INSTITUTO DE HIGIENE E
MEDICINA TROPICAL
DESDE 1902

Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Wolbachia in medically important mosquitoes from Cape Verde: prevalence, genetic diversity and role in vector competence

Aires Januário Fernandes da Moura

DISSERTATION SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD) IN BIOMEDICAL SCIENCES, SPECIALIZING IN CELLULAR AND MOLECULAR BIOLOGY

JANUARY 2025

Universidade Nova de Lisboa
Instituto de Higiene e Medicina Tropical

Wolbachia in medically important mosquitoes from Cape Verde: prevalence, genetic diversity and role in vector competence

Author: Aires Januário Fernandes da Moura

Supervisor: Prof. João Pedro Soares da Silva Pinto

Co-supervisor: Prof. Carla Alexandra Gama Carrilho da Costa Sousa

Dissertation submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Biomedical Sciences.

Funded by Fundação para a Ciência e Tecnologia (FCT) and Camões I.P

Dissertation Publications

The thesis presented herein is the result of the compilation of the following publications:

Journal article 1

Title:
Screening of natural *Wolbachia* infection in mosquitoes (Diptera: Culicidae) from the Cape Verde islands.

Authors and affiliations:
Aires Januário Fernandes da Moura[1,2], Vera Valadas[1], Silvania Da Veiga Leal[3], Eddyson Montalvo Sabino[1,4], Carla A. Sousa [1], João Pinto[1].

[1] Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal.
[2] Unidade de Ciências da Natureza, da Vida e do Ambiente, Universidade Jean Piaget de Cabo Verde, Praia, Cape Verde.
[3] Laboratório de Entomologia Médica, Instituto Nacional de Saúde Pública, Praia, Cape Verde.
[4] Laboratório de Simulidos, Universidad Nacional Hermilio Valdizan, Huánuco, Peru.

Journal: Parasites & Vectors
Accepted: 17 March 2023
Published online: 25 April 2023
DOI: https://doi.org/10.1186/s13071-023-05745-w

Journal article 2

Title:
Vector Competence of *Culex quinquefasciatus* from Santiago Island, Cape Verde, to West Nile Virus: Exploring the Potential Effect of the Vector Native *Wolbachia.*

Authors and affiliations:
Aires Januário Fernandes da Moura[1,2], Filipe Tomaz[1], Tiago Melo[1], Gonçalo Seixas[1], Carla A. Sousa[1], João Pinto[1].

[1] Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal.
[2] Unidade de Ciências da Natureza, da Vida e do Ambiente, Universidade Jean Piaget de Cabo Verde, Praia, Cape Verde.

Journal: Parasites & Vectors
Accepted: 28 November 2024
Published online: 23 December 2024
DOI: https://doi.org/10.1186/s13071-024-06609-7

Acknowledgments

First and foremost, I express my gratitude to God for granting me the strength, perseverance, and guidance necessary throughout this demanding and transformative journey. I am also profoundly grateful to my family, whose unconditional support and constant encouragement were instrumental in overcoming the numerous challenges encountered during the completion of this degree.

To my supervisors, Professors João Pinto and Carla Sousa, I extend my sincere gratitude for their insightful feedback, thoughtful guidance, and commitment to excellence, all of which were instrumental in the development of this thesis.

I acknowledge with gratitude Camões I.P. for awarding the doctoral scholarship that made this research possible. Additionally, I extend my appreciation to the R&D Unit GHTM for providing the financial support essential for advancing key phases of this study.

I extend my gratitude to the National Institute of Public Health of Cape Verde and to the entomological surveillance technicians from health departments across the islands for their logistical and technical support during the collection of mosquito samples.

Lastly, I would like to thank the staff of VIASEF (the *in vivo* Arthropod Security Facility from IHMT), for their support, technical assistance, and for providing such a collaborative and welcoming environment.

To all those who contributed, in one way or another, to this journey, I sincerely thank you.

Abstract

Wolbachia pipientis is an endosymbiotic bacterium currently employed in the control of vector-borne diseases due to its capacity to induce cytoplasmic incompatibility and suppress arbovirus replication in mosquito vectors. The objective of this study was to evaluate the prevalence and genetic diversity of *Wolbachia* across different mosquito species (Diptera: Culicidae) in Cape Verde and to assess the potential influence of the endosymbiont on the transmission of West Nile virus (WNV) by the mosquito vector *Culex quinquefasciatus*.

Mosquito samples were collected from six islands in Cape Verde and *Wolbachia* detection was performed by amplifying a fragment of the surface protein gene (*wsp*). Strain identification was conducted by multilocus sequence typing (MLST) using housekeeping genes (*gatB, coxA, hcpA, fbpA* and *ftsZ*) and the *wsp* hypervariable region (HVR). Classification of *w*Pip groups was determined by PCR-RFLP, targeting the ankyrin-domain gene *pk1*. To evaluate the impact of native *Wolbachia* on vector competence, *Wolbachia*-infected and uninfected *Cx. quinquefasciatus* from Santiago Island were exposed to the PT6.39 strain of WNV using a Hemotek membrane feeding system. WNV infection was subsequently evaluated through One-Step RT-qPCR at 7, 14 and 21 days post-infection (dpi).

Wolbachia was detected in *Culex tigripes* (100% prevalence), *Cx. quinquefasciatus* (98.3%), *Cx. pipiens s.s.* (100%) and *Cx. pipiens/quinquefasciatus* hybrids (100%). MLST and phylogenetic analysis revealed that *Wolbachia* from *Cx. pipiens* complex belongs to Sequence Type (ST) 9, *w*Pip clade and Supergroup B. *Wolbachia* isolated from *Cx. tigripes* was also classified under Supergroup B but formed a unique lineage separate from the *w*Pip clade, with no assigned MLST profile. Results from the PCR-RFLP showed the presence of *w*Pip-II, *w*Pip-III and *w*Pip-IV *Wolbachia* in Cape Verde. Vector competence assessments demonstrated that *Cx. quinquefasciatus* females lacking native *Wolbachia* exhibited significantly higher WNV loads in their bodies and increased virus dissemination compared to their wild-type counterparts harboring *Wolbachia*.

The high diversity of *w*Pip groups observed in this study suggests multiple introductions of *Cx. pipiens s.l.* in Cape Verde and provides insights for the design of future *Wolbachia*-based control strategies in the archipelago. The occurrence of *Wolbachia* in *Culex tigripes* is reported for the first time, which likely represents a novel strain of the endosymbiont with promising implications for vector control strategies. Native *Wolbachia* from *Cx. quinquefasciatus* appears to restrict WNV dissemination and further studies will be needed to elucidate the mechanism underlying this *Wolbachia*-mediated protection.

Keywords: *Wolbachia*; *Culex*; Cape Verde; Genetic diversity; Vector competence; West Nile virus (WNV)

Resumo

Wolbachia pipientis é uma bactéria endossimbiótica atualmente utilizada no controlo de doenças transmitidas por vetores, devido à sua capacidade de induzir incompatibilidade citoplasmática e suprimir a replicação de arbovírus em mosquitos vetores. O objetivo deste estudo foi avaliar a prevalência e a diversidade genética de *Wolbachia* em diferentes espécies de mosquitos (Diptera: Culicidae) em Cabo Verde, bem como avaliar a influência deste endossimbionte na transmissão do vírus do Nilo Ocidental (WNV) pelo vetor *Culex quinquefasciatus*.

As amostras de mosquitos foram colhidas em seis ilhas de Cabo Verde. A deteção de *Wolbachia* nos mosquitos foi realizada através da amplificação de um segmento do gene da proteína de superfície (*wsp*). A identificação da estirpe foi efetuada por Tipagem de Sequência Multilocus (*Multilocus Sequence Typing,* MLST), utilizando genes constitutivos (*gatB, coxA, hcpA, fbpA e ftsZ*) e a região hipervariável do *wsp* (HVR). A classificação dos grupos *w*Pip foi determinada por PCR-RFLP, direcionada para o gene da anquirina (*pk1*). Para avaliar o impacto da *Wolbachia* nativa na competência vetorial, fêmeas de *Cx. quinquefasciatus*, infetadas e não infetadas com *Wolbachia*, provenientes da ilha de Santiago, foram expostas à estirpe PT6.39 do WNV utilizando um sistema de alimentação por membrana. Posteriormente, a infeção por WNV foi avaliada por One-step RT-qPCR aos 7, 14 e 21 dias pós-infeção (dpi).

Wolbachia foi detetada em *Culex tigripes* (100% de prevalência), *Cx. quinquefasciatus* (98,3%), *Cx. pipiens s.s.* (100%) e em híbridos *Cx. pipiens/quinquefasciatus* (100%). As análises MLST e filogenética revelaram que a *Wolbachia* do complexo *Cx. pipiens* pertence à *Sequence Type* (ST) 9, ao clado *w*Pip e ao Supergrupo B. A *Wolbachia* isolada de *Cx. tigripes* também foi classificada no Supergrupo B, mas formou uma linhagem única, distinta do clado *w*Pip, sem perfil MLST atribuído. Os resultados PCR-RFLP indicaram a presença de *w*Pip-II, *w*Pip-III e *w*Pip-IV em Cabo Verde. As avaliações de competência vetora demonstraram que as fêmeas de *Cx. quinquefasciatus* desprovidas da sua *Wolbachia* nativa apresentaram títulos de WNV significativamente mais elevadas nos seus corpos e uma disseminação aumentada do vírus, em comparação com os seus homólogos de tipo selvagem com *Wolbachia.*

A elevada diversidade dos grupos *w*Pip observada sugere múltiplas introduções de *Cx. pipiens s.l.* em Cabo Verde e fornece informações cruciais para o desenvolvimento de futuras estratégias de controlo baseadas em *Wolbachia* no arquipélago. Relata-se a primeira ocorrência de *Wolbachia* na espécie *Culex tigripes*, que provavelmente representa uma nova estirpe do endossimbionte, com implicações promissoras para as estratégias de controlo vetorial. A *Wolbachia* nativa de *Cx. quinquefasciatus* aparenta restringir a disseminação do vírus do Nilo Ocidental (WNV), sendo necessários estudos adicionais para elucidar o mecanismo subjacente a esta proteção mediada por *Wolbachia*.

