COI and ND4 across *Ae. aegypti* samples from Madeira Island.

Haplotype	**N**	**Polymorphic positions**													
ND4		**0**	**0**	**0**	**1**	**1**	**1**	**1**	**2**	**2**	**2**	**2**	**2**	**3**	
		1	**2**	**7**	**3**	**4**	**5**	**9**	**1**	**3**	**6**	**7**	**7**	**1**	
		6	**2**	**3**	**3**	**2**	**5**	**0**	**7**	**5**	**8**	**1**	**7**	**3**	
H1	171	T	T	T	C	T	G	T	A	T	A	T	T	C	
H2	16	C	C	C	T	C	.	C	G	C	G	A	C	T	
H3	1	.	.	.	.	.	A	.	.	.	.	.	.	.	
H4	3	.	.	.	.	.	.	.	G	.	.	A	.	.	
COI															
		0	**0**	**0**	**0**	**1**	**1**	**1**	**3**	**3**	**4**	**4**	**4**	**4**	**6**
		0	**6**	**7**	**7**	**5**	**6**	**8**	**1**	**3**	**1**	**6**	**6**	**9**	**8**
		1	**7**	**0**	**9**	**1**	**3**	**4**	**3**	**4**	**2**	**3**	**9**	**3**	**2**
H1	180	A	C	T	A	G	G	C	A	T	G	A	T	C	C
H2	19	G	T	C	G	A	A	T	G	C	A	G	C	T	T
H3	2	.	.	.	.	.	.	.	.	C	.	.	.	.	.

N corresponds to the number of sample belonging to this haplotype.



Supplementary Figure S1. Proportion (in percentage) of related and unrelated pairs of individuals as determined by ML-RELATE. Legend: U - unrelated, HS - half-siblings, FS - full-siblings and PO - parent-offspring. NS refers to a non-significant difference between adult and larval samples using Chi-square test ($p > 0.05$).



Supplementary Figure S2. Graphics of Evanno's *ΔK* for the different Bayesian clustering analysis implemented by STRUCTURE. (a) All Madeira samples (Figure 2), (b) Worldwide dataset and Madeira (Figure 4a), (c) *Ae. aegypti aegypti* and Madeira samples (Figure 4b), (d) Madeira and South America samples (Figure 4c).

Chapter 4.

Zika virus, a new threat for Europe?

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Abstract

Background:

Since its emergence in 2007 in Micronesia and Polynesia, the arthropod-borne flavivirus Zika virus (ZIKV) has spread in the Americas and the Caribbean, following first detection in Brazil in May 2015. The risk of ZIKV emergence in Europe increases as imported cases are repeatedly reported. Together with chikungunya virus (CHIKV) and dengue virus (DENV), ZIKV is transmitted by *Aedes* mosquitoes. Any countries where these mosquitoes are present could be potential sites for future ZIKV outbreak. We assessed the vector competence of European *Aedes* mosquitoes (*Aedes aegypti* and *Aedes albopictus*) for the currently circulating Asian genotype of ZIKV.

Methodology/Principal Findings:

Two populations of *Ae. aegypti* from the island of Madeira (Funchal and Paul do Mar) and two populations of *Ae. albopictus* from France (Nice and Bar-sur-Loup) were challenged with an Asian genotype of ZIKV isolated from a patient in April 2014 in New Caledonia. Fully engorged mosquitoes were then maintained in insectary conditions (28°±1°C, 16h:8h light:dark cycle and 80% humidity). 16-24 mosquitoes from each population were examined at 3, 6, 9 and 14 days post-infection to estimate the infection rate, disseminated infection rate and transmission efficiency. Based on these experimental infections, we demonstrated that *Ae. albopictus* from France were not very susceptible to ZIKV.

Conclusions/Significance:

In combination with the restricted distribution of European *Ae. albopictus*, our results on vector competence corroborate the low risk for ZIKV to expand into most parts of Europe with the possible exception of the warmest regions bordering the Mediterranean coastline.

Keywords: *Aedes albopictus*, *Aedes aegypti*, Europe, Zika virus, emergence risk.

Author summary

In May 2015, local transmission of Zika virus (ZIKV) was reported in Brazil and since then, more than 1.5 million human cases have been reported in Latin America and the Caribbean. This arbovirus, primarily found in Africa and Asia, is mainly transmitted by *Aedes* mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Viremic travelers returning from America to European countries where *Ae. albopictus* is established could become the source for local transmission of ZIKV. In order to estimate the risk of seeding ZIKV into local mosquito populations, the susceptibility of European *Ae. aegypti* and *Ae. albopictus* to ZIKV was measured using experimental infections. We demonstrated that *Ae. albopictus* and *Ae. aegypti* from Europe were not very susceptible to ZIKV. The threat for a Zika outbreak in Europe should be limited.

Introduction

Zika virus (ZIKV) (genus *Flavivirus*, family *Flaviviridae*) is an emerging arthropod-borne virus transmitted to humans by *Aedes* mosquitoes. ZIKV infection in humans was first observed in Africa in 1952 [1], and can cause a broad range of clinical symptoms presenting as a "dengue-like" syndrome: headache, rash, fever, and arthralgia. In 2007, an outbreak of ZIKV on Yap Island resulted in 73% of the total population becoming infected [2]. Following this, ZIKV continued to spread rapidly with outbreaks in French Polynesia in October 2013 [3], New Caledonia in 2015 [4], and subsequently, Brazil in May 2015 [5, 6]. During this expansion period, the primary transmission vector is considered to have been *Aedes aegypti*, although *Aedes albopictus* could potentially serve as a secondary transmission vector [7] as ZIKV detection has been reported in field-collected *Ae. albopictus* in Central Africa [8]. As Musso et al. [9] observed, the pattern of ZIKV emergence from Africa, throughout Asia, to its subsequent arrival in South America and the Caribbean closely resembles the emergence of Chikungunya virus (CHIKV). In Europe, returning ZIKV-viremic travelers may become a source of local transmission in the presence of *Aedes* mosquitoes, *Ae. albopictus* in Continental Europe and *Ae. aegypti* in the Portuguese island of Madeira. *Ae. albopictus* originated from Asia was recorded for the first time in Europe in Albania in 1979 [10], then in Italy in 1990

[11]. It is now present in all European countries around the Mediterranean Sea [12]. This mosquito was implicated as a vector of CHIKV and DENV in Europe [13]. On the other hand, *Ae. aegypti* disappeared after the 1950s with the improvement of hygiene and anti-malaria vector control. This mosquito reinvaded European territory, Madeira island, in 2005 [14], and around the Black Sea in southern Russia, Abkhazia, and Georgia in 2004 [12]. The species was responsible for outbreaks of yellow fever in Italy in 1804 [15] and dengue in Greece in 1927–1928 [16]. To assess the possible risk of ZIKV transmission in Europe, we compared the relative vector competence of European *Ae. aegypti* and *Ae. albopictus* populations to the Asian genotype of ZIKV.

Materials and Methods

Ethics Statement

The Institut Pasteur animal facility has received accreditation from the French Ministry of Agriculture to perform experiments on live animals in compliance with the French and European regulations on care and protection of laboratory animals. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at the Institut Pasteur. No specific permits were required for the described field studies in locations that are not protected in any way and did not involve endangered or protected species.

Mosquitoes

Four populations of mosquitoes (two populations of *Ae. aegypti*: Funchal (32°40’N, 16°55’W) and Paul do Mar (32°45’N, 17°13’W), collected on island of Madeira and two populations of *Ae. albopictus*: Nice (43°42’N, 7°15’E) and Bar-sur-Loup (43°42’N, 6°59’E) in France) were collected using ovitraps. Eggs were immersed in dechlorinated tap water for hatching. Larvae were distributed in pans of 150-200 individuals and supplied with 1 yeast tablet dissolved in 1L of water every 48 hours. All immature stages were maintained at 28°C ± 1°C. After emergence, adults were given free access to a 10% sucrose solution and maintained at 28°C ± 1°C with 70% relative humidity and a 16:8 light/dark cycle. The F1 generation of *Ae. aegypti* from Madeira and F7-8 generation of *Ae. albopictus* from France were used for experimental infections.

Viral strain

The ZIKV strain (NC-2014-5132) originally isolated from a patient in April 2014 in New Caledonia was used to infect mosquitoes. The viral stock used was subcultured five times on Vero cells prior to the infectious blood-meal. The NC-2014-5132 strain is phylogenetically closely related to the ZIKV strains circulating in the South Pacific region, Brazil [5] and French Guiana [17].

Oral Infection of Mosquitoes

Infectious blood-meals were provided using a titer of 10^7 $TCID_{50}$/mL. Seven-day old mosquitoes were fed on blood-meals containing two parts washed rabbit erythrocytes to one part viral suspension supplemented with ATP at a final concentration of 5 mM. Rabbit arterial blood was collected and erythrocytes were washed five times with Phosphate buffered saline (PBS) 24 h before the infectious blood-meal. Engorged females were transferred to cardboard containers with free access to 10% sucrose solution and maintained at 28°C and 70% relative humidity with a 16:8 light/dark cycle. 16-24 female mosquitoes from each population were analyzed at 3, 6, 9, and 14 days post-infection (dpi) to estimate the infection rate, disseminated infection rate and transmission efficiency. Briefly, legs and wings were removed from each mosquito followed by insertion of the proboscis into a 20 µL tip containing 5 µL FBS for 20 minutes. The saliva-containing FBS was expelled into 45 µL serum free L-15 media (Gibco), and stored at -80°C. Following salivation, mosquitoes were decapitated and head and body (thorax and abdomen) were homogenized separately in 300 µL L-15 media supplemented with 3% FBS using a Precellys homogenizer (Bertin Technologies) then stored at -80°C. Infection rate was measured as the percentage of mosquitoes with infected bodies among the total number of analyzed mosquitoes. Disseminated infection rate was estimated as the percentage of mosquitoes with infected heads (i.e., the virus had successfully crossed the midgut barrier to reach the mosquito hemocoel) among the total number of mosquitoes with infected bodies. Transmission efficiency was calculated as the overall proportion of females with infectious saliva among the total number of tested mosquitoes. Samples were titrated by plaque assay in Vero cells.