Palavras-chave: *Wolbachia*; *Culex*; Cabo Verde; Diversidade genética; Competência vetora; Vírus do Nilo Ocidental (WNV)

Table of contents

CHAPTER 1: General Introduction 1
1.1 Mosquito-borne diseases 1
1.2 Mosquito-borne diseases in Cape Verde 3
1.3 Control of mosquito-borne diseases 6
1.4 *Wolbachia* as a strategy for controlling mosquito-borne diseases 9
1.4.1 The endosymbiont *Wolbachia* 9
1.4.2 *Wolbachia* Genetic diversity 10
1.4.3 *Wolbachia*-induced reproductive manipulations and role on vector competence 12
1.4.4 Natural *Wolbachia* infection in major mosquito vectors 17
1.4.4.1 *Wolbachia* in *Culex* species 17
1.4.4.2 *Wolbachia* in *Anopheles spp.* 18
1.4.4.3 *Wolbachia* in *Aedes* species 19
1.4.5 The Application of *Wolbachia* as a Vector Control Tool 20
1.5 Research Questions 24
1.6 Objectives 24
1.7 Workflow outline 25
1.8 References 26
CHAPTER 2: Prevalence and Genetic Diversity of *Wolbachia* in Mosquitoes (Diptera: Culicidae) from Cape Verde (article 1) 43
2.1 Background 45
2.2 Methods 46
2.3 Results 50
2.4 Discussion 55
2.5 Conclusion 58
2.6 References 60
2.7 Supplementary Information 66
CHAPTER 3: The Role of Native *Wolbachia* on Vector Competence (article 2) 70
3.1 Background 72
3.2 Methods 73

3.3 Results 79
3.4 Discussion 83
3.5 Conclusion 86
3.6 References 88
3.7 Supplementary information 97
CHAPTER 4. General Discussion 99
Conclusion 106
4.1 References 107

List of abbreviations and acronyms

BSL3: Biosafety Level 3

CHIKV: Chikungunya virus

CI: Cytoplasmic Incompatibility

CTAB: Cetyltrimethylammonium bromide

DENV: Dengue virus

DNA: Deoxyribonucleic Acid

dpi: days post-infection

MLST: Multilocus Sequence Typing

PCR: Polymerase Chain Reactions

PFU: Plaque forming Units

RT-qPCR: Quantitative real-time PCR

RFLP: Restriction Fragment Length Polymorphism

ST: Sequence Type

VIASEF: In Vivo Arthropod Security Facility

WNV: West Nile virus

Wsp: *Wolbachia* Surface Protein

wsp: *Wolbachia* surface protein gene

ZIKV: Zika virus

CHAPTER 1: General introduction

1.1Mosquito-borne diseases

Mosquitoes act as vectors of numerous pathogens responsible for diseases collectively known as mosquito-borne diseases. Mosquito-borne diseases represent a substantial share of the overall burden of infectious diseases and pose a significant challenge to global public health. Among the most prevalent and emerging mosquito-borne diseases are malaria, dengue fever, chikungunya, Zika, yellow fever, Japanese encephalitis, and West Nile fever. Together, these infections account for nearly 700 million cases and over one million deaths annually [1].

Malaria continues to be one of the most devastating mosquito-borne diseases, heavily contributing to the global disease burden. Transmitted by *Anopheles* mosquitoes, malaria affects approximately 249 million people annually, with over 608,000 lives lost each year, predominantly impacting children under five years of age [2]. Over 90% of malaria-attributed deaths occur in sub-Saharan Africa.

Malaria is caused by five distinct *Plasmodium* species (*Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, and Plasmodium knowlesi*), each contributing to a spectrum of clinical manifestations. The disease may range from asymptomatic cases to uncomplicated malaria, often characterized by symptoms such as fever, chills, sweating, anemia, and nausea. Severe malaria, most commonly due to *P. falciparum*, can lead to life-threatening conditions, including severe anemia, multiple organ failure, and cerebral malaria, usually leading to coma [3].

Dengue virus (DENV) is currently the most prevalent arbovirus worldwide, endangering the health of 3.9 billion people across 132 countries. Each year, nearly 96 million individuals suffer from symptomatic dengue fever cases and 40,000 deaths occur [2]. The virus comprises four distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), transmitted primarily through the bites of *Aedes* mosquitoes. Among these vectors, *Aedes aegypti* and *Aedes albopictus* are the key species involved in transmission [4].

Dengue fever is highly endemic in tropical and subtropical regions around the world, mainly thriving in urban and semi-urban areas. The clinical spectrum of dengue fever is

remarkably broad, encompassing a range of conditions from mild cases to the far more severe form known as dengue hemorrhagic fever (DHF). DHF is distinguished by increased vascular permeability, leading to plasma leakage and an elevated risk of hemorrhage, underscoring the potentially life-threatening nature of the disease [5].

Another mosquito-borne virus of significant global concern is the Zika virus (ZIKV). Zika is primarily transmitted through the bite of infected *Aedes* mosquitoes, particularly *Ae. aegypti* [6]. In 2016, Zika emerged as a severe international health crisis, largely due to its association with complications such as microcephaly and other neurological disorders. Although the global incidence of Zika has declined since 2017, it continues to persist at low levels in many endemic regions [7]. The lack of a vaccine for prevention ensures that Zika remains a critical challenge, demanding ongoing vigilance and research efforts.

Chikungunya virus (CHIKV), an alphavirus transmitted by *Ae. aegypti* and *Ae. albopictus* mosquitoes poses a significant health threat across numerous regions. Chikungunya fever typically presents with symptoms such as high fever, intense muscle pain, and debilitating polyarthralgia, which can, in some cases, escalate to severe neurological complications and even result in death [8]. Over one hundred countries have reported the circulation of CHIKV, with more than 10 million cumulative cases of chikungunya fever recorded to date. Today, it is estimated that 1.3 billion people worldwide are at risk of contracting this disease, underscoring its broad impact and ongoing threat to public health [9].

Another emerging arbovirus of great concern is the West Nile virus (WNV). Currently, WNV is the most prevalent mosquito-borne viral infection and the leading cause of viral encephalitis in the United States [10]. In 2018, Europe faced a significant outbreak, with 2,083 reported cases resulting in 181 fatalities across the continent [11]. More recently, in 2023, 728 human cases of WNV were documented in Europe, including 709 locally acquired infections and 19 linked to travel [12].

Around 80% of individuals infected with WNV remain asymptomatic. However, the remaining 20% may manifest a range of symptoms, going from mild, flu-like discomfort to severe neurological complications, including meningitis and encephalitis. Severe illness develops in approximately 1% of cases, predominantly affecting older adults or those with compromised immune systems, where the risk is significantly heightened. In

such severe cases, mortality rates can exceed 10%, emphasizing the potentially life-threatening nature of the virus [13,14].

Mosquito-borne infections are becoming increasingly prevalent in endemic regions as well as in areas that were previously unaffected. Determinants responsible for the emergence or reemergence of vector-borne diseases include: i) climate changes, which have modified vector ecology and distribution while also affecting the life cycles of pathogens, and ii) non-climatic factors, such as unplanned urbanization, international trade and travel, human-induced ecological changes (*e.g.* agricultural activities and construction of dams), development of vector insecticide resistance and mutations in the genomes of pathogens due to exposure to antimicrobial agents [15,16].

Vector-borne diseases pose a significant health threat across African nations, making a substantial contribution to both illness and mortality throughout the continent. The recent outbreaks of diseases caused by arboviruses underscore the challenges faced by both mainland and island African regions in their efforts to control the spread of these illnesses [17]. These outbreaks serve as reminders of the persistent vulnerabilities and obstacles inherent in combating vector-borne diseases across diverse geographical landscapes in Africa.

1.2 Mosquito-borne diseases in Cape Verde

To date, approximately eleven mosquito species have been identified in Cape Verde, including four known vectors of human pathogens: *Ae. aegypti*, *Anopheles arabiensis*, *Culex quinquefasciatus* and *Culex pipiens sensu stricto* (*s.s*) [18,19]. These findings highlight the potential risk of mosquito-borne disease transmission in the archipelago and emphasize the need for a continuous and integrated entomological surveillance system to effectively control or mitigate such threats.

Aedes aegypti was initially identified on the island of São Vicente in 1931 and later expanded to the remaining islands of Cape Verde [20,21]. Today, it stands as one of the primary arbovirus vectors in the archipelago, thriving in high density across all inhabited islands [22]. Thus far, it remains the sole vector linked to the transmission of DENV and ZIKV in Cape Verde, which have, in recent years, placed a substantial burden on the country.

In 2009, Cape Verde experienced its first dengue epidemic, resulting in more than 21,000 infections, of which 174 were cases of hemorrhagic fever and four led to fatalities [23]. Subsequently, *Ae. aegypti* mosquitoes collected from the field in 2014 and 2015 were found to be infected with DENV-2 and DENV-4, despite the absence of any diagnosed human dengue cases during that period, suggesting a silent circulation of DENV in Cape Verde [24].

More recently, cases of dengue have been reported across all municipalities of Cape Verde, with approximately 13,827 confirmed cases and five fatalities recorded between November 2023 and November 2024. These cases were attributed to DENV-1 and DENV-3, with the highest incidence occurring on the islands of Santiago and Fogo [25].

An additional arboviral disease of considerable relevance to Cape Verde is Zika. From 2015 to 2016, the country experienced a Zika outbreak, reporting 7,580 suspected cases and 18 occurrences of microcephaly [26]. Notably, it represented the first documented occurrence of ZIKV-associated microcephaly cases in Africa [27].

Genomic analysis indicated that Cape Verdean ZIKV isolates belonged to the Asian lineage, presumably originating from northeastern Brazil and introduced before the outbreak between June 2014 and August 2015 [26]. These findings underscore the need for rigorous arbovirus surveillance in both mosquito vectors and human populations in Cape Verde, as well as improvements in travel safety protocols for highly endemic regions. Given Cape Verde's strategic position as a trans-Atlantic route linking West African countries with Europe and the Americas, these measures are essential for safeguarding public health.

Anopheles arabiensis is a primary malaria vector and its presence in Cape Verde carries substantial implications for public health in the country. Genetic analyses revealed that *An. arabiensis* is the sole representative of the *Anopheles gambiae* complex in Cape Verde [19,20]. Despite its widespread distribution throughout the archipelago, its population density remains notably low and most breeding sites are transient, mainly comprising small water collections that arise from water storage practices by the communities [28].

Malaria has been reported in Cape Verde since the 16th century and until the 1940s, it posed a serious public health issue, causing over 200 fatalities annually. However,

following the implementation of entomological surveillance and control measures, the incidence significantly declined, with seasonal outbreaks subsequently being reported only on the islands of Santiago and Boavista, typically peaking during the rainy season [29].

The last malaria outbreak occurred in 2017, when approximately 423 autochthonous cases were reported in the country [29]. On January 12, 2024, the World Health Organization certified Cape Verde as a malaria-free country after six consecutive years without local cases, highlighting the effectiveness of its malaria control program [30]. Nevertheless, the persistence of *An. arabiensis* in the archipelago and the substantial movement of individuals between Cape Verde and endemic African countries heighten the risk of new malaria cases reemerging. In this context, it is essential to uphold strong surveillance measures to avert the re-establishment of transmission following the importation of malaria cases.

Mosquitoes belonging to the *Cx. pipiens* complex are found extensively across the Cape Verde archipelago, inhabiting all populated islands, with their initial documentation dating back to the period between the 1950s and 1980s [21]. Although no current records indicate pathogen transmission by these species, historical evidence points to past local transmission of WNV [31]. Moreover, the occurrence of hybridization between *Cx. quinquefasciatus* and *Cx. pipiens s.s.* in four islands of the archipelago (Maio, Fogo, Santo Antão, and São Nicolau) promotes an opportunistic feeding behavior, thereby increasing the risk of pathogen transmission in the country [19,32].

After the outbreaks of DENV and ZIKV in Cape Verde, vector control measures were strengthened, incorporating public education initiatives along with the application of insecticides (temephos for larvae and indoor residual spraying of deltamethrin for adult mosquitoes), adulterated diesel and mosquito fish (*Gambusia sp.*) [33]. Coincidently, recent research has indicated a growing resistance to insecticides in *Ae. aegypti*, *Cx. pipiens sensu lato* (*s.l.*) and *An. arabiensis* [34–36], suggesting that chemical treatment may not be effective in the future. This emerging challenge underscores the urgent need for Cape Verde to embrace more innovative and effective strategies for vector control, ensuring sustainable public health outcomes in the future.

1.3 Control of mosquito-borne diseases

Vector control remains the most effective strategy for preventing vector-borne diseases. Since its origins in the late 19th century, it has undergone a remarkable evolution from basic environmental management practices to the adoption of advanced genetic and biological approaches. However, despite some notable successes, progress has often been hindered by several challenges, such as the absence of widely accessible and effective vaccines, the growing problem of insecticide resistance and delays in the development of antiviral treatments for most arboviruses.

Before the advent of chemical insecticides (late 1800s to early 1900s), vector control relied heavily on environmental management, which involved practices such as draining wetlands, removing water collections, introducing natural predators, and applying oil to standing water [37]. Later, in the mid-20th century, the discovery of DDT's insecticidal properties marked the beginning of a new chapter in vector control, leading to its extensive use in malaria eradication efforts.

However, resistance to DDT emerged a decade after its introduction, accompanied by growing social concerns about the harmful effects of the insecticide on both the environment and human health. DDT faced bans in the USA, Japan, and Western Europe, leading to a decline in its popularity. It was ultimately classified as a priority pollutant under the 2001 Stockholm Convention [38,39]. Nonetheless, its production and application are still allowed for vector control in regions of Africa and Southeast Asia, especially where locally safe, effective and affordable alternatives are unavailable [40].

After DDT, other insecticides have emerged, including organophosphates, carbamates, and pyrethroids. However, over time, their extensive use has raised alarms over the growing spread of resistance genes, particularly in vectors of malaria and dengue. The primary mechanisms driving the development of insecticide resistance included: i) enhanced metabolic detoxification of insecticides resulting from gene overexpression, as well as structural mutations in P450, esterase and GST genes, and ii) reduced sensitivity of target-site proteins such as acetylcholinesterases (AChEs), sodium channels and GABA receptors, due to structural changes or point mutations [41].

In response to the limitations associated with chemical control measures, the World Health Organization (WHO) promoted the implementation of Integrated Vector Management (IVM), which was formalized in the early 2000s with the publication of the "Global Strategic Framework for Integrated Vector Management" [42]. IVM promotes community empowerment, cross-sector collaboration and efficient resource use by integrating chemical and non-chemical vector control methods with other health measures. It emphasizes evidence-based decision-making, supported by research and surveillance, and invests in human resource development through training and career opportunities [43].

Despite these efforts, current vector control still predominantly depends on chemical methods, such as larvicides, insecticide-treated bed nets, indoor residual spraying, fogging, and space spraying. According to the World Health Organization, the total annual global use of insecticides for vector control during the period from 2010 to 2019 included 3,314 metric tons of organochlorines (DDT), 1,625 metric tons of organophosphates, 677 metric tons of carbamates and 194 metric tons of pyrethroids [44]. In parallel, non-chemical approaches were also employed, including the use of microbial larvicides, the introduction of predatory species, and habitat modification [37].

Although vaccines for mosquito-borne diseases such as dengue, malaria, Japanese encephalitis, and yellow fever have been developed and are now available, the effective management of outbreaks of such emerging diseases remains a considerable challenge. This is mainly attributable to the absence of highly effective vaccines, as well as the restricted access in low-income regions, which are disproportionately affected by these illnesses [45]. Moreover, in the case of the dengue vaccine, its recommendation is limited exclusively to regions with high transmission rates, primarily due to concerns regarding the increased risk of severe illness in individuals without prior exposure to the virus [46].

In this context, innovative vector control strategies have recently emerged, embracing a range of cutting-edge methods, including gene editing and gene drive technology, irradiation or sterilizing chemicals and the manipulation of the mosquito endosymbiont *Wolbachia* [39]. These novel approaches have shown promising results, inspiring significant hope for their potential in the control of vector-borne diseases.

To date, three main gene editing tools have been developed and employed in mosquitoes: zinc-finger nuclease (ZFN) technology, transcription activator-like effector nuclease (TALEN) technology, and clustered regulatory interspaced short palindromic repeats (CRISPR) technology. Both ZFN and TALEN recognize target DNA sites, utilizing the Fok-I endonuclease to cleave double-stranded DNA, thus inducing specific genetic mutations in mosquitoes. The CRISPR-Cas9 system employs a single-guide RNA (sgRNA) to target specific DNA sequences, followed by the cleavage of the double strand by the Cas9 endonuclease [47]. CRISPR-Cas9 stands out for its superior efficiency, convenience, and cost-effectiveness, allowing for random deletions and insertions or precise incorporation of gene sequences, making it an invaluable innovation in the field of mosquito genetic engineering [48].

Gene drive represents an advanced gene-editing technology that uses homing endonuclease genes or CRISPR-Cas9 to ensure that specific genes are transmitted to nearly all offspring, thereby circumventing the conventional Mendelian principles of inheritance and allowing the propagation of advantageous traits throughout interbreeding mosquito populations [49,50].

Gene-editing initiatives have been employed to develop constructs with multiple potential targets, with recent research efforts primarily focused on *Anopheles gambiae* and *Ae. aegypti*. Research conducted on these two species has successfully induced mutations in specific genes, leading to a range of impactful outcomes: i) resistance to *Plasmodium* and arboviruses; ii) sterilization or reproductive impairments (used in Sterile Insect Technique-SIT); iii) reduction in hatching and pupation rates; iv) decreased adult activity levels; and v) modifications in insecticide resistance [47].

Beyond gene editing, the Sterile Insect Technique (SIT) employs radiation or sterilizing chemicals, leading to chromosomal disruption or the induction of dominant lethal mutations in exposed male mosquitoes. Once sterilized, these males are released into the natural environment, where, upon mating with wild females, they are unable to produce viable offspring, thereby gradually reducing the population [51]. The Sterile Insect Technique (SIT) has shown great potential in lowering vector populations, with ongoing trials worldwide primarily focusing on *Ae. aegypti*, *Ae. albopictus* and *An. arabiensis* [52].

Among the innovative strategies, the application of *Wolbachia* stands out as one of the most effective tools for controlling mosquito-borne diseases. This endosymbiotic bacterium may play a dual role: i) suppress mosquito populations through cytoplasmic incompatibility; and ii) enhance mosquito vector resistance against specific pathogens, thereby significantly decreasing their vector competence [53]. Vector control programs employing *Wolbachia* are currently undergoing extensive field trials, with their efficacy already demonstrated on a global scale [54,55]. These encouraging results have elevated hopes for the transformative potential of this endosymbiont in combating mosquito-borne diseases.

1.4 *Wolbachia* as a strategy for controlling mosquito-borne diseases

1.4.1 The endosymbiont Wolbachia

Wolbachia pipientis (Alphaproteobacteria, Rickettsiales) is a gram-negative bacterium, obligate intracellular and proteobacterial symbiont that infects a wide range of invertebrates, including insects, arachnids, crustaceans, spiders, scorpions, collembolans and nematodes [56,57].

The endosymbiont was first discovered in *Cx. pipiens* in 1924 by Hertig and Wolbach and, therefore, designated as *Wolbachia pipientis* [53]. *Wolbachia* primarily spreads through vertical transmission, passing across generations via the female oocytes. Beyond this main route, the bacterium can also transfer between different hosts through a less frequent process called host shift or horizontal transmission [58]. In its host cells, *Wolbachia* is closely associated with the endoplasmic reticulum and cytoskeletal components, including dynein, kinesin, microtubules, and actin. The bacterium exhibits a series of distinct morphologies, ranging from spheroidal, bacillary and intermediate forms, which may represent different developmental stages [59].

In arthropods, *Wolbachia* is primarily found in reproductive tissues and is known to induce several reproductive modifications, including feminization, parthenogenesis, male-killing, and cytoplasmic incompatibility (CI) [60,61]. Beyond these reproductive manipulations, *Wolbachia* also contributes to its hosts by providing nutritional benefits and enhancing resistance to pathogens. This protective capability, in particular, highlights *Wolbachia* as a promising tool for controlling mosquito-borne diseases [62].