Virus Quantification

For head/body homogenates and saliva samples, Vero E6 cell monolayers were inoculated with serial 10-fold dilutions of virus-containing samples and incubated for 1 hour at 37°C followed by an overlay consisting of DMEM 2X, 2% FBS, antibiotics and 1% agarose. At 7 dpi, overlay was removed and cells were fixed with crystal violet (0.2% Crystal Violet, 10% Formaldehyde, 20% ethanol) and positive/negative screening was performed for cytopathic effect (body and head homogenates) or plaques were enumerated (head and saliva samples). Vero E6 cells (ATCC CRL-1586) were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (Eurobio), Penicillin and Streptomycin, and 0.29 mg/mL l-glutamine.

Statistical analysis

All statistical tests were conducted with the STATA software (StataCorp LP, Texas, USA) using 1-sided Fisher's exact test and *P*-values>0·05 were considered non-significant.

Results

Aedes aegypti from Madeira transmit ZIKV efficiently

To test whether *Ae. aegypti* from a European territory were able to transmit ZIKV, we analyzed the vector competence of two *Ae. aegypti* populations collected on the island of Madeira based on three parameters: viral infection of the mosquito midgut, viral dissemination to secondary organs, and transmission potential, analyzed at 3, 6, 9, and 14 dpi. Only mosquitoes presenting an infection (i.e. infected midgut) were analyzed for viral dissemination. The two populations presented similar infection (*P* = 0.50 (3 dpi), 0.17 (6), 0.36 (9), 0.50 (14); Figure 1) and disseminated infection (*P* = 0.59 (3 dpi), 0.63 (6), 0.43 (9), 0.06 (14); Figure 1) with the highest rates measured at 9 dpi and 9-14 dpi, respectively. When examining transmission efficiency, only *Ae. aegypti* Funchal were able to transmit ZIKV at 9 (1 individual among 20 tested) and 14 dpi (1 among 20) (Figure

1). When considering the number of viral particles in heads, no significant difference was detected between *Ae. aegypti* Funchal and *Ae. aegypti* Paul do Mar (P = 1 (3 dpi), 0.22 (6), 0.60 (9), 0.38 (14); Figure 2). When examining viral loads in saliva, only *Ae. aegypti* Funchal exhibited 1550 particles at 9 dpi and 50 at 14 dpi (Figure 2).

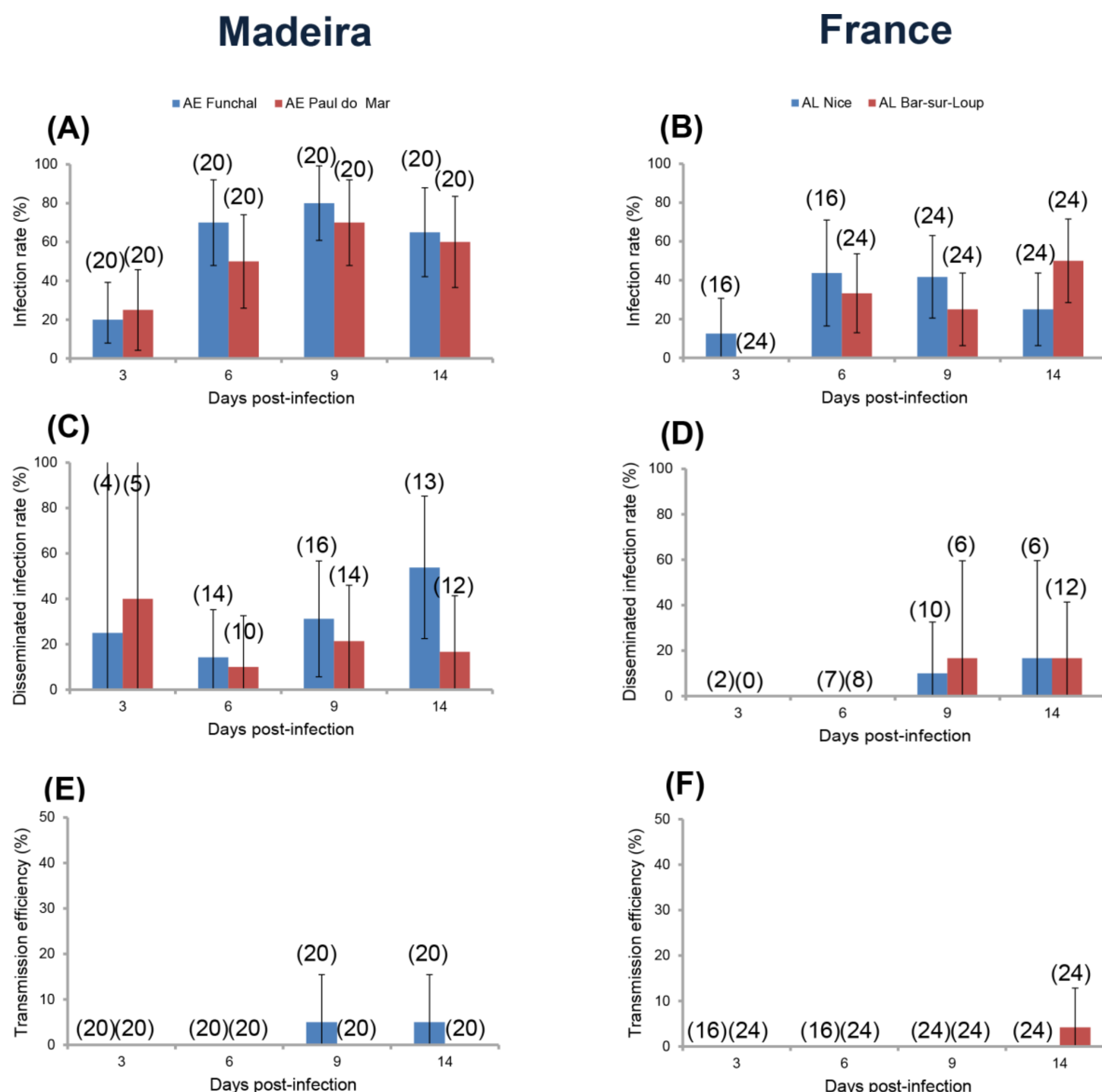

Figure 1. *Ae. aegypti* from Madeira Island and *Ae. albopictus* from France were assessed for viral infection (A, B), dissemination (C, D), and transmission (E, F) at days 3, 6, 9, 14 after infection with ZIKV provided at a titer of 10^7 $TCID_{50}$/mL. 16-24 mosquitoes were sampled each day. Infection rates were measured as the percentage of mosquitoes with infected bodies among the total number of analyzed mosquitoes. Disseminated infection rates were estimated as the percentage of mosquitoes with infected heads (i.e., the virus has successfully crossed the midgut barrier to reach the

hemocoel) among the total number of mosquitoes with infected bodies. The transmission efficiency was calculated as the overall proportion of females with infectious saliva among the total number of tested mosquitoes. Arrows refer to scenarios of ZIKV introduction into Europe from the Americas *via* transmission by *Aedes albopictus* (in blue) and/or *Ae. aegypti* (in grey). AE = *Ae. aegypti*; AL = *Ae. albopictus*. In red, countries where ZIKV has been isolated. Error bars show the confidence intervals (95%). In brackets, the number of mosquitoes tested.

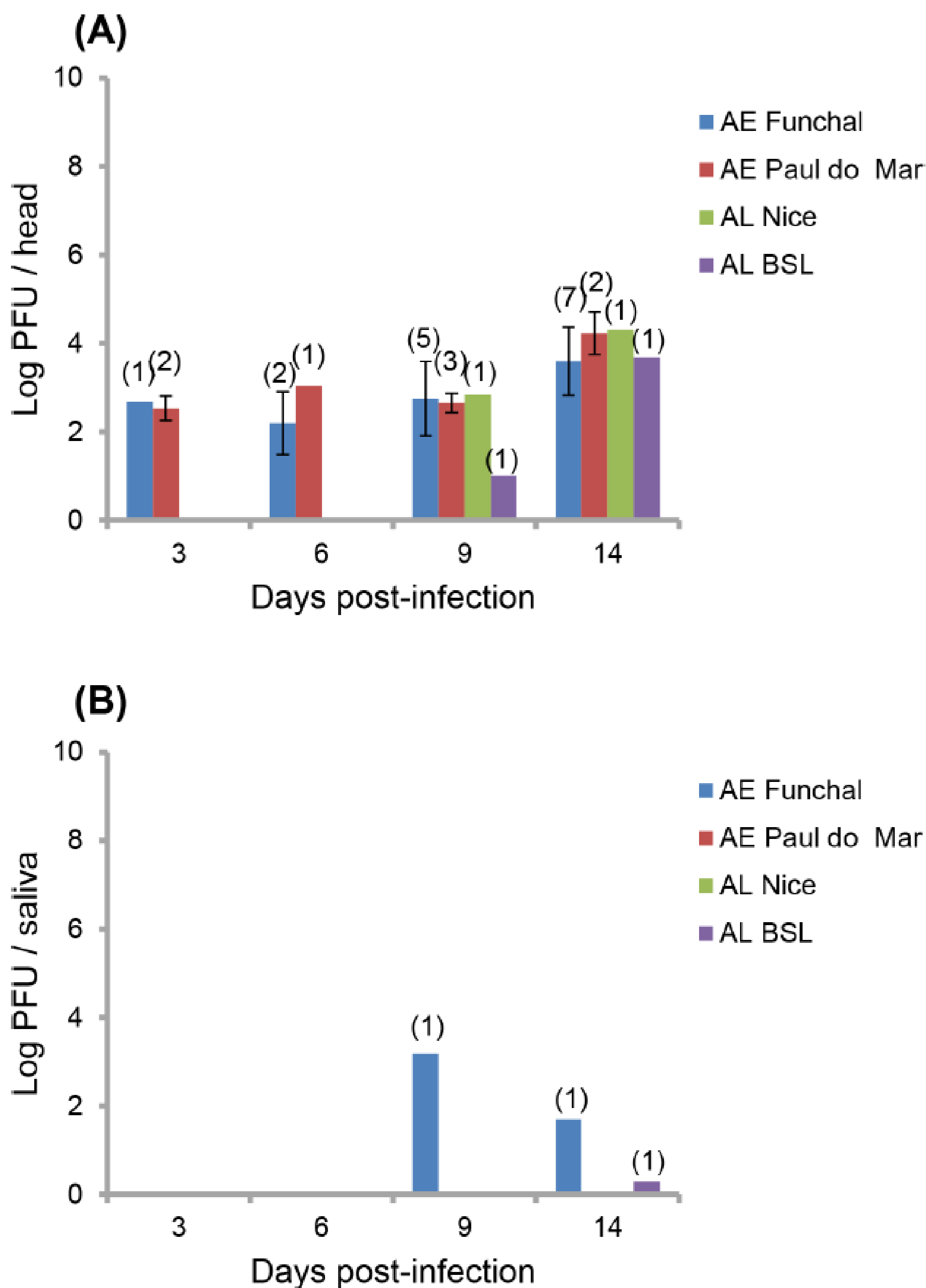

Figure 2. Viral loads in heads (A) and saliva (B) for mosquitoes infected with ZIKV provided at a titer of 10^7 $TCID_{50}$/mL. The number of infectious particles per head homogenate and saliva was estimated by plaque assays on Vero cells. Titers were expressed as PFU (plaque-forming unit). AE = *Ae. aegypti*; AL = *Ae. albopictus*. Error

bars refer to the standard error. In brackets, the number of mosquitoes tested.