Over the past decade, *Wolbachia* has garnered significant attention, primarily due to its extensive prevalence and potential for controlling vector-borne diseases. A pivotal historical milestone that marked the beginning of *Wolbachia´*s use in tackling mosquito-borne pathogen transmission was the discovery of its ability to inhibit viral replication in *Drosophila melanogaster* [63,64]. This finding prompted experimental embryonic microinjections of *Wolbachia* into major mosquito vectors to assess its potential to inhibit arbovirus transmission.

The ability of *Wolbachia* to inhibit pathogens such as the ZIKV, DENV, WNV, and CHIKV in transinfected *Ae. aegypti* has been validated in laboratory experiments [65–67]. In the field, *Wolbachia*-based vector control initiatives have led to significant reductions in dengue cases in several countries worldwide, demonstrating the efficacy of this approach in controlling arbovirus transmission [68,69].

1.4.2 Wolbachia Genetic diversity

The *Wolbachia* genome has been the focus of extensive research due to its unique genetic characteristics. The endosymbiont harbors a small genome, ranging between 1.08 to 1.7 Mb, notable for its abundance of repetitive elements and prophage WO sequences (segments of the *Wolbachia* genome that contains the genetic material of the bacteriophage WO), which are actively transcribed and expressed through a lytic cycle [61]. The *Wolbachia* genome also has a high level of recombination across various strains, impacting the *Wolbachia* surface protein (*wsp*) gene, housekeeping genes, prophage genes, and intergenic regions. Such extensive recombination contributes to the remarkable genetic diversity observed within the genus and supports the hypothesis of a chimeric origin for *Wolbachia* strains [70,71].

The genetic diversity of *Wolbachia* was initially explored using single-gene markers, such as 16S rRNA and the *wsp* gene [72,73]. However, this traditional phylogenetic approach faced significant challenges in capturing the full extent of *Wolbachia*'s phylogenetic diversity. The 16S rRNA gene evolves at a notably slow rate, limiting its ability to resolve differences among strains, while the *wsp* gene is subject to extensive recombination between supergroups, complicating the differentiation of *Wolbachia* strains and obscuring their evolutionary relationships [74].

Currently, the genetic diversity of *Wolbachia* is most effectively assessed through Multilocus Sequence Typing (MLST), which utilizes a set of conserved housekeeping genes, including *gatB*, *coxA*, *hcpA*, *fbpA,* and *ftsZ*. In MLST analysis, each *Wolbachia* strain is defined by the unique allelic profile of these five genes, which collectively determine its sequence type (ST) [75]. These particular genes were chosen for their consistent presence across sequenced genomes and their relative stability under strong selective pressures [76]. Additionally, the *Wolbachia* surface protein (Wsp), which is segmented into four hypervariable regions (HVRs), is used as an additional marker for assessing genetic variation in *Wolbachia* MLST analyses and a dedicated database has been established to facilitate the classification of alleles and specific HVR peptides [61]. As a result, MLST offers a more precise and comprehensive understanding of *Wolbachia* diversity and phylogeny.

MLST analyses have revealed that *Wolbachia* possesses remarkable genetic diversity, reflected in its classification into numerous supergroups. Although considered a single species, *Wolbachia* is subdivided into 19 distinct supergroups, labeled from A to U [58]. Among arthropods, the most commonly observed supergroups are A, B, E, H, I and K, while in nematodes, *Wolbachia* is primarily found within Supergroups C, D and J. Notably, Supergroup L is unique to plant-parasitic nematodes, whereas Supergroup F stands apart as the only group detected in both arthropods and nematodes, illustrating the complex and diverse nature of this endosymbiont's evolutionary relationships [57].

Each *Wolbachia* supergroup encompasses a variety of distinct strains, with each strain typically named after its host species (e.g., *w*Alb in *Ae. albopictus* and *w*Pip in *Cx. pipiens*). In certain cases, additional details, such as the collection site or specific phylogenetic classification, are included in the strain's name to reflect genetic variation within a previously identified strain [77]. For instance, *w*AlbA and *w*AlbB are both present in the host *Ae. albopictus*, yet they belong to different supergroups, with *w*AlbA being classified under Supergroup A and *w*AlbB under Supergroup B.

Most *Wolbachia* strains characterized to date belong to Supergroups A and B, which are exclusively found in arthropods. Across these two supergroups, 44 distinct *Wolbachia* strains have been identified, with 36% classified under Supergroup A and 45% under Supergroup B [58,78].

In arthropods, the observed phylogenetic discrepancies between *Wolbachia* strains and their hosts imply that the endosymbiont is capable of both vertical and horizontal transmission. Through vertical transmission, *Wolbachia* is integrated into the host's eggs, persists throughout embryonic development and ultimately becomes embedded in the germ-line stem cell precursors [79]. In horizontal transmission, *Wolbachia* spreads between hosts via close tissue-level interactions, such as predator-prey dynamics and host-parasitoid relationships [80].

Unlike in arthropods, the phylogenetic congruence between nematode hosts and their associated *Wolbachia* strains suggests a strictly vertical mode of transmission, passed exclusively through infected female hosts. This relationship appears to be the outcome of an extended period of co-evolution, resulting in an obligate symbiosis [57]. In adult filarial nematodes, the loss of *Wolbachia* leads to infertility and, eventually, the death of the host, underscoring the essential nature of this relationship. Recognition of this obligate association has paved the way for innovative antibiotic-based strategies designed to target *Wolbachia*, thereby providing a promising approach to eradicating adult filarial nematodes [59].

1.4.3 Wolbachia-induced reproductive manipulations and role on vector competence

Wolbachia-induced reproductive manipulations include male killing, feminization, parthenogenesis, and cytoplasmic incompatibility (CI). These manipulations grant the infected females a distinct reproductive advantage, promoting the spread of *Wolbachia* into previously uninfected populations [81].

Cytoplasmic incompatibility (CI) is the most extensively studied *Wolbachia*-induced phenotype in arthropods, especially in mosquitoes. This biological process is characterized by embryonic death in crosses between *Wolbachia*-infected males and uninfected females or between individuals harboring incompatible *Wolbachia* strains [51] (Figure 1).

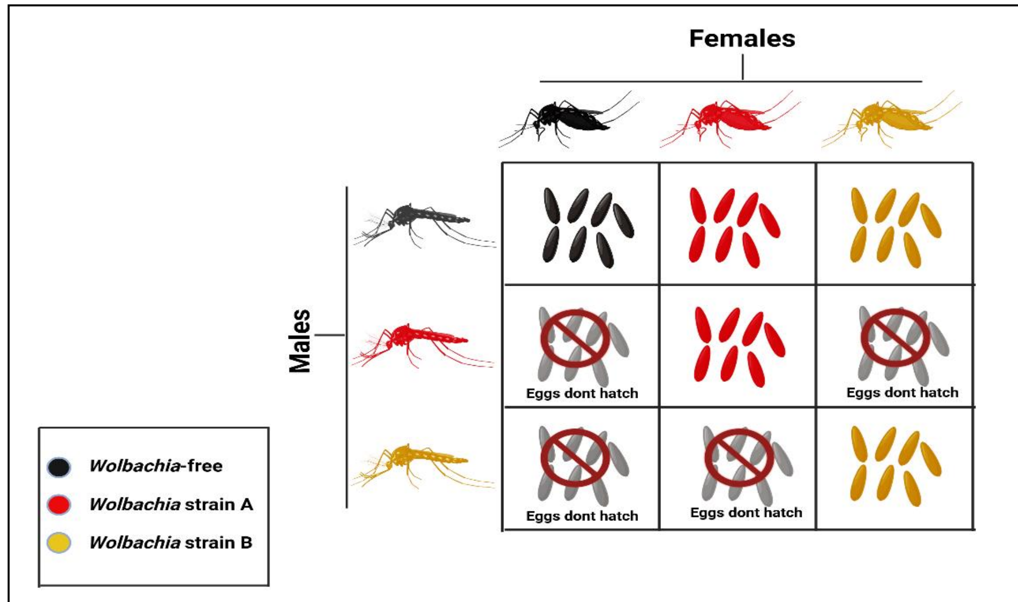

Figure 1- Illustration of cytoplasmic incompatibility (CI) in mosquitoes due to *Wolbachia* infection. Unidirectional CI occurs in crosses between *Wolbachia*-infected males and uninfected females. Bidirectional CI arises from crosses between males and females infected with incompatible *Wolbachia* strains. *Wolbachia*-uninfected adults and eggs are depicted in black, while those harboring incompatible *Wolbachia* strains A and B are shown in red and yellow, respectively. This figure was created with BioRender.com.

Cytoplasmic incompatibility is classified as unidirectional when embryonic lethality arises from crosses between *Wolbachia*-infected males and uninfected females and as bidirectional when these males mate with females carrying incompatible *Wolbachia* strains [82] (Figure 1). In specific scenarios, known as asymmetric unidirectional CI, females infected with one particular *Wolbachia* strain can rescue the offspring of males infected with a different strain, yet this ability is not reciprocal, giving rise to the term "asymmetric" [83].

While CI has been linked to the presence of *Wolbachia* for several decades [84], only in recent years researchers have begun to unravel the molecular mechanisms underlying this phenomenon. Cytoplasmic incompatibility is commonly described as a "modification-rescue" system in which paternal *Wolbachia* introduces "modification" factors that lead to embryonic abnormalities unless corresponding "rescue" factors (or compatible "rescue" factors) are provided by maternal *Wolbachia* [85].

The genes primarily responsible for CI are *cidA* and *cidB*, both situated within the prophage WO region. The *cidA* gene encodes a protein that serves as a "rescue" factor, while *cidB* encodes a protein that functions as a toxin. The toxin produced by *cidB* induces

chromosomal abnormalities in the male host's gametes, impairing successful embryonic development unless the corresponding rescue protein, present in *Wolbachia*-infected females, is available to counteract the effects [86,87].

Two general classes of models explain the interaction between *cidA* and *cidB*: the toxin-antitoxin model and the host-modification model. In the toxin-antitoxin model, the rescue mechanism is based on the direct binding of *cidA* to *cidB* within the embryo, effectively neutralizing the toxic effects of *cidB*. In contrast, the host-modification model suggests that there is no direct interaction between *cidA* and *cidB*. Instead, *cidB* induces a harmful modification to a factor derived from the sperm (host), while cidA provides a compensatory modification, either directly or indirectly, to mitigate the damage [59,88].

Cytoplasmic incompatibility gives *Wolbachia*-infected females a significant reproductive advantage, allowing them to mate successfully with both infected and uninfected males. This capability enhances the spread of *Wolbachia*-infected individuals across generations, making it a crucial mechanism for introducing *Wolbachia* into naïve mosquito populations in vector control programs [89]. The identification of *Wolbachia* genes involved in CI, along with their associated rescue mechanisms, holds the potential to inspire innovative strategies for controlling vector populations.

Wolbachia can also trigger a fascinating reproductive phenotype known as parthenogenesis induction (PI), where female hosts reproduce asexually, giving rise exclusively to female offspring. This phenomenon often leads to populations composed entirely of females and is most commonly observed in species of the order Hymenoptera, particularly parasitoid wasps [90]. By ensuring that all offspring develop into reproductively capable females, this form of reproductive manipulation maximizes the transmission of the endosymbiont [91].

Feminization is another phenotypic trait induced by *Wolbachia*, in which genetic males are transformed to develop as females. The mechanism behind feminization in insects remains elusive. In isopods, it has been linked to *Wolbachia*-induced hypertrophy of the androgenic gland, leading to its functional inhibition [61]. Male-killing is yet another intriguing way in which *Wolbachia* influences the biology and ecology of its hosts. This phenotype, observed across several orders of the phylum Arthropoda (including Diptera), is characterized by the death of infected male embryos [92]. Both feminization and male-

killing provide *Wolbachia*-infected females with a reproductive advantage, enabling them to produce more female offspring that carry the bacterium. This selective benefit allows *Wolbachia* to spread more effectively throughout host populations.

Another *Wolbachia*-induced phenotype of importance for vector control is the endosymbiont's ability to reduce vector competence [93]. Vector competence refers to a vector's intrinsic ability to transmit infectious agents. This ability is influenced by physical, immunological, cellular, and microbial barriers within the vector's body, which are mainly shaped by its genetic background [94].

When an arbovirus is acquired through a blood meal, it first invades the midgut, establishing an initial site of infection. From there, the virus spreads beyond the midgut, disseminating to other tissues and undergoing secondary amplification. Eventually, the virus infects the salivary glands and is released into the salivary ducts, enabling transmission to an uninfected vertebrate host [95]. Therefore, for an infectious agent to reach the vector's saliva, it must first overcome several internal barriers within the mosquito, including the midgut infection barrier (MIB), the midgut escape barrier (MEB), the salivary gland infection barrier (SGIB) and the salivary gland escape barrier (SGEB) [96] (Figure 2).

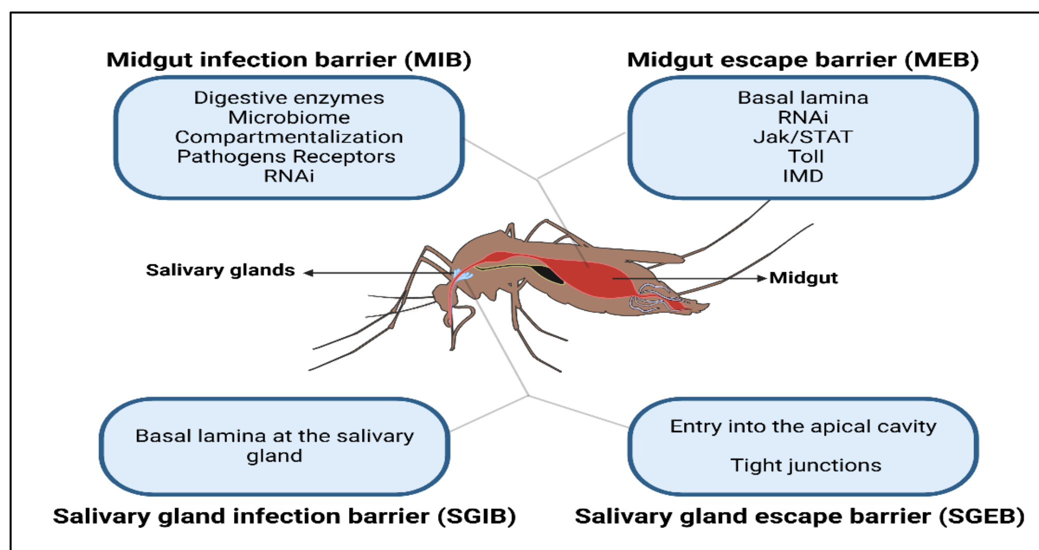

Figure 2- Barriers to arbovirus infection in mosquitoes and their components. The midgut infection barrier (MIB) prevents the establishment or replication of the pathogen in the mosquito's midgut. The midgut escape barrier (MEB) inhibits the dissemination of the agent, even if it is already established in the midgut. The salivary gland infection barrier (SGIB) prevents the pathogen from infecting the salivary gland, while the salivary gland escape barrier (SGEB) blocks its passage to the saliva of the vector. This figure was created with BioRender.com.

The midgut infection barrier (MIB) consists of several components that prevent pathogen establishment, including digestive enzymes that deactivate the virus, the compartmentalization of the virus by the peritrophic membrane, the absence of viral receptors on the intestinal epithelium and the influence of the mosquito's microbiome [93]. Additionally, the RNA interference (RNAi) pathway has been identified as a defense mechanism in the gut, as suggested by studies on *Ae. aegypti* experimentally infected with DENV [97]. The midgut escape barrier (MEB) acts as a defense mechanism, preventing pathogens replicating within the midgut from spreading to other tissues. This barrier includes various components, such as the dense, fibrous matrix of the basal lamina beneath the midgut epithelium, as well as the activation of genes involved in multiple immune pathways, including RNAi, Janus kinase–signal transducer and activator of transcription (Jak/STAT), Toll and immune deficiency (IMD) [98] (Figure 2).

The salivary gland infection barrier (SGIB) prevents the salivary glands from becoming infected, even in mosquitoes exhibiting a disseminated infection. On the other hand, the salivary gland escape barrier (SGEB) restricts pathogens from entering the saliva, even when the salivary glands themselves are already infected [96]. Although the exact components of SGIB are not yet fully understood, it is believed that the basal lamina surrounding the salivary glands plays a critical role in blocking infection [99]. Potential salivary gland escape barriers include the pathogen's ability to enter the apical cavity of the gland and the tight junctions between acinar cells, which may limit the pathogen's access to saliva [96] (Figure 2).

Wolbachia colonization in mosquitoes has a significant impact on vector competence. In transinfected mosquito strains, the extent to which DENV, ZIKV, and CHIKV are inhibited is closely linked to the density of the endosymbiont in somatic tissues, including the midgut and salivary glands [100]. Despite this relationship, the precise mechanisms by which *Wolbachia* suppresses viral replication are still not fully understood. Two potential mechanisms have been proposed. The first suggests that *Wolbachia* competes directly with the virus for essential cellular resources and the second proposes that *Wolbachia* stimulates the host's immune system (known as immune priming), thereby enhancing its ability to inhibit the pathogen [101].

Regarding resource availability, competition for cholesterol between *Wolbachia* and the virus seems to be a key limiting factor for viral replication. Experimental studies have demonstrated that cholesterol availability is crucial for *Wolbachia*'s ability to inhibit the replication of ZIKV and DENV in *Ae. albopictus* and *Ae. aegypti* cells [102,103]. These studies suggest that *Wolbachia* sequesters cholesterol within host cells, disrupting the conditions necessary for viral replication and spread.

The second potential mechanism by which *Wolbachia* influences vector competence is through stimulation of the vector's immune response. It is believed that the presence of *Wolbachia* enhances the mosquito's immune defenses, making it more resistant to viral infection [104]. The antiviral immune response in mosquitoes involves several signaling pathways, including JAK/STAT, Toll, IMD) and RNAi pathways [105]. Research has demonstrated that *Wolbachia* can upregulate immune genes within the host, particularly those involved in the Toll, IMD, and JAK/STAT pathways, thereby strengthening the mosquito's ability to combat viral pathogens [106–108].

Wolbachia seems to be less effective at activating the immune system in mosquito species where it naturally resides, likely due to the long co-evolutionary relationship between the endosymbiont and its host [109–111]. However, recent research indicates that native *Wolbachia* strains can still interfere with RNA virus replication in mosquitoes [112–114], though the precise mechanism by which the endosymbiont exerts this effect in its natural hosts remains unclear.

1.4.4 Natural Wolbachia infection in major mosquito vectors

Wolbachia does not occur naturally in all mosquito species. This endosymbiont is commonly found in major mosquito vectors like *Cx. pipiens* and *Ae. albopictus*, but it is rarely detected in other major vectors such as *An. gambiae s.l.* and *Ae. aegypti* [77].

1.4.4.1 Wolbachia in Culex species

Several species within the *Culex* genus harbor *Wolbachia*, including members of the *Cx. pipiens* complex, as well as in *Culex gelidus*, *Culex theileri*, *Culex restuans*, *Culex sitiens* and *Culex nigripalpus*, among others [115]. *Wolbachia* strains found in the *Cx. pipiens* complex have garnered the most attention, largely due to this group's critical role in transmitting significant pathogens, such as the WNV and parasitic nematodes responsible for lymphatic filariasis.

Mosquitoes of the *Cx. pipiens* complex host a wide variety of *Wolbachia* strains from the *w*Pip clade. Using rapidly evolving markers such as *ank2* and *pk1* genes, researchers have identified over 100 genetically distinct *w*Pip strains, which are categorized into five groups (*w*Pip-I to *w*Pip-V) [116]. Despite their diversity, all five groups share a monophyletic origin within Supergroup B, as confirmed by MLST analyses [117]. These *w*Pip strains exhibit a remarkable diversity of unidirectional and bidirectional CI patterns, where strains within the same group are generally compatible, while those from different groups tend to be incompatible [118].

The different *w*Pip groups show distinct geographical distributions. *w*Pip-I is primarily found in sub-Saharan Africa, South America, and Southeast Asia, whereas *w*Pip-III is most prevalent in North America. *w*Pip-II and *w*Pip-V are mainly confined to Western Europe and Asia, respectively, while *w*Pip-IV has a broader, sporadic presence across Europe, North Africa, and Asia [116,119]. These findings highlight a distinct pattern of the geographical distribution of *w*Pip groups across *Cx. pipiens s.l.* populations, which may reflect their adaptation to different environmental and ecological conditions.