French *Ae. albopictus* showed significantly reduced competence to transmit ZIKV

To determine if *Ae. albopictus* present in continental Europe were able to sustain local transmission of ZIKV as previously observed with CHIKV and DENV, we evaluated the vector competence of two *Ae. albopictus* populations collected in Nice and Bar-sur-Loup in the South of France. When compared with *Ae. aegypti*, the two *Ae. albopictus* populations showed similar infection rates at 3 dpi ($P = 0.08$) and 6 dpi ($P = 0.11$) and disseminated infection rates at 9 dpi ($P = 0.62$) and 14 dpi ($P = 0.10$) (Figure 1). Only one individual among 24 *Ae. albopictus* Bar-sur-Loup tested at 14 dpi was able to transmit ZIKV (Figure 1). When analyzing the number of viral particles in heads, only few mosquitoes were infected (Figure 2). When examining saliva, one *Ae. albopictus* Bar-sur-Loup exhibited 2 viral particles at 14 dpi (Figure 2).

In summary, ZIKV dissemination through *Ae. aegypti* was noticeably superior and the virus in saliva was detected earlier in *Ae. aegypti* than in *Ae. albopictus*. However both mosquito species showed similar transmission efficiencies at 9-14 dpi.

Discussion

ZIKV could be transmitted, spread and maintained in Europe either via (i) Madeira where the main vector *Ae. aegypti* has been established since 2005 or (ii) Continental Europe where *Ae. albopictus* is known to have been present since 1979 [12]. We demonstrated that ZIKV was amplified and expectorated efficiently in saliva by European *Ae. aegypti* from Madeira. This contrasts with the lower vector competence for ZIKV of French *Ae. albopictus*. Taking these observations and the overall average lower temperatures of most regions of Europe into account, the risk of major outbreaks of Zika fever in most areas of Europe, at least for the immediate future, appears to be relatively low.

Our results highlight the potential risk for ZIKV transmission on Madeira where two main factors are present: the presence of the main vector, *Ae. aegypti* introduced in 2005 [18] and imported cases from Brazil with which Madeira, an autonomous region of Portugal, maintains active exchanges of goods and people sharing the same language.

Thus Madeira Island could be considered as a stepping stone for an introduction of ZIKV into Europe.

Autochthonous cases of CHIKV and DENV have been reported in Europe since 2007: CHIKV in Italy in 2007, South France in 2010, 2014, and DENV in South France in 2010, 2013, 2015, and Croatia in 2010 [19]. The invasive species *Ae. albopictus* first detected in Europe in 1979 [10] has played a central role in this transmission [19]. Thus, there might be a risk of a similar establishment of ZIKV in Europe upon the return of viremic travelers [20, 21]. We showed that *Ae. albopictus* from South France were less competent for ZIKV infection requiring 14 days to be expectorated in the mosquito saliva after infection. Therefore, we can suggest that the Asian tiger mosquito from Southern France and more widely, Europe, are less suitable to sustain local transmission of ZIKV compared to CHIKV and perhaps, DENV. *Ae. albopictus* Nice were not able to expectorate ZIKV in saliva at day 14 post-infection like *Ae. albopictus* Bar-sur-Loup suggesting two populations genetically differentiated.

Considering the extensive airline travel between Latin America and Europe, the risk for local transmission of ZIKV in the European area where the mosquito *Ae. albopictus* is widely distributed, is assumed to be minimal based on our studies of vector competence. Nevertheless, reinforcement of surveillance and control of mosquitoes should remain a strong priority in Europe since *Aedes* mosquitoes also transmit DENV and CHIKV and virus adaptation to new vectors cannot be excluded, as previously observed with CHIKV in La Reunion [22, 23].

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Competing interests

We declare that we have no competing interests.

References

1. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg **1952**; 46:509-20.

2. Duffy MR, Chen TH, Hancock WT, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med **2009**; 360:2536-43.

3. Cao-Lormeau VM, Roche C, Teissier A, et al. Zika virus, French polynesia, South pacific, 2013. Emerg Infect Dis **2014**; 20:1085-6.

4. Dupont-Rouzeyrol M, O'Connor O, Calvez E, et al. Co-infection with Zika and dengue viruses in 2 patients, New Caledonia, 2014. Emerg Infect Dis **2015**; 21:381-2.

5. Zanluca C, de Melo VC, Mosimann AL, Dos Santos GI, Dos Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz **2015**; 110:569-72.

6. Campos GS, Bandeira AC, Sardi SI. Zika Virus Outbreak, Bahia, Brazil. Emerg Infect Dis **2015**; 21:1885-6.

7. Wong PS, Li MZ, Chong CS, Ng LC, Tan CH. *Aedes* (Stegomyia) *albopictus* (Skuse): a potential vector of Zika virus in Singapore. PLoS Negl Trop Dis **2013**; 7:e2348.

8. Grard G, Caron M, Mombo IM, et al. Zika virus in Gabon (Central Africa)--2007: a new threat from *Aedes albopictus*? PLoS Negl Trop Dis **2014**; 8:e2681.

9. Musso D, Cao-Lormeau VM, Gubler DJ. Zika virus: following the path of dengue and chikungunya? Lancet **2015**; 386:243-4.

10. Adhami J, Reiter P. Introduction and establishment of *Aedes* (Stegomyia) *albopictus* skuse (Diptera: Culicidae) in Albania. J Am Mosq Control Assoc **1998**; 14:340-3.

11. Sabatini A, Raineri V, Trovato G, Coluzzi M. [Aedes albopictus in Italy and possible diffusion of the species into the Mediterranean area]. Parassitologia **1990**; 32:301-4.

12. Medlock JM, Hansford KM, Schaffner F, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis **2012**; 12:435-47.

13. Vega-Rua A, Zouache K, Caro V, et al. High efficiency of temperate *Aedes albopictus* to transmit chikungunya and dengue viruses in the Southeast of France. PLoS One **2013**; 8:e59716.
14. Nene V, Wortman JR, Lawson D, et al. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science **2007**; 316:1718-23.
15. Levre E. The yellow fever outbreak of 1804 in Leghorn. Ann Ig **2002**; 14:153-7.
16. Rosen L. Dengue in Greece in 1927 and 1928 and the pathogenesis of dengue hemorrhagic fever: new data and a different conclusion. Am J Trop Med Hyg **1986**; 35:642-53.
17. Antoine Enfissi JC, Jimmy Roosblad, Mirdad Kazanji, Dominique Rousset Zika virus genome from the Americas. The Lancet **2015**; 7 janvier 2016.
18. Margarita Y SGA, Lencastre I, Silva AC, Novo T, Sousa C, et al. First record of *Aedes* (Stegomia) *aegypti* (Linnaeus, 1762) (Diptera, Culicidae) in Madeira Island – Portugal. Acta Parasitológica Portuguesa **2006**; 13:59-61.
19. Rezza G. Dengue and chikungunya: long-distance spread and outbreaks in naive areas. Pathog Glob Health **2014**; 108:349-55.
20. Tappe D, Rissland J, Gabriel M, et al. First case of laboratory-confirmed Zika virus infection imported into Europe, November 2013. Euro Surveill **2014**; 19.
21. Zammarchi L, Tappe D, Fortuna C, et al. Zika virus infection in a traveller returning to Europe from Brazil, March 2015. Euro Surveill **2015**; 20.
22. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog **2007**; 3:e201.
23. Vazeille M, Moutailler S, Coudrier D, et al. Two Chikungunya isolates from the outbreak of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. PLoS One **2007**; 2:e1168.

Chapter 5.

Potential of *Aedes aegypti* populations in Madeira Island to transmit dengue and chikungunya viruses

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Potential of *Aedes aegypti* populations in Madeira Island to transmit dengue and chikungunya viruses

Abstract

Background: *Aedes* spp. mosquitoes mainly transmit the arboviruses dengue virus (DENV) and chikungunya virus (CHIKV) in urban areas, causing a severe public health problem. In 2012–2013, a major dengue outbreak occurred on Madeira Island where the mosquito *Aedes aegypti* was the only vector. Up to now, the competence of *Ae. aegypti* populations from Madeira to transmit DENV or CHIKV remains unknown. This study aimed to assess experimentally the ability of *Ae. aegypti* populations from Madeira to transmit these viruses.

Results: By orally exposing mosquitoes to CHIKV (NC/2011-568) and DENV-2 (Bangkok), the vector competence of two field-collected *Ae. aegypti* populations, i.e. Funchal and Paúl do Mar, was evaluated. We found that both populations were similarly infected and ensured the dissemination and transmission of CHIKV at the same rates. With DENV-2, viral dissemination was significantly higher in the Funchal population compared to Paúl do Mar. We found no significant differences in transmission rates between populations.

Conclusions: To our knowledge, this study has demonstrated for the first time the ability of temperate European *Ae. aegypti* populations from Madeira to transmit DENV and CHIKV. As our results suggest, there is a potential risk for the local transmission of DENV and CHIKV if introduced to Madeira or continental Europe where *Aedes albopictus* is present. Our results highlight the need for continuing vector surveillance and control on Madeira Island to future-proof the Island against mosquito-borne epidemics.