1.4.4.2 <u>Wolbachia in Anopheles spp.</u>

For decades, *Anopheles* species were believed to be resistant to *Wolbachia* infection. However, recent research has successfully detected the endosymbiont in numerous *Anopheles* species. Baldini *et al*. [120] were the first to identify *Wolbachia* DNA sequences in a natural population of *An. gambiae*, collected in 2011 in Burkina Faso. Gene sequencing revealed that the *Wolbachia* detected in these mosquitoes represented a new strain named *w*Anga [120].

Wolbachia has also been detected in *An. gambiae* and *An. coluzzii* populations from Mali, identified as the *w*Anga-Mali strain [121]. Additionally, Jeffries *et al.* [122] also reported *Wolbachia* in *An. gambiae* from the Democratic Republic of Congo and *An. coluzzii* from Ghana.

Baldini *et al*. [123] reported the presence of the *Wolbachia* strain *w*Anga in *An. arabiensis* from the Kilombero Valley, Tanzania. In Senegal, Niang *et al.* [124] documented the first detection of *Wolbachia* in *Anopheles funestus s.s*, the second most significant malaria vector in Africa. Phylogenetic analysis revealed two distinct *Wolbachia* genotypes in this

species, designated as *w*Anfu-A and *w*Anfu-B, corresponding to their closer affiliations with Supergroups A and B, respectively [124].

As part of a more extensive study, Ayala *et al.* [125] examined the natural occurrence of *Wolbachia* in 25 *Anopheles* species collected in Gabon, discovering that 16 of these species were positive for the endosymbiont. Among the positive species were major malaria vectors in Africa, including *An. gambiae, An. coluzzii, An. funestus* and *Anopheles moucheti*.

Natural *Wolbachia* infection in *Anopheles* appears to influence *Plasmodium* development. Shaw *et al.* [126] observed an inverse association between *Wolbachia* infection and *Plasmodium* presence in blood-fed *An. coluzzii* females collected from households. Similarly, Gomes *et al.* [121] found that the prevalence and load of *P. falciparum* sporozoites were significantly lower in wild-caught *An. coluzzii* females infected with *Wolbachia*. Moreover, experimental infections conducted in the same study confirmed that *Wolbachia* significantly reduced both the prevalence and load of sporozoite infection, mirroring the results observed in field studies [121].

Despite these findings, some researchers still question the natural occurrence of *Wolbachia* in *Anopheles* species. Walker *et al*. [127] highlighted that most studies detecting *Wolbachia* in *An. gambiae* have relied solely on highly sensitive nested PCR, underscoring the need for more rigorous evidence to determine whether *Wolbachia* strains are truly established as endosymbionts in this species. Sawadogo *et al.* [128] re-evaluated *Wolbachia* prevalence in *An. gambiae* populations from Burkina Faso, where a high prevalence had previously been reported and found it to be only 0.54% (29 out of 5341 individuals). According to the authors, such a low prevalence does not eliminate the possibility that the positive results from preceding studies could be attributed to environmental contamination or false positives [128].

1.4.4.3 <u>Wolbachia in Aedes species</u>

The *Aedes* genus includes some of the most globally widespread mosquito species, with *Ae. aegypti* and *Ae. albopictus* standing out as the most significant vectors. These species are responsible for transmitting arboviruses such as DENV, ZIKV, CHIKV, and Yellow Fever virus (YFV), all of which pose serious threats to human health [129].

Wolbachia is a common endosymbiont in *Ae. albopictus*. These mosquitoes are naturally infected with two distinct *Wolbachia* strains, *w*AlbA and *w*AlbB, belonging to supergroups A and B, respectively [77]. In natural populations of *Ae. albopictus*, individuals can be infected with either *w*AlbA or *w*AlbB, or they may carry a superinfection involving both strains [130,131].

Regarding *Ae. aegypti*, Kulkarni *et al*. [132] reported the presence of native *Wolbachia* in populations from New Mexico and Florida, USA. In addition, Carvajal *et al*. [133] and Reyes *et al.* [134] detected *Wolbachia* in wild populations of *Ae. aegypti* in Manila and the Philippines. *Wolbachia*-infected *Ae. aegypti* have also been documented in Panama, Malaysia, and India [135–137]. Despite these findings, the natural presence of *Wolbachia* in *Ae. aegypti* remains a topic of debate within the scientific community. In fact, most studies have been unable to detect the bacterium in this species, raising doubt on whether *Wolbachia* is truly established in this vector [138,139].

Given *Wolbachia*'s capacity to induce CI and reduce vector competence, efforts have been made over the years to use the endosymbiont as a means to inhibit arbovirus transmission by *Ae. aegypti.* The development of techniques for introducing *Wolbachia* strains into mosquitoes via embryonic microinjection [140,141] has enabled the production of *Ae. aegypti* laboratory lines harboring *Wolbachia* strains capable of resisting infection by several viruses, including DENV, ZIKV, and CHIKV [142]. Currently, many of these *Wolbachia*-transinfected lines are being released into the field as a promising strategy to prevent arbovirus transmission by *Ae. aegypti*.

1.4.5 The Application of Wolbachia as a Vector Control Tool

The effects of *Wolbachia* on its hosts, particularly its ability to inhibit pathogens and induce CI, have been leveraged to develop innovative *Wolbachia*-based methods for controlling vector-borne diseases. Currently, vector control strategies using *Wolbachia* fall into two main strategies (Figure 3): 1- suppression of vector populations through CI (Incompatible Insect Technique - IIT) and 2- modification of vector populations to make them resistant to pathogens [77].

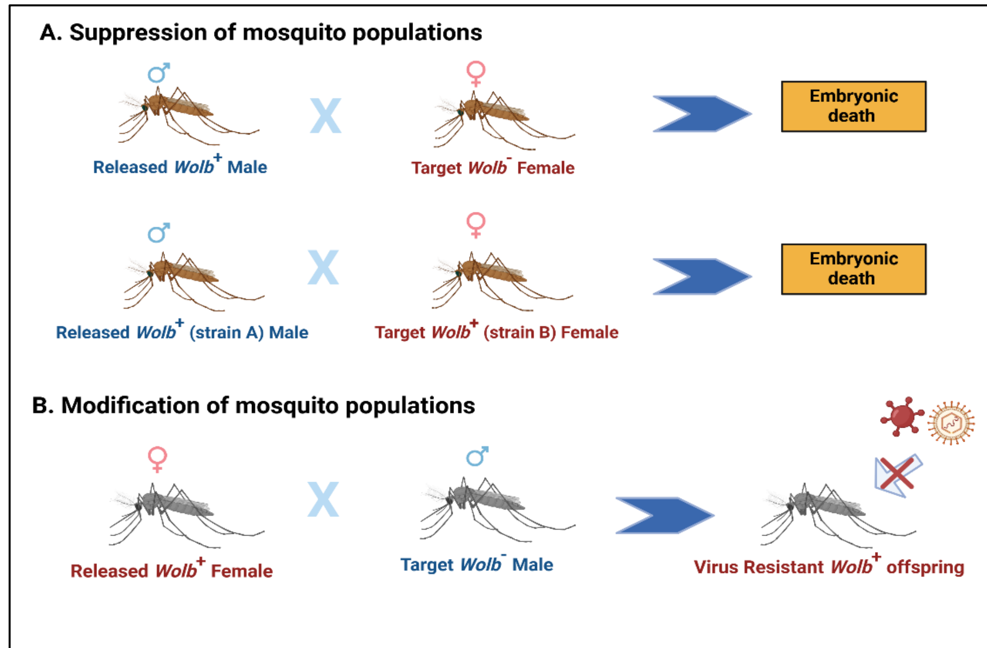

Figure 3- Vector control initiatives using *Wolbachia*. A. Suppression of the mosquito population by the Incompatible Insect Technique (IIT): male mosquitoes harboring *Wolbachia* are released in the wild and when mating with uninfected females or females carrying an incompatible strain of *Wolbachia*, the resulting embryo dies. B. Modification of mosquito population: female mosquitoes infected with *Wolbachia* strains that confer protection against pathogens are released into the wild and, after mating with local males, their offspring inherit the protective endosymbiont. This figure was created with BioRender.com.

In the Incompatible Insect Technique (IIT), male mosquitoes carrying a *Wolbachia* strain that is absent from the target population are released into the wild. When these males mate with females that are either uninfected or carry a different *Wolbachia* strain, the resulting offspring do not survive, effectively reducing the mosquito population [51].

Wolbachia-based IIT was first successfully implemented in 1967 to control *Cx. quinquefasciatus* in Burma [143]. More recently, mass releases of *Wolbachia*-infected *Ae. aegypti* males have led to significant reductions in mosquito populations across various trial locations, including in Australia [144], Singapore [145], Mexico [146], Thailand [65], and China [147].

The IIT approach has also been used to manage *Ae. albopictus* populations. In 2014, male *Ae. albopictus* mosquitoes transinfected with *Wolbachia* from *Cx. pipiens* (*w*Pip) were released in Lexington, USA. These males were unable to produce viable offspring when mating with wild females carrying *w*AlbA and *w*AlbB, resulting in a decline in the *Ae. albopictus* population [148]. More recently, this same method was applied in Rome, Italy, to control the local *Ae. albopictus* population [149]. Results from field trials assessing the

effectiveness of *w*Pip-harboring *Ae. albopictus* males in sterilizing wild females have been promising, suggesting that this approach could be widely adopted for controlling this species.

Unlike other sterilization techniques, such as radiation or genetic modification, the *Wolbachia*-based Incompatible Insect Technique (IIT) avoids the fitness costs associated with those methods and it bypasses the complexities of regulatory requirements [150]. However, a key limitation of IIT lies in the challenge of separating male and female mosquitoes before release, which raises the risk of accidentally releasing fertile females and potentially altering the local population. To mitigate this issue, new strategies are being explored, including the use of low-dose radiation of adult mosquitoes in conjunction with the IIT and the application of artificial intelligence for more efficient automated mosquito sexing [147].

The second *Wolbachia*-based control strategy aims to modify mosquito populations to make them resistant to specific pathogens, thereby reducing their vector competence. In this approach, female mosquitoes are artificially infected with *Wolbachia* strains that confer protection against viruses and are subsequently released into the wild. When these females mate with local males, their offspring inherit the protective endosymbiont. Moreover, CI gives *Wolbachia*-infected females a reproductive advantage, promoting the spread and establishment of Wolbachia within the population [53].