Keywords: Arbovirus, Europe, Vector competence, *Aedes aegypti.*

Background

Aedes aegypti (Linnaeus, 1762) is known to be the vector of several arboviruses [1]. While originally native to Africa, this species has continuously expanded its range during the last centuries [2], including to the European territories such as Madeira Island (Portugal), Georgia and occasionally in the Netherlands [3, 4]. First detected in 2005 in the city of Funchal on Madeira, this mosquito is now widely distributed throughout the southern coast of the island [5] and was responsible for a major dengue outbreak in

October 2012 with thousands of dengue cases [6], representing the first autochthonous cases in a Portuguese territory.

Beside the dengue virus (DENV; genus *Flavivirus*, family *Flaviviridae*), *Ae. aegypti* is also experimentally competent for chikungunya virus (CHIKV; genus *Alphavirus*, family *Togaviridae*) [7, 8]. Dengue and chikungunya are serious public health issues in tropical regions, and each virus family has different serotypes, lineages and genotypes [9, 10]. Dengue is caused by four genetically distinct DENV serotypes (1, 2, 3 and 4) and generally lead to a self-limited febrile illness characterised by a headache, fever and rash. CHIKV causes an acute febrile illness characterised by severe arthralgia [11]. Phylogenetic analysis suggests that CHIKV lineages can be classified into three distinct genotypes: Asian, West African and Eastern/Central/Southern African (ECSA). Both DENV and CHIKV infections have a large proportion of asymptomatic cases contributing actively to virus dissemination and transmission [12].

The recent emergence of dengue and chikungunya in Europe, such as the 2012 outbreaks of dengue in Madeira and chikungunya in France [13, 14] and Italy [15], have raised concerns of arbovirus transmission in countries infested by mosquito species that could sustain epidemics, especially *Ae. aegypti* and/or *Aedes albopictus* (Skuse, 1894) [16]. Due to the intense social and commercial relations with Brazil and Venezuela, Madeira Island could serve as a source for the introduction of *Ae. aegypti* and/or arboviruses to continental Europe [17]. The risk of arboviral outbreaks in Madeira is real since imported cases of DENV and Zika virus (ZIKV) were detected in citizens returning from DENV- and ZIKV-infected countries [18] and local *Ae. aegypti* populations from Madeira were experimentally susceptible to ZIKV [19]. This study aims to assess the ability of *Ae. aegypti* populations from Madeira Island to experimentally transmit CHIKV and DENV. The results obtained will provide a solid basis for decisions regarding disease prevention and control for the Madeira Health Authorities and decision-makers in Europe.

Methods

Mosquitoes

Two *Ae. aegypti* populations from Madeira were used in vector competence assays: the Funchal population, collected in the major urban area and island's capital city, and Paúl do Mar population collected in the most western point of the species distribution on the

island, *c.*42 km away from Funchal, and considered a rural area. Mosquito eggs were collected in 2014 using widely distributed ovitraps [20] and hatched in insectaries. The larvae were split as 200–300 individuals per pan and fed with yeast tablets. Emerging adults were maintained in cages at 28 ± 1 °C with a 14 h light/10 h dark photocycle, 80% relative humidity, and supplied with a 10% sucrose solution *ad libitum*. The F1 generation was used for experimental infections.

Viral strains

CHIKV (NC/2011-568) was isolated in 2011 from a patient by the Institut Pasteur of New Caledonia (kindly provided by Dr Myrielle Dupont-Rouzeyrol); this isolate belongs to the Asian genotype and possesses an alanine at base position 226 in the E1 envelope glycoprotein (GenBank: HE806461). DENV belonging to serotype 2 (DENV-2) was isolated in 1974 from a patient in Bangkok, Thailand [21]. Both viral stocks were produced following 2–3 passages on C6/36 *Ae. albopictus*-derived cells.

Mosquito oral infections

Four batches of 60 one-week-old female adults were fed on an infectious blood-meal that consisted of 1400 µl of washed rabbit erythrocytes, 700 µl of viral suspension, supplemented with 5 mM adenosine triphosphate (ATP), a phagostimulant. Two feeders were prepared per virus, and a feeder was available to two batches of mosquito (successively) for 20 min. The viral titre of the infectious blood-meal was determined at 2×10^7 focus-forming units (ffu)/ml for DENV-2 and 2×10^7 ffu/ml for CHIKV. After exposure, fully engorged females were transferred to cardboard containers and maintained with 10% sucrose at 28 ± 1 °C and 80% relative humidity.

Dissemination and transmission analysis

Twenty mosquitoes from each population were analysed at different time-points: 3, 6, 9 and 14 days post-infection (dpi) for CHIKV and 7 and 14 dpi for DENV-2. To estimate the infection and dissemination, the virus in bodies (including thorax and abdomen) and heads was analysed, respectively. Mosquito samples were grounded in 300 µl of Leibovitz L15 medium (Invitrogen, Carlsbad, USA) supplemented with 3% fetal bovine serum (FBS). Samples were then centrifuged for 5 min at 10,000× *rpm*, and the

supernatant obtained was used for virus quantification. To estimate transmission, saliva was collected from each mosquito as previously described [22]. Briefly, legs and wings were removed from each mosquito, and the proboscis was inserted into a 20 µl tip containing 5 µl of FBS. After 20 min, saliva containing FBS was expelled into 45 µl of Leibovitz L15 medium for titration. Infection rate (IR) was used as a measure of susceptibility to each virus and corresponds to the number of mosquitoes with the infected body among the tested ones. The percentage of mosquitoes with infected heads among mosquitoes with an infected body is the dissemination rate (DR). The transmission rate (TR) is defined as the percentage of mosquitoes with infectious saliva among mosquitoes with positive viral dissemination. The number of viral particles per saliva and head was determined by titration using focus fluorescent assay on C6/36 cells. Briefly, 10-fold serial dilutions were performed for each sample and inoculated onto C6/36 cell culture in 96-well plates. After incubation at 28 °C during three days (CHIKV) or 5 days (DENV), plates were stained using hyper-immune ascetic fluid specific to CHIKV or DENV as the primary antibody. Alexa Fluor 488 goat anti-mouse IgG was used as the second antibody (Life Technologies, Carlsbad, USA).

Statistical analysis

Statistical analyses were performed with GraphPad Prism v 6.03. Proportions were compared using Chi-square test and sample distributions with the Mann-Whitney test ($n = 2$) or Kruskal-Wallis test ($n > 2$). *P*-values > 0.05 were considered non-significant.

Results

***Aedes aegypti* from Madeira Island is highly susceptible to CHIKV infection**

The susceptibility of *Ae. aegypti* from Madeira Island for CHIKV was studied using a viral strain belonging to Asian lineage, as the current circulating lineage in the Americas [10]. Our results showed that local *Ae. aegypti* can transmit CHIKV very efficiently: Funchal and Paúl do Mar populations were both highly susceptible to CHIKV infection, with similar infection rates [Chi-square test: $P > 0.05$; 3 dpi ($\chi^2 = 1.02$, $df = 1$, $P = 0.31$); 6 dpi ($\chi^2 = 1.02$, $df = 1$, $P = 0.31$)] ranging from 95 to 100% after 3 dpi (Table 1).

Table 1 Infection, dissemination and transmission rates (in %) estimated at different days after exposure of *Ae. aegypti* from Madeira to CHIKV NC/2011-568 strain

Days post-infection	**Funchal**			**Paúl do Mar**		
	IR	**DR**	**TR**	**IR**	**DR**	**TR**
	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
3	95 (20)	84.2 (19)	25 (16)	100 (20)	90 (20)	27.7 (18)
6	95 (20)	94.7 (19)	33.3 (18)	100 (20)	100 (20)	55 (20)
9	100 (20)	95 (20)	57.9 (19)	100 (20)	100 (20)	50 (20)
14	100 (20)	100 (20)	40 (20)	100 (20)	100 (20)	25 (20)

Abbreviations: IR, infection rate; DR, dissemination rate; TR, transmission rate; *n*, the number of mosquitoes analysed

To measure the ability of CHIKV to cross the mosquito midgut barrier, dissemination rate (DR) was assessed at 3, 6, 9 and 14 dpi. According to the results, 100% DR was reached at 6 dpi for the Paúl do Mar population and 14 dpi for the Funchal population. No difference of DR was detected between the two populations [Chi-square test: $P > 0.05$; 3 dpi ($\chi^2 = 0.29$, $df = 1$, $P = 0.59$); 6 dpi ($\chi^2 = 1.08$, $df = 1$, $P = 0.30$); 9 dpi ($\chi^2 = 1.02$, $df = 1$, $P = 0.31$)]. The intensity of viral dissemination was evaluated by estimating the number of viral particles in head homogenates. Virus in heads was detectable from 3 dpi in both populations. The number of viral particles (Fig. 1) varied significantly throughout the time course in both populations [Kruskal-Wallis test: $P < 0.05$; Funchal ($\chi^2 = 21.80$, $df = 3$, $P < 0.0001$); Paúl do Mar ($\chi^2 = 12.72$, $df = 3$, $P = 0.005$)]. When taking into account sequential method of Bonferroni, allowing to adjust the significance level of each test to the number of tests run, both *P*-values remain significant. Significant differences were found at 6 dpi between the two populations (Mann-Whitney U-test: $Z = 2.62$, $P = 0.009$). The maximum number of CHIKV was detected at 6 dpi with 5.77 ± 0.53 $\log_{10}$ ffu/ml for the Funchal strain and 5.42 ± 0.75 $\log_{10}$ ffu/ml for the Paúl do Mar strain.

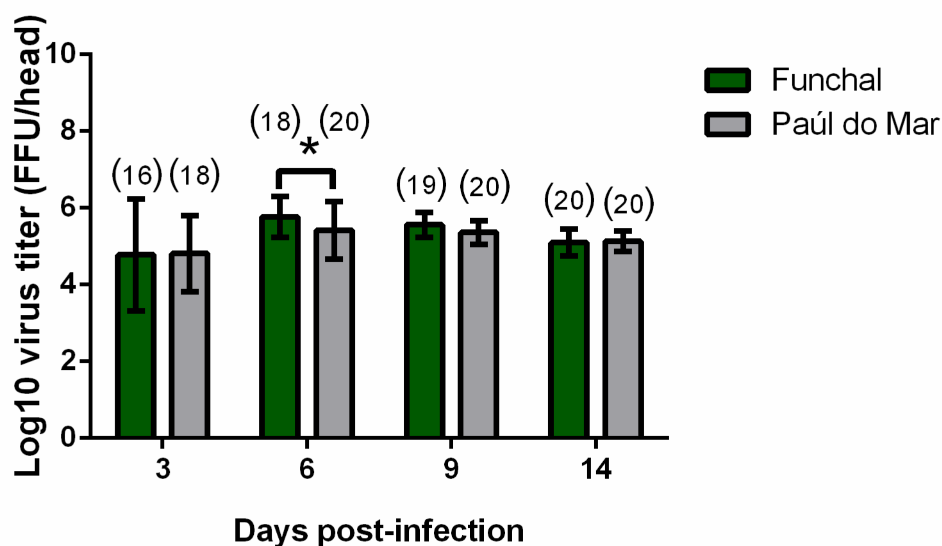

Fig. 1 Dissemination of CHIKV in *Ae. aegypti* from Madeira Island. Mosquitoes were sacrificed, and heads were removed for viral titration at days 3, 6, 9 and 14 after infection on C6/36 cells. The numbers of analysed mosquitoes are given in parentheses. An asterisk refers to significant difference (P-value < 0.05). Error bars refer to the standard deviation.

To evaluate the ability of CHIKV to reach the salivary glands and be transmitted through the mosquito bite, transmission rate (TR) was assessed at 3, 6, 9 and 14 dpi. Although only 20–25% of mosquitoes were able to transmit at 3 dpi, TR increased after 6 dpi for both populations. When comparing TR between the two populations at a given dpi, no significant differences were detected [Chi-square test: $P > 0.05$; 3 dpi ($\chi^2 = 0.03$, $df = 1$, $P = 0.85$); 6 dpi ($\chi^2 = 1.79$, $df = 1$, $P = 0.18$); 9 dpi ($\chi^2 = 0.24$, $df = 1$, $P = 0.62$); 14 dpi ($\chi^2 = 1.02$, $df = 1$, $P = 0.31$)]. The intensity of viral transmission was evaluated by quantifying the viral load in mosquito saliva. CHIKV particles reached its maximum at 14 dpi for both populations, with Funchal presenting a 2.62 ± 0.79 $\log_{10}$ ffu/ml and Paúl do Mar with 2.96 ± 1.14 $\log_{10}$ ffu/ml. At a given dpi, no significant difference was detected between populations [Mann-Whitney test: $P > 0.05$; 3 dpi ($Z = 0.0$, $P = 1.0$); 6 dpi ($Z = -1.71$, $P = 0.09$); 9 dpi ($Z = -0.32$, $P = 0.74$); 14 dpi ($Z = -0.74$, $P = 0.46$)]. In addition, the number of viral particles in saliva (Fig. 2) did not vary along with the dpi for both populations [Kruskal-Wallis test: $P > 0.05$; Funchal ($\chi^2 = 0.98$, $df = 3$, $P = 0.80$); Paúl do Mar ($\chi^2 = 3.61$, $df = 3$, $P = 0.30$)].

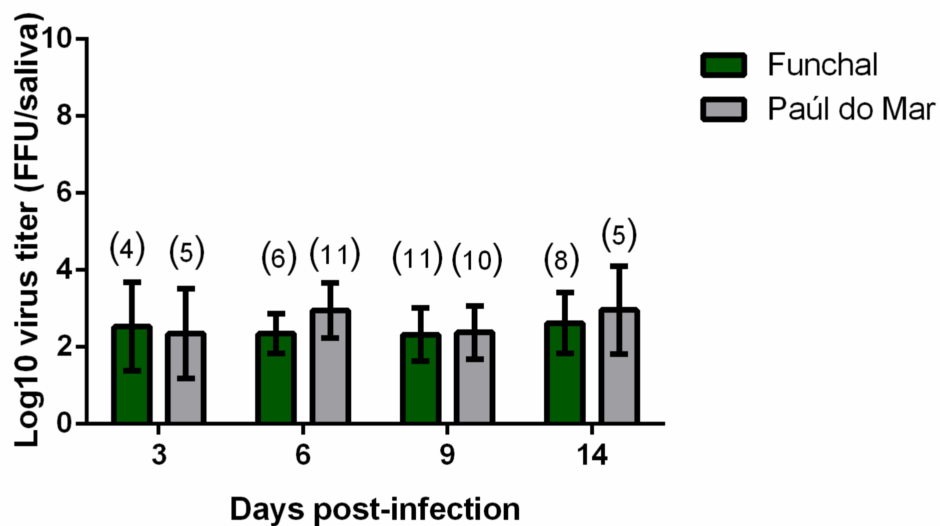

Fig. 2 Transmission of CHIKV in the saliva of *Ae. aegypti* from Madeira Island. Mosquitoes were sacrificed, and saliva was collected individually and titrated at days 3, 6, 9 and 14 after infection on C6/36 cells. The numbers of analysed mosquitoes are given in parentheses. Error bars refer to the standard deviation.

***Aedes aegypti* from Funchal and Paúl do Mar transmit DENV-2 at different rates**

The potential of DENV-2 transmission by *Ae. aegypti* from Madeira was measured by infecting mosquitoes with a DENV-2 strain from Bangkok. IR, DR and TR were assessed at 7 and 14 dpi (Table 2).

Table 2 Infection, dissemination and transmission rates (in %) calculated at different days after infection of *Ae. aegypti* from Madeira with DENV-2 Bangkok strain

Days post-infection	Funchal			Paúl do Mar		
	IR	DR	TR	IR	DR	TR
	n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
7	95 (20)	52.6 (19)	0 (10)	80 (20)	18.7 (16)	0 (3)
14	95 (20)	94.7 (19)	27.7 (18)	75 (20)	80 (15)	8.3 (12)

Abbreviations: IR, infection rate; DR, dissemination rate; TR, transmission rate; *n*, the number of mosquitoes analysed

Our study indicated a different pattern of susceptibility to dengue infection compared to chikungunya infection. While both populations presented similar IR [Chi-square test *P* >

0.05; 7 dpi (χ^2 = 2.05, *df* = 1, *P* = 0.15); 14 dpi (χ^2 = 3.13, *df* = 1, *P* = 0.08)], the Funchal population ensured a better dissemination of DENV-2 than Paúl do Mar at 7 dpi [Chi-square test: $P < 0.05$; 7 dpi (χ^2 = 4.27, *df* = 1, *P* = 0.04)] Virus titer in heads was slightly higher at 14 dpi for the Funchal strain (Mann-Whitney test: *Z* = 2.11, *P* = 0.03). The maximum number of DENV-2 particles in mosquito heads was detected at 14 dpi for both populations: 4.51 ± 0.63 $\log_{10}$ ffu/ml for Funchal population and 3.98 ± 0.88 $\log_{10}$ ffu/ml for Paúl do Mar population (Fig. 3).

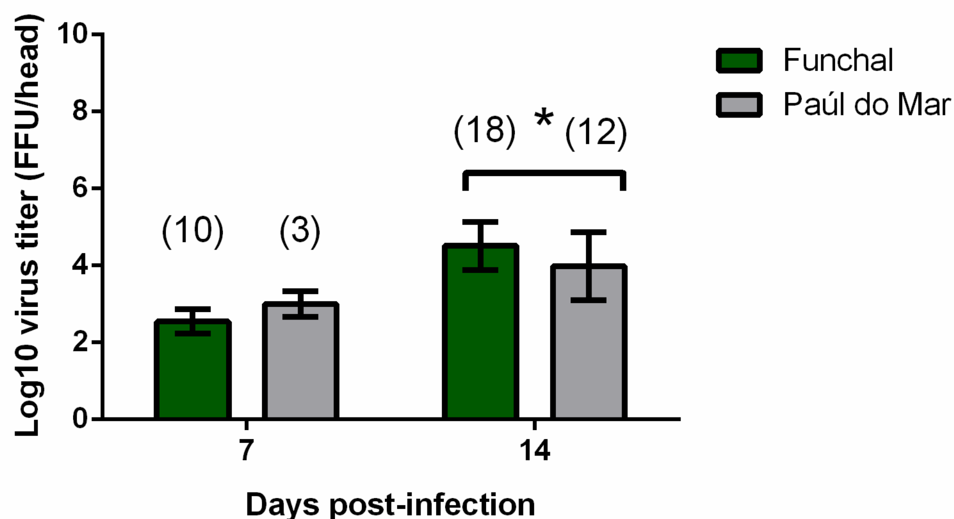

Fig. 3 Dissemination of DENV in *Ae. aegypti* from Madeira Island. Mosquitoes were sacrificed, and heads were removed for viral titration at days 7 and 14 after infection on C6/36 cells. The numbers of analysed mosquitoes are given in parentheses. An asterisk refers to significant difference (*P*-value < 0.05). Error bars refer to the standard deviation.

When examining TR, transmission with DENV-2 was lower than with CHIKV. TR reached a maximum at 14 dpi: 27.7% for the Funchal population and 8.3% for the Paúl do Mar population. No significant differences were detected between populations at each dpi (Chi-square test: χ^2 = 1.70, *df* = 1, *P* = 0.19 at 14 dpi). As observed with CHIKV, the number of viral particles in saliva was lower than in heads (Figs. 3, 4). The maximum number of DENV particles in saliva was reached at 14 dpi: 1.81 ± 0.34 $\log_{10}$ ffu/ml for the Funchal population and 1.60 ffu/ml for the Paúl do Mar population (Fig. 4). Both populations presented a similar number of viral particles in saliva at 14 dpi (Mann-Whitney test: *Z* = 0.69, *P* = 0.49).

Fig. 4 Transmission of DENV in the saliva of *Ae. aegypti* from Madeira Island. Mosquitoes were sacrificed, and saliva was collected individually and titrated at days 7 and 14 after infection on C6/36 cells. The numbers of analysed mosquitoes are given in parentheses. Error bars refer to the standard deviation.