Stable symbiotic associations between *Ae. aegypti* and *Wolbachia* have been successfully established through embryonic microinjection. Currently, the *Wolbachia* strains that are used for *Ae. aegypti* transinfection include *w*AlbB (originating from *Ae. albopictus*), *w*MelPop, and *w*Mel (both derived from *D. melanogaster*) [151]. *Aedes aegypti* lines carrying these *Wolbachia* strains exhibit a reduced ability to transmit arboviruses and are now being tested in field trials as a strategy to control arboviral diseases [142].

Currently, most field trials employ *Ae. aegypti* infected with the *w*Mel strain to control DENV transmission [152]. However, comparative studies have demonstrated that *w*AlbB is equally effective in suppressing DENV in *Ae. aegypti* [153]. Moreover, *w*AlbB imposes lower fitness costs on the vector and shows greater resilience to cyclic thermal stress compared to *w*Mel [154]. In contrast, the *w*MelPop strain significantly reduces the fitness of *Ae. aegypti*, leading to decreased longevity and fecundity. Field trials conducted in

Australia and Vietnam with the *w*MelPop strain failed to replace local *Ae. aegypti* populations, largely due to the severe fitness costs associated with this strain [151].

The first large-scale release of *Ae. aegypti* mosquitoes transinfected with the *w*Mel strain took place in 2011 in northern Australia as part of the Dengue Elimination Program [55]. Since then, similar releases have been carried out in numerous other countries, including Brazil, Colombia, Indonesia, and Malaysia [51]. These trials have shown that *Aedes aegypti* populations carrying *Wolbachia* have successfully established in many test areas, leading to a notable reduction in dengue incidence in both Australia and Malaysia [68,69]. Utarini *et al.* [155] further reported that the introduction of *Ae. aegypti* mosquitoes infected with the *w*Mel strain significantly reduced the incidence of symptomatic dengue and decreased dengue-related hospitalizations in Yogyakarta, Indonesia.

In Australia, *Ae. aegypti* has successfully retained the *w*Mel strain years after its initial introduction, consistently demonstrating reduced rates of DENV transmission compared to their *Wolbachia*-free wild-type counterparts [156]. Furthermore, the mosquito population has also sustained its fertility fitness and genome analysis has identified only minor genomic mutations over seven years [55].

In Malaysia, field trials with *Ae. aegypti* infected with the *w*AlbB strain resulted in an average reduction of 62.4% in dengue cases. The reduction in dengue incidence was closely linked to the endosymbiont prevalence [54].

The *Wolbachia*-based population replacement approach is also being assessed for *Ae. albopictus*. Blagrove *et al.* [157] demonstrated that the *w*Mel strain induces strong CI in *Ae. albopictus* that have been cured of their native *Wolbachia*. Additionally, the same strain effectively inhibited DENV and CHIKV transmission under laboratory conditions [157,158].

Recently, *Ae. albopictus* mosquitoes carrying a triple *Wolbachia* infection (*w*AlbA, *w*AlbB, and *w*Au, the latter originating from *Drosophila simulans*) were evaluated under laboratory conditions. This mosquito line showed complete resistance to ZIKV and DENV infections, demonstrating significant potential for future use in *Wolbachia*-based population replacement strategies [159]. Despite these encouraging results, the

application of *Wolbachia* strains for controlling arboviruses transmitted by *Ae. albopictus* has not yet been evaluated in field trials.

To date, no field trials have been conducted to control *Anopheles* species and malaria transmission. However, a few studies involving transinfected *Anopheles* species have yielded promising results. Bian *et al.* [160] established the first stable line of *Anopheles stephensi* (named LB1 line) infected with the *w*AlbB strain, achieving successful maternal transmission and CI. Furthermore, the *An. stephensi* LB1 line showed significant resistance to *P. falciparum* compared to their wild-type counterparts [160]. Joshi *et al.* [161] also revealed that the LB1 line is resistant to *Plasmodium berghei*, underscoring the potential of this transinfected mosquito line as a sustainable tool in vector control programs targeting *An. stephensi.*

1.5 Research Questions

What is the prevalence and genetic diversity of *Wolbachia* among different mosquito species (Diptera: Culicidae) from Cape Verde and how does the endosymbiont affect the vector competence of its mosquito hosts?

1.6 Objectives

The purpose of this study is to explore the main research questions through the following general and specific objectives:

- 1.6.1 To detect *Wolbachia* and characterize its genetic diversity in the mosquito fauna of Cape Verde;

- To estimate the prevalence of *Wolbachia* in different mosquito species;

- To genetically characterize *Wolbachia* strains in each species and assess their distribution across the archipelago of Cape Verde.

- 1.6.2 To assess the influence of naturally occurring *Wolbachia* from Cape Verde on mosquito vector competence.

- To perform artificial infections with WNV in both *Wolbachia*-infected and *Wolbachia*-uninfected *Cx. quinquefasciatus* mosquitoes;

- To conduct comparative analyses of infection, dissemination, and transmission rates between *Wolbachia*-infected and uninfected mosquitoes.

1.7 Workflow outline

The next two chapters correspond to two scientific articles that collectively explore the research questions outlined in the Introduction and Objectives sections. Together, these studies offer an in-depth exploration of natural *Wolbachia* infection in Cape Verdean mosquito populations, highlighting its potential impact on vector competence.

The first paper, entitled "Screening of natural *Wolbachia* infection in mosquitoes (Diptera: Culicidae) from the Cape Verde islands," presents a detailed examination of *Wolbachia* prevalence and genetic diversity across different mosquito species found on this archipelago. These findings provide insights on the origins of mosquito populations and the potential application of native endosymbiotic bacteria in future *Wolbachia*-based mosquito control strategies in Cape Verde.

The second paper, entitled "Vector Competence of *Culex quinquefasciatus* from Santiago Island, Cape Verde, to West Nile Virus: Exploring the Potential Effect of the Vector Native *Wolbachia*," examines the role of native *Wolbachia* in influencing the ability of *Cx. quinquefasciatus* mosquitoes to transmit WNV. Focusing specifically on populations from Santiago Island, the study employs controlled laboratory infection experiments to determine how native *Wolbachia* affects the infection, dissemination and transmission rates of WNV. These results enhance our understanding of how these native endosymbionts may influence the dynamics of WNV within their natural hosts and provide insights to leverage this protective effect for the development of novel vector control initiatives.

The final chapter of the thesis presents a comprehensive discussion that integrates the key findings of both studies. The integrated discussion offers valuable insights for vector control programs in Cape Verde, particularly by leveraging the natural presence of *Wolbachia* and its distribution within local mosquito populations. It also highlights how natural patterns of *Wolbachia* infection can shape the dynamics of WNV transmission by mosquitoes.

1.8 References

1. Chilakam N, Lakshminarayanan V, Keremutt S, Rajendran A, Thunga G, Poojari PG, *et al*. Economic burden of mosquito-borne diseases in low- and middle-income countries: Protocol for a Systematic Review. JMIR Res Protoc. 2023;12:e50985.

2. World Health Organization. Vector-borne diseases. 2024. Available from: https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases. Accessed 14 November 2024.

3. Tripathi H, Bhalerao P, Singh S, Arya H, Alotaibi BS, Rashid S, *et al*. Malaria therapeutics: are we close enough? Parasit Vectors. 2023;16:130.

4. Khan MB, Yang ZS, Lin CY, Hsu MC, Urbina AN, Assavalapsakul W, *et al*. Dengue overview: An updated systemic review. J Infect Public Health. 2023;16:1625–42.

5. Kularatne SA, Dalugama C. Dengue infection: Global importance, immunopathology and management. Clin Med. 2022; 22:9–13.

6. Ferreira QR, Lemos FFB, Moura MN, Nascimento JOS, Novaes AF, Barcelos IS, *et al*. Role of the Microbiome in *Aedes spp*. Vector Competence: What Do We Know? Viruses. 2023;15:779.

7. World Health Organization. Zika virus. 2022. Available from: https://www.who.int/news-room/fact-sheets/detail/zika-virus. Accessed 11 November 2024.

8. de Souza WM, Ribeiro GS, de Lima STS, de Jesus R, Moreira FRR, Whittaker C, *et al*. Chikungunya: a decade of burden in the Americas. Lancet Reg Heal. 2024;30:100673.

9. Bartholomeeusen K, Daniel M, LaBeaud DA, Gasque P, Peeling RW, Stephenson KE, *et al*. Chikungunya fever. Nat Rev. 2023;9:1–21.

10. Hadfield J, Brito AF, Swetnam DM, Vogels CBF, Tokarz RE, Andersen KG, *et al*. Twenty years of West Nile virus spread and evolution in the Americas visualized by Nextstrain. PLoS Pathog. 2019;15:e1008042.

11. Garrigós M, Garrido M, Panisse G, Veiga J, Martínez-de la Puente J. Interactions between West Nile Virus and the Microbiota of *Culex pipiens* Vectors: A Literature Review. Pathog. 2023;12:1287.

12. ECDC. Epidemiological update: West Nile virus transmission season in Europe, 2023. 2024. Available from: https://www.ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-virus-transmission-season-europe-2023-0. Accessed 29 October 2024.

13. Mencattelli G, Ndione MHD, Rosà R, Marini G, Diagne CT, Diagne MM, *et al*. Epidemiology of West Nile virus in Africa: An underestimated threat. PLoS Negl Trop Dis. 2022;16:e0010075.

14. Ulbert S. West Nile virus vaccines–current situation and future directions. Hum Vaccines Immunother. 2019;15:2337–42.

15. Chala B, Hamde F. Emerging and Re-emerging Vector-Borne Infectious Diseases and the Challenges for Control: A Review. Front. Public Heal. 2021;9:715759.

16. Paz S. Climate change: A driver of increasing vector-borne disease transmission in non-endemic areas. PLOS Med. 2024;21: e1004382.

17. Amuda Tajudeen Y, Jayeola Oladipo H, Olatunji Oladunjoye I, Onyinoyi Yusuf R, Sodiq H, Olawale Omotosho A, *et al*. Emerging Arboviruses of Public Health Concern in Africa: Priorities for Future Research and Control Strategies. Challenges. 2022; 13:60.