Discussion

To our knowledge, this study represents the first evaluation of the vector competence of European *Ae. aegypti* populations for the transmission of two arboviruses of medical importance, CHIKV and DENV. Since its arrival in 2005, *Ae. aegypti* has been spreading throughout Madeira Island, increasing the risk of emergence of mosquito-borne diseases. The risk became a reality in 2012 when autochthonous cases of DENV-1 were reported in Funchal [6, 23]; Venezuela in South America was the most probable origin of virus importation [24]. After this event, vector competence studies with *Ae. aegypti* from Madeira became pivotal in evaluating the risk of arboviral disease outbreak.

Our data demonstrate that the local *Ae. aegypti* populations are very susceptible to CHIKV and DENV-2 infections. Regarding CHIKV, our results showed that only three days after infection, *Ae. aegypti* from Madeira was able to transmit this virus suggesting that the extrinsic incubation period of CHIKV with this vector population was short, as expected [7]. Despite high levels of viral dissemination (84–100%), *Ae. aegypti* from both localities displayed quite similar and moderate transmission rates (25–55%), with Funchal ensuring a slightly higher virus transmission at 14 dpi compared to the Paúl do

Mar population. Similar results were also obtained with other populations of *Ae. aegypti* from the Americas, with transmission ranging between 20–80% [8, 25], and Africa [26]. In the case of a possible chikungunya outbreak on the island, the onset of cases would be alarmingly fast, particularly in Funchal city, where most of the inhabitants live and work.

In addition to CHIKV risk assessment, vector competence for DENV-2 transmission was also evaluated. The reason DENV-2 was chosen for the vector competence study was related to the increasing concern that a new serotype will arrive in Madeira. Dengue secondary infection might lead to severe clinical symptoms and potential fatalities [9]. Our results underline a significantly higher dissemination efficiency of DENV-2 in *Ae. aegypti* from Funchal when comparing to Paúl do Mar. However, we observed no significant differences in transmission rate between the two populations or in the number of virus particles in mosquito saliva. This suggests that higher dissemination of DENV-2 in *Ae. aegypti* may not be correlated with the higher transmission in saliva. It would be interesting to verify if this result can be found with another serotype [27]. Funchal city differs from Paúl do Mar in possessing higher human and *Ae. aegypti* densities favourable to arbovirus transmission as illustrated by the 2012 dengue outbreak caused by DENV-1. Funchal was the central hotspot for DENV-1 transmission, and no DENV-1 cases were observed in Paúl do Mar [23]. Similar viral midgut infection and dissemination rates were observed in other studies with *Ae. aegypti* from the Americas, Australia and, surprisingly, from Africa, even using different methodologies than the one used in this study [7, 28–32].

Madeira Island could be a stepping-stone for the introduction of ZIKV into Europe. The main factors are present: the vector *Ae. aegypti*, imported cases from Brazil and Venezuela [19], and a naïve human population. Vector competence studies for ZIKV were also performed using the same *Ae. aegypti* population described in this study [19]. It has been demonstrated that the Funchal strain was the only population showing viral particles in saliva samples [19]. One should also note that the level of vector competence mostly depends on mosquito population genetics and the viral genotype used in the oral infections [25]. To provide a complete risk assessment of arboviral emergence, more studies should be implemented using additional viral strains or genotypes circulating in areas neighbouring Madeira Island.

CHIKV and DENV are two arboviruses with the highest potential to be introduced to Madeira Island. Based on genetic markers (mtDNA and *kdr* mutations), it has been shown that *Ae. aegypti* from Madeira originated from Brazil or Venezuela [33]. Owing to the extensive exchanges of goods and people with the two South American countries, the risk of CHIKV autochthonous cases on Madeira Island remains high. As previously stated, the Asian genotype of CHIKV was used for the oral infections in *Ae. aegypti* from Madeira. CHIKV has had a severe impact in the Americas since 2014, particularly in Venezuela with the highest number of cases recorded in the Andean region [34]. As with CHIKV, all DENV serotypes can be introduced to Madeira by a viremic traveller returning to Funchal from Caracas [35]. Caracas is connected to the island by weekly direct flights [24].

This study also highlights the need for further studies to define the genetic background of the *Ae. aegypti* populations of Madeira. Differences observed in DENV dissemination between *Ae. aegypti* from Funchal and Paúl do Mar could suggest population-based differences. Attention should be given to differential gene expression related to insecticide resistance [36] or immunity genes which may explain the differences observed. Moreover, the natural habitat of both populations presents distinct environmental and typological conditions: Funchal is considered an urban area, with vector control activities whereas Paúl do Mar is mainly a rural area, geographically isolated from the rest of the island, and with a higher mean temperature along the year. Therefore, the role of environmental and genetic factors should be considered. Additional population genetic studies are being performed with polymorphic DNA markers to refine our knowledge about the origin, genetic differentiation, and stability of the species in the island.

The temperate climate on Madeira Island can also play a key role in modulating *Ae. aegypti* vector competence for arbovirus transmission. It has been shown that temperature affects the vector competence in a tripartite interaction between mosquito genotype, viral genotype, and environment [25, 37]. Considering the Madeira climate, it would be of great importance to assess the vector competence under lower incubation temperature regimes, such as 20 °C, in contrast to the usual incubation temperature of 28 °C.

Conclusions

Based on our results, we strongly recommend that a robust and strengthened vector surveillance program be maintained on Madeira Island. There is an urgent need for new control strategies since the local *Ae. aegypti* populations are considered resistant to several insecticide classes [36] and this could lead to the complete failure of the vector control programmes. Our results with CHIKV and DENV suggest that it is crucial for Madeira Island to be prepared for more mosquito-borne disease epidemics. If mosquito densities reach levels like those observed during the dengue outbreak in 2012, immediate control measures, such as intensive community-based campaigns or using alternative non-chemical strategies, should be triggered to prevent arbovirus transmission. Our results are also of great importance for European countries where another species, *Ae. albopictus*, has been implicated in the last chikungunya and dengue outbreaks [13–15]. Coordination of vector control strategies between all European countries should be implemented as globalisation will contribute to the growing expansion of vector-borne pathogens, mosquito vectors and viremic people.

Abbreviations

CHIKV: chikungunya virus; DENV: dengue virus; ZIKV: Zika virus; ATP: adenosine triphosphate; FBS: fetal bovine serum; dpi: days post-infection; ffu: focus forming units; IR: infection rate; DR: dissemination rate; TR: transmission rate

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

Data supporting the conclusions of this article are included within the article. The raw datasets used and/or analysed during this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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References

1. Leta S, Beyene TJ, De Clercq EM, Amenu K, Kraemer MUG, Revie CW. Global risk mapping for major diseases transmitted by *Aedes aegypti* and *Aedes albopictus*. Int J Infect Dis. 2018;67:25–35.
2. Brown JE, Evans BR, Zheng W, Obas V, Barrera-Martinez L, Egizi A, et al. Human impacts have shaped historical and recent evolution in *Aedes aegypti*, the dengue and yellow fever mosquito. Evolution. 2014;68:514–25.
3. Almeida APG, Gonçalves YM, Novo MT, Sousa CA, Melim M, Gracio AJ. Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. Euro Surveill. 2007;12:E071115.6.
4. Ibañez-Justicia A, Gloria-Soria A, Den Hartog W, Dik M, Jacobs F, Stroo A. The first detected airline introductions of yellow fever mosquitoes (*Aedes aegypti*) to

Europe, at Schiphol International airport, the Netherlands. Parasit Vectors. 2017;10:603.

5. Margarita Y, Santos Grácio AJ, Lencastre I, Silva AC, Novo T, Sousa C. First record of *Aedes* (Stegomyia) *aegypti* (Linnaeus, 1762) (Diptera, Culicidae) in Madeira Island – Portugal (Portuguese, English abstract). Acta Parasitol Port. 2006;13: 59–61.
6. Alves MJ, Fernandes PL, Amaro F, Osório H, Luz T, Parreira P, et al. Clinical presentation and laboratory findings for the first autochthonous cases of dengue fever in Madeira island, Portugal, October 2012. Euro Surveill. 2013;18:20398.
7. Vega-Rua A, Zouache K, Caro V, Diancourt L, Delaunay P, Grandadam M, et al. High efficiency of temperate *Aedes albopictus* to transmit chikungunya and dengue viruses in the Southeast of France. PLoS One. 2013;8:e59716.
8. Vega-Rua A, Zouache K, Girod R, Failloux A-B, Lourenco-de-Oliveira R. High level of vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor in the spread of chikungunya virus. J Virol. 2014;88:6294–306.
9. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. Nat Rev Microbiol. 2010;8:S7–16.
10. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014;383:P514.
11. Diagne CT, Faye O, Guerbois M, Knight R, Diallo D, Faye O, et al. Vector competence of *Aedes aegypti* and *Aedes vittatus* (Diptera: Culicidae) from Senegal and Cape Verde archipelago for West African lineages of chikungunya virus. Am J Trop Med Hyg. 2014;91:635–41.
12. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol. 2010; 8:491–500.
13. Gould EA, Gallian P, De Lamballerie X, Charrel RN. First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality! Clin Microbiol Infect. 2010;16:1702–4.
14. Calba C, Guerbois-Galla M, Franke F, Jeannin C, Auzet-Caillaud M, Grard G, et al. Preliminary report of an autochthonous chikungunya outbreak in France, July to September 2017. Euro Surveill. 2017;22(39): pii=17-00647.

15. Venturi G, Di Luca M, Fortuna C, Elena Remoli M, Riccardo F, Severini F, et al. Detection of a chikungunya outbreak in Central Italy, August to September 2017. Euro Surveill. 2017;22(39):pii=17-00646..
16. Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H, et al. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector-Borne Zoonotic Dis. 2012;12:435–47.
17. Nazareth T, Teodósio R, Porto G, Gonçalves L, Seixas G, Silva AC, et al. Strengthening the perception-assessment tools for dengue prevention: a cross-sectional survey in a temperate region (Madeira, Portugal). BMC Public Health. 2014;14:39.
18. Instituto de Administração da Saúde, IP-RAM. http://iasaude.sras.gov-madeira.pt/Documentos/WEB/Anexos/Boletim%20epidemiologico%201-2016.pdf. Accessed 17 Jul 2018.
19. Jupille H, Seixas G, Mousson L, Sousa CA, Failloux AB. Zika virus, a new threat for Europe? PLoS Negl Trop Dis. 2016;10:e0004901.
20. NÃO MOSQUITO platform. http://iasaude.sras.gov-madeira.pt/naomosquito/. Accessed 17 Jul 2018.
21. Vazeille-Falcoz M, Mousson L, Rodhain F, Chungue E, Failloux AB. Variation in oral susceptibility to dengue type 2 virus of populations of *Aedes aegypti* from the islands of Tahiti and Moorea, French Polynesia. Am J Trop Med Hyg. 1999;60:292–9.
22. Dubrulle M, Mousson L, Moutailler S, Vazeille M, Failloux A-B. Chikungunya virus and *Aedes* mosquitoes: saliva is infectious as soon as two days after oral infection. PLoS One. 2009;4:e5895.
23. Sousa CA, Clairouin M, Seixas G, Viveiros B, Novo MT, Silva AC, et al. Ongoing outbreak of dengue type 1 in the Autonomous Region of Madeira, Portugal: preliminary report. Euro Surveill. 2012;17(49)
24. Wilder-Smith A, Quam M, Sessions O, Rocklov J, Liu-Helmersson J, Franco L, et al. The 2012 dengue outbreak in Madeira: exploring the origins. Euro Surveill. 2014;19:20725.

25. Vega-Rúa A, Lourenço-de-Oliveira R, Mousson L, Vazeille M, Fuchs S, Yébakima A, et al. Chikungunya virus transmission potential by local *Aedes* mosquitoes in the Americas and Europe. PLoS Negl Trop Dis. 2015;9:e0003780.
26. Ngoagouni C, Kamgang B, Kazanji M, Paupy C, Nakouné E. Potential of *Aedes aegypti* and *Aedes albopictus* populations in the Central African Republic to transmit enzootic chikungunya virus strains. Parasit Vectors. 2017;10:164.
27. Moura A, de Melo Santos M, Oliveira C, Guedes D, de Carvalho-Leandro D, da Cruz Brito, M. et al. Vector competence of the *Aedes aegypti* population from Santiago Island, Cape Verde, to different serotypes of dengue virus. Parasit Vectors. 2015;8:114.
28. Lourenço-de-Oliveira R, Rua A, Vezzani D, Willat G, Vazeille M, Mousson L, et al. *Aedes aegypti* from temperate regions of South America are highly competent to transmit dengue virus. BMC Infect Dis. 2013;13:610.
29. Gonçalves CM, Melo FF, Bezerra JM, Chaves BA, Silva BM, Silva LD, et al. Distinct variation in vector competence among nine field populations of *Aedes aegypti* from a Brazilian dengue-endemic risk city. Parasit Vectors. 2014;7:320.
30. Ye YH, Ng TS, Frentiu FD, Walker T, Van Den Hurk AF, O'Neill SL, et al. Comparative susceptibility of mosquito populations in North Queensland, Australia to oral infection with dengue virus. Am J Trop Med Hyg. 2014;90:422–30.
31. Dickson LB, Sanchez-Vargas I, Sylla M, Fleming K, Black WC. Vector competence in West African *Aedes aegypti* is flavivirus species and genotype dependent. PLoS Negl Trop Dis. 2014;8:e3153.
32. Calvez E, Guillaumot L, Girault D, Richard V, O'Connor O, Paoaafaite T. et al. Dengue-1 virus and vector competence of *Aedes aegypti* (Diptera: Culicidae) populations from New Caledonia. Parasit Vectors. 2017;10 :381.
33. Seixas G, Salgueiro P, Silva AC, Campos M, Spenassatto C, Reyes-lugo M, et al. *Aedes aegypti* on Madeira Island (Portugal): genetic variation of a recently introduced dengue vector. Mem Inst Oswaldo Cruz. 2013;vol. 108:3-10.
34. Rodríguez-Morales AJ, Paniz-Mondolfi AE. Venezuela: Far from the path to dengue and chikungunya control. J Clin Virol. 2015;66:60–1.

35. Torres J, Orduna T, Piña-Pozas M, Vázquez-Vega D, Sarti E. Epidemiological characteristics of dengue disease in Latin America and in the Caribbean: a systematic review of the literature. J Trop Med 2017;2017:8045435.
36. Seixas G, Grigoraki L, Weetman D, Vicente JL, Silva AC, Pinto J, et al. Insecticide resistance is mediated by multiple mechanisms in recently introduced *Aedes aegypti* from Madeira Island (Portugal). PLoS Negl Trop Dis. 2017;11:e0005799.
37. Zouache K, Fontaine a, Vega-Rua A, Mousson L, Thiberge J, Lourenco-De-Oliveira R, et al. Three-way interactions between mosquito population, viral strain and temperature underlying chikungunya virus transmission potential. Proc Biol Sci. 2014;281:20141078.

Chapter 6.

Concluding remarks and perspectives

Concluding remarks and perspectives

This study has produced relevant data on important traits of *Ae. aegypti* in Madeira, including a comprehensive assessment of the population genetics of this mosquito and its competence for arboviruses transmission.

The first part of the project presented in this thesis (Chapter 2) explored insecticide susceptibility and underlying resistance mechanisms present in *Ae. aegypti* from Madeira Island. High levels of insecticide resistance were found for all insecticide classes in the two island populations tested. The only exception was the population of Paúl do Mar where susceptibility to fenitrothion was observed. Pre-exposure to synergists, biochemical assays and microarray-based gene expression analysis all agree with presence of metabolic resistance mechanisms, involving overexpression of detoxifying enzymes (esterases, GSTs and oxidases). In addition, overexpression at genes coding for cuticular proteins suggest that cuticle thickening may also contribute to the resistance phenotype. Genotyping of *kdr* mutations showed fixation of the F1534C mutation and the presence of the V1016I mutation at moderate frequencies. Worryingly, the frequency of the resistance associated 1016I alleles has significantly increased since 2009. Continued monitoring of this locus is recommended, as continuous selection pressure may lead to eventual fixation of resistance alleles.

While the major resistance mechanisms were disclosed for the Madeiran *Ae. aegypi* populations, there are still a few aspects that require further investigation. Monitoring of the newly described V410L *kdr* mutation (Haddi et al. 2017) is recommended. The presence of this mutation together with V1016I and F1534C was associated with high levels of pyrethroid resistance in Latin American *Ae. aegypti* populations (Saaverda-Rodriguez et al. 2018). Furthermore, population genetic analysis (Chapter 3) confirmed the Venezuelan origin of *Ae. aegypti* in Madeira, stressing the need to genotype, in Madeira populations, newly described *kdr* mutations found in mosquito populations from this region. In addition to novel target-site mutations, genomic studies confirmed the role of CNVs in the overexpression of detoxification enzymes associated with insecticide resistance and have provided a set of genomic markers to track metabolic resistance in *Ae. aegypti* (Faucon et al. 2015, 2017). Therefore, the search for CNVs in Madeira

populations and its functional validation, through RNAi or CRISPR/cas9 systems (Itokawa et al. 2016; Homem and Davies, 2018), is required in order to complete the species insecticide resistance profile in the island. Finally, electron microscopy studies (Balabinidou et al. 2018) are needed to confirm if in fact *Ae. aegypti* from Madeira have a thicker cuticle when compared to reference susceptible populations. Previous studies, in insectary conditions, revealed that when insecticide selection pressure is removed, susceptibility could be restored after 10 generations (Grossman et al. 2018). The results obtained in Chapter 2 also raise questions on how the resistance profile is being kept under a scenario where no insecticides are being used in current vector control strategies. For this reason, the impact of household insecticides should be evaluated (Gray et al. 2018).

Presence of multiple mechanisms of insecticide resistance in the same mosquito population poses serious obstacles to the implementation and sustainability of insecticide-based vector control. Specifically, it remains to be addressed whether the use of insecticides can still be considered an option for Madeira, for instance, during an arbovirus outbreak. The WHO (2012) Global Plan for Insecticide Resistance Management recommends four major strategies for managing insecticide resistance: rotations of insecticides, mixtures of insecticides and/or insecticides/synergists, use of interventions in combination and mosaic spraying. All of these strategies rely on the expectation that field populations are resistant to a limited number of insecticides available for public health. Judging from the bioassays data and the complex combination of resistance mechanisms found in Madeira, these expectations are unlikely to be met. Such argument plays in favor of the adoption of non-insecticidal methods, as the mainstay for vector control in Madeira.

Another argument in favor of the adoption of alternative non-insecticidal vector control methods in Madeira comes from the results obtained by the population genetic analysis (Chapter 3). Effective population size estimates indicate that the *Ae. aegypti* population has increased until 2012, when the dengue outbreak occurred in the island. This population growth trend suggests that the 2005-2008 insecticide-based campaign was insufficient to reduce *Ae. aegypti* abundance and contain its expansion. On the other hand, the 10-fold reduction in N_e that was observed after the outbreak may be regarded as a

promising result that encourages the use of educational campaigns to promote community-based vector control measures to reduce *Ae. aegypti* abundance.

The emergence of DENV and re-emergence of yellow fever viruses (YFV), as well as the occurrence of epidemics due to novel arboviruses such as CHIKV and ZIKV, reinforce the need to better understand the evolutionary origin and population genetic structure of *Ae. aegypti*. The results obtained in Chapters 2 and 3 suggest that the resistance phenotype was already an established trait in the colonizing specimens that invaded Madeira in 2005. Therefore, the South American genetic background had a major influence in the invasion and the establishment success of this species. While low genetic diversity and effective size may induce extinction, the same does not seem to be the case for invasive species such as *Ae. aegypti* (Estoup et al. 2016). Despite the initial low genetic variability, suggesting a low number of founder mosquitoes, the invasive population was able to expand and thrive in a new habitat. The underlying mechanisms of successful local adaptation to a new environment are of great interest, not only to Madeira but also for Europe, where *Ae. aegypti* invasion may also occur. Newly developed genomic tools, such as SNP chips (Evans et al. 2015) or RADseq (Rašić et al. 2014), are now available to study the genetic basis of *Ae. aegypti* biological traits. The newly sequenced *Ae. aegypti* genome assembly (AaegL5) was improved (Mathews et al. 2018) and revealed that 65% of the genome is composed by transposable elements (TEs), repetitive DNA that cause genetic variation (Salgueiro et al. 2013). These may have a major role in ecological plasticity and successful adaptation to a new environment of *Aedes* species (Arensburger et al. 2011; Goubert et al. 2017), such as the case of *Ae. aegypti* in Madeira. Genomic tools could also help to refine population origin, genetic differentiation and better elucidate the demographic history of *Ae. aegypti* in Madeira (Rašić et al. 2015; Crawford et al. 2017; Gloria-Soria et al. 2018; Sherpa et al. 2018). Moreover, these genomic approaches may help to clarify the genetic basis of other important traits, such as vector competence (Evans et al. 2015).