18. Gonçalves AALM, da Silva Lopes LFV, Monteiro DDS, da Moura AJF, da Costa Sousa CAGC, da Veiga Leal S. Finding unveiled: *Culex thalassius* (Diptera: Culicidae), a new mosquito species emerges in the Cabo Verde archipelago. J Med Entomol. 2024; 61:791–7.

19. Leal SDV, Varela IBF, Monteiro DDS, Ramos de Sousa CM, da Luz Lima Mendonça M, De Pina AJ, *et al*. Update on the composition and distribution of the mosquito fauna (Diptera: Culicidae) in Cabo Verde, a country at risk for mosquito-borne diseases. J Med Entomol; 2024; 61:919-24.

20. Alves J, Gomes B, Rodrigues R, Silva J, Arez AP, Pinto J, *et al*. Mosquito fauna on the Cape Verde Islands (West Africa): An update on species distribution and a new finding. J Vector Ecol. 2010; 35:307–12.

21. Ribeiro H, Ramos H, Capela R, Pires C. Os Mosquitos de Cabo Verde (Diptera, Culicidae): Sistemática, distribuição, bioecologia e importância médica. Lisboa: Junta de Investigações Científicas do Ultramar, Lisboa; 1980.

22. Leal SDV, Varela IBF, Gonçalves AAL, Monteiro DDS, de Sousa CMR, Mendonça M da LL, *et al*. Abundance and Updated Distribution of *Aedes aegypti* (Diptera: Culicidae) in Cabo Verde Archipelago: A Neglected Threat to Public Health. Int J Environ Res Public Health. 2020;17:1291.

23. Ministry of Health of Cape Verde. Relatório Estatístico 2009. Praia; 2010.

24. Guedes DRD, Gomes ETB, Paiva MHS, de Melo-Santos MAV, Alves J, Gomez LF, *et al*. Circulation of DENV2 and DENV4 in *Aedes aegypti* (Diptera: Culicidae) mosquitoes from Praia, Santiago Island, Cabo Verde. J Insect Sci. 2017; 17:86.

25. INSP. Boletim Epidemiológico da dengue-Cabo Verde. Semana Epidemiológica 45 de 2024. Praia; 2024. Available from: https://insp.gov.cv/wp-content/uploads/2024/11/BO-dengue-43.pdf. Accessed 28 November 2024.

26. Faye O, de Lourdes Monteiro M, Vrancken B, Prot M, Lequime S, Diarra M, *et al*. Genomic Epidemiology of 2015-2016 Zika Virus Outbreak in Cape Verde. Emerg Infect Dis. 2020; 26:1084-1090.

27. Lourenço J, Monteiro M de L, Valdez T, Rodrigues JM, Pybus O, Faria NR. Epidemiology of the Zika Virus Outbreak in the Cabo Verde Islands, West Africa. PLoS Curr. 2018;10: ecurrents.outbreaks.19433b1e4d007451c691f138e1e67e8c.

28. Leal SDV, Sousa C de, Monteiro DDSR, Mendonça M da LL, Gonçalves AALM, DePina AJ. The geographical distribution of the malaria vector *Anopheles arabiensis* in Cabo Verde, 2016–2023. Front Trop Dis. 2024; 5:1353839.

29. Depina AJ, Dia AK, De Ascenção Soares Martins A, Ferreira MC, Moreira AL, Leal SV, *et al*. Knowledge, attitudes and practices about malaria in Cabo Verde: A country in the pre-elimination context. BMC Public Health. 2019;19:850.

30. World Health Organization. WHO certifies Cabo Verde as malaria-free, marking a historic milestone in the fight against malaria. 2024. Available from:

https://www.who.int/news/item/12-01-2024-who-certifies-cabo-verde-as-malaria-free--marking-a-historic-milestone-in-the-fight-against-malaria. Accessed 25 September 2024.

31. IHMT. Grupo de Trabalho de Luta Contra o Paludismo e Erradicação dos Culicídeos na República de Cabo Verde em Colaboração com a Brigada de Luta Contra o Paludismo de Cabo Verde. Lisboa; 1986.

32. Gomes B, Alves J, Sousa CA, Santa-Ana M, Vieira I, Silva TL, *et al*. Hybridization and population structure of the *Culex pipiens* complex in the islands of Macaronesia. Ecol Evol. 2012; 2:1889–902.

33. Campos M, Ward D, Morales RF, Gomes AR, Silva K, Sepúlveda N, *et al*. Surveillance of *Aedes aegypti* populations in the city of Praia, Cape Verde: Zika virus infection, insecticide resistance and genetic diversity. Parasit Vectors. 2020; 13:481.

34. da Cruz DL, Paiva MHS, Guedes DRD, de Souza Gomes EC, Pires SG, Gomez LF, *et al*. First report of the L1014F *kdr* mutation in wild populations of *Anopheles arabiensis* in Cabo Verde, West Africa. Parasit Vectors. 2021; 14:582.

35. Pires S, Alves J, Dia I, Gómez LF. Susceptibility of mosquito vectors of the city of Praia, Cabo Verde, to Temephos and *Bacillus thuringiensis var israelensis*. PLoS One. 2020;15: e0234242.

36. Rocha HDR, Paiva MHS, Silva NM, de Araújo AP, de Azevedo Camacho D dos R da R, da Moura AJF, *et al*. Susceptibility profile of *Aedes aegypti* from Santiago Island, Cabo Verde, to insecticides. Acta Trop. 2015;152:66–73.

37. Wilson AL, Courtenay O, Kelly-Hope LA, Scott TW, Takken W, Torr SJ, *et al*. The importance of vector control for the control and elimination of vector-borne diseases. PLoS Negl Trop Dis. 2020; 14:1–31.

38. Araújo MF, Castanheira EMS, Sousa SF. The Buzz on Insecticides: A Review of Uses, Molecular Structures, Targets, Adverse Effects and Alternatives. Molecules. 2023;28:3641.

39. Macias VM, Ohm JR, Rasgon JL. Gene Drive for Mosquito Control: Where Did It Come from and Where Are We Headed? Int J Environ Res Public Heal. 2017;14:1006.

40. Van Den Berg H, Manuweera G, Konradsen F. Global trends in the production and use of DDT for control of malaria and other vector-borne diseases. Malar J. 2017;16:1–8.

41. Liu N. Insecticide resistance in mosquitoes: impact, mechanisms, and research directions. Annu Rev Entomol. 2015;60:537–59.

42. World Health Organization. Global strategic framework for integrated vector management. Geneva: World Health Organization; 2004. Available from: https://iris.who.int/bitstream/handle/10665/68624/WHO_CDS_CPE_PVC_2004_10.pdf . Accessed 5 November 2024.

43. World Health Organization. Handbook for Integrated Vector Management. Geneva: World Health Organization; 2012. Available from: https://iris.who.int/bitstream/handle/10665/44768/9789241502801_eng.pdf;sequence=1. Accessed 5 November 2024.

44. World Health Organization. Global insecticide use for vector-borne diseases control: a 10-year assessment (2010-2019). Geneva: World Health Organization; 2021. Available from: https://iris.who.int/bitstream/handle/10665/345573/9789240032033-eng.pdf. Accessed 6 November 2024.

45. Principi N, Esposito S. Development of Vaccines against Emerging Mosquito-Vectored Arbovirus Infections. Vaccines. 2024;12:87.

46. World Health Organization. WHO position paper on dengue vaccines – May 2024. 2024. Available from: https://www.who.int/publications/i/item/who-wer-9918-203-224. Accessed 6 November 2024.

47. Liu X, Zhou X, Xie X, Li C. Advances in CRISPR/Cas9-Based Gene Editing Technology in Mosquitoes. Zoonoses. 2024;4.

48. Kistler KE, Vosshall LB, Matthews BJ. Genome Engineering with CRISPR-Cas9 in the Mosquito *Aedes aegypti.* Cell. 2015;11:51–60.

49. Ferguson NM. Challenges and opportunities in controlling mosquito-borne infections. Nat. 2018;559:490–7.

50. James S, Tountas KH. Using Gene Drive Technologies to Control Vector-Borne Infectious Diseases. Sustain. 2018;10:4789.

51. Flores HA, O'Neill SL. Controlling vector-borne diseases by releasing modified mosquitoes. Nat Rev Microbiol. 2018;16:508–18.

52. Bouyer J. Current status of the sterile insect technique for the suppression of mosquito populations on a global scale. Infect Dis poverty. 2024;13:68.

53. Yen PS, Failloux AB. A Review: *Wolbachia*-Based Population Replacement for Mosquito Control Shares Common Points with Genetically Modified Control Approaches. Pathog. 2020;9:404.

54. Hoffmann AA, Ahmad NW, Keong WM, Ling CY, Ahmad NA, Golding N, *et al*. Introduction of *Aedes aegypti* mosquitoes carrying *w*AlbB *Wolbachia* sharply decreases dengue incidence in disease hotspots. iScience. 2024;27:108942.

55. Ross PA, Robinson KL, Yang Q, Callahan AG, Schmidt TL, Axford JK, *et al*. A decade of stability for *w*Mel *Wolbachia* in natural *Aedes aegypti* populations. PLOS Pathog. 2022;18:e1010256.

56. Glaser RL, Meola MA. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One. 2010;5:e11977.

57. Manoj RRS, Latrofa MS, Epis S, Otranto D. *Wolbachia*: endosymbiont of onchocercid nematodes and their vectors. Parasit Vectors. 2021;14:1–24.

58. Gomes T, Wallau GL, Loreto ELS. Multiple long-range host shifts of major *Wolbachia* supergroups infecting arthropods. Sci Reports. 2022;12:1–8.

59. Porter J, Sullivan W. The cellular lives of *Wolbachia*. Nat Rev Microbiol. 2023;21:750–66.

60. Hu Y, Xi Z, Liu X, Wang J, Guo Y, Ren D, *et al*. Identification and molecular characterization of *Wolbachia* strains in natural populations of *Aedes albopictus* in China. Parasit and Vectors. 2020;13:1–14.

61. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008;6:741–51.

62. Chrostek E, Gerth M. Is *Anopheles gambiae* a natural host of *Wolbachia*? MBio. 2019;10:1–10.

63. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. *Wolbachia* and virus protection in insects. Science. 2008;322:702.

64. Teixeira L, Ferreira Á, Ashburner M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 2008;6:2753–63.

65. Kittayapong P, Chansang C, Chansang U, Mongkalangoon