Re-introduction of dengue as well as importation of other arbovirus is possible due to Madeira connections with endemic countries (Lourenço and Recker, 2014). The experimental infections with arboviruses described in chapters 4 and 5 demonstrated that *Ae. aegypti* from Madeira is a competent vector for all the viruses tested under laboratory

conditions. These results are fundamental for the design of effective control strategies and for general public health decision making, in areas where vector-borne diseases may emerge, such as the case of Madeira Island. The relevance of this data is associated to the fact that Madeira is a tourism region, visited by hundreds of thousands of tourists every year. This fact, coupled with optimal climatic factors for *Ae. aegypti* breeding and a non-immune human population, puts in evidence the potential vulnerability of Madeira and Europe regarding future emergence of dengue or other arboviral infections transmitted by *Ae. aegypti*. In the context, further vector competence studies should be undertaken to test the ability of *Ae. aegypti* from Madeira to transmit other DENV, CHIKV and ZIKV serotypes/lineages. Moreover, previous studies showed that *Ae. aegypti* can transmit these viruses simultaneously in double or triple infections (Rückert et al. 2017). It is also known that different temperature regimes may also influence vector competence results (Vega-Rúa et al. 2015; Brustolin et al. 2018). Therefore, it is recommended that additional experimental infections are performed using one or several viruses under different temperature regimes in order to assess the competence of the Madeira island *Ae. aegypti* population for co-transmission of different arboviruses under the Mediterranean temperate climate that characterizes this island. In addition, information about vector competence for other (re)emerging arboviruses would be also necessary. New emerging arboviruses such as Mayaro virus or Ross River virus and re-emergence of the YFV (Wilder-Smith et al. 2017) put in evidence the need to assess the ability of *Ae. aegypti* to transmit these viruses.

Another remaining line to explore is the role of the microbiota of *Ae. aegypti* from Madeira in shaping vector competence for arbovirus transmission. Previous studies detected the presence of Insect Specific Flaviviruses (ISFVs) in the Madeiran *Ae. aegypti* population (Calzolari et al. 2016). These viruses are known for interfering in vector competence by competing with the pathogenic virus for resources inside the mosquito (Hedge et al. 2015; Zakrzewski et al. 2018**).** Therefore, exploring the relationships between ISFVs and pathogenic arboviruses in experimental infections may help in the design of new tools to control arboviral transmission.

In addition to contributing to new knowledge on mechanisms of insecticide resistance and on the genetic structure and evolutionary biology of a recently introduced *Ae. aegypti*

population in an island setting, the research conducted also aimed at providing Madeira Health Authorities with informative data from an operational perspective, for the design and implementation of contingency plans against *Ae. aegypti* and *aegypti*-borne infections. Overall, the results here described not only answered the proposed objectives but also represent a basis for future research, in order to establish more efficient vector control programs in Madeira and other regions of similar ecological settings.

References

Arensburger P, Hice RH, Wright J a, Craig NL, Atkinson PW. The mosquito *Aedes aegypti* has a large genome size and high transposable element load but contains a low proportion of transposon-specific piRNAs. BMC Genomics. 2011;12(1):606.

Balabanidou V, Grigoraki L, Vontas J. Insect cuticle: a critical determinant of insecticide resistance. Curr Opin Insect Sci. 2018;27:68–74.

Brustolin M, Santamaria C, Napp S, Verdún M, Rivas R, Pujol N, et al. Experimental study of the susceptibility of a European *Aedes albopictus* strain to dengue virus under a simulated Mediterranean temperature regime. Med Vet Entomol. 2018.

Calzolari M, Zé-Zé L, Vázquez A, Sánchez Seco MP, Amaro F, Dottori M. Insect-specific flaviviruses, a worldwide widespread group of viruses only detected in insects. Infect Genet Evol. 2016;40:381–8.

Crawford JE, Alves JM, Palmer WJ, Day JP, Sylla M, Ramasamy R, et al. Population genomics reveals that an anthropophilic population of *Aedes aegypti* mosquitoes in West Africa recently gave rise to American and Asian populations of this major disease vector. BMC Biol; 2017;15(1):1–16.

Evans BR, Gloria-Soria A, Hou L, McBride C, Bonizzoni M, Zhao H, et al. A Multipurpose high throughput SNP chip for the dengue and yellow fever mosquito, *Aedes aegypti*. G3; 2015;5:711–8.

Estoup A, Ravigné V, Hufbauer R, Vitalis R, Gautier M, Facon B. Is there a genetic paradox of biological invasions? Annu Rev Ecol Evol Syst. 2016;47:51–72.

Faucon F, Dusfour I, Gaude T, Navratil V, Boyer F, et al. Unravelling genomic

changes associated with insecticide resistance in the dengue mosquito *Aedes aegypti* by deep targeted sequencing. Genome Res. 2015:1–13.

Faucon F, Gaude T, Dusfour I, Navratil V, Corbel V, Juntarajumnong W, et al. In the hunt for genomic markers of metabolic resistance to pyrethroids in the mosquito *Aedes aegypti*: An integrated next-generation sequencing approach. PLoS Negl Trop Dis. 2017;11(4):1–20.

Gloria-Soria A, Lima A, Lovin DD, Cunningham JM, Severson DW, Powell JR. Origin of a high-latitude population of *Aedes aegypti* in Washington, DC. Am J Trop Med Hyg. 2018;98(2):445–52.

Gray L, Dzib Florez S, Medina Barreiro A, Vadillo-Sánchez J, González-Olvera G, Lenhart A, et al. Experimental evaluation of the impact of household aerosolized insecticides on pyrethroid resistant *Aedes aegypti*. 2018;1–11.

Grossman MK, Uc-Puc V, Rodriguez J, Cutler DJ, Morran LT, Manrique-Saide P, et al. Restoration of pyrethroid susceptibility in a highly resistant *Aedes aegypti* population. Biol Lett. 2018;14(6):18–22.

Goubert C, Henri H, Minard G, Valiente Moro C, Mavingui P, Vieira C, et al. High-throughput sequencing of transposable element insertions suggests adaptive evolution of the invasive Asian tiger mosquito towards temperate environments. Mol Ecol. 2017;26(15):3968–81.

Haddi K, Tomé HVV, Du Y, Valbon WR, Nomura Y, Martins GF, et al. Detection of a new pyrethroid resistance mutation (V410L) in the sodium channel of *Aedes aegypti*: A potential challenge for mosquito control. Sci Rep. 2017;7:1–9.

Hegde S, Rasgon JL, Hughes GL. The microbiome modulates arbovirus transmission in mosquitoes. Curr Opin Virol. 2015;15:97–102.

Homem RA, Davies TGE. An overview of functional genomic tools in deciphering insecticide resistance. Curr Opin Insect Sci. 2018;27:103–10.

Itokawa K, Komagata O, Kasai S, Ogawa K, Tomita T. Testing the causality between CYP9M10 and pyrethroid resistance using the TALEN and CRISPR/Cas9 technologies. Sci Rep. 2016;6(April):1–10.

Lourenço J, Recker M. The 2012 Madeira Dengue Outbreak: Epidemiological Determinants and Future Epidemic Potential. PLoS Negl Trop Dis. 2014;8(8).

Matthews BJ, Dudchenko O, Kingan S, Koren S, Antoshechkin I, Crawford JE, et al. Improved *Aedes aegypti* mosquito reference genome assembly enables biological discovery and vector control. bioRxiv. 2017;240747.

Rašić G, Filipović I, Weeks AR, Hoffmann A. Genome-wide SNPs lead to strong signals of geographic structure and relatedness patterns in the major arbovirus vector, *Aedes aegypti*. BMC Genomics. 2014;15:275.

Rašić G, Endersby-Harshman N, Tantowijoyo W, Goundar A, White V, Yang Q, et al. *Aedes aegypti* has spatially structured and seasonally stable populations in Yogyakarta, Indonesia. Parasit Vectors. 2015;8:610.

Rückert C, Weger-Lucarelli J, Garcia-Luna SM, Young MC, Byas AD, Murrieta RA, et al. Impact of simultaneous exposure to arboviruses on infection and transmission by *Aedes aegypti* mosquitoes. Nat Commun. 2017;8:1–9.

Saavedra-Rodriguez K, Maloof FV, Campbell CL, Garcia-Rejon J, Lenhart A, Penilla P, et al. Parallel evolution of Vgsc mutations at domains IS6, IIS6 and IIIS6 in pyrethroid resistant *Aedes aegypti* from Mexico. Sci Rep. 2018;8(1):1–9.

Salgueiro P, Moreno M, Simard F, O'Brochta D, Pinto J. New insights into the population structure of *Anopheles gambiae* s.s. in the Gulf of Guinea Islands revealed by herves transposable elements. PLoS One. 2013;8(4).

Sherpa S, Rioux D, Goindin D, Fouque F, François O, Després L. At the origin of a worldwide invasion: unraveling the genetic makeup of the Caribbean bridgehead populations of the dengue vector *Aedes aegypti*. Genome Biol Evol. 2018;10(1):56–71.

Vega-Rúa A, Lourenço-de-Oliveira R, Mousson L, Vazeille M, Fuchs S, Yébakima A, et al. Chikungunya virus transmission potential by local *Aedes* mosquitoes in the Americas and Europe. PLoS Negl Trop Dis. 2015;9(5):e0003780.

Wilder-Smith A, Gubler DJ, Weaver SC, Monath TP, Heymann DL, Scott TW. Epidemic arboviral diseases: priorities for research and public health. Lancet Infect Dis. 2017;17(3):e101–6.

World Health Organization. Global plan for insecticide resistance management in malaria vectors (GPIRM). 2012.

Zakrzewski M, Rašić G, Darbro J, Krause L, Poo YS, Filipović I, et al. Mapping the virome in wild-caught *Aedes aegypti* from Cairns and Bangkok. Sci Rep. 2018;8(1):1–12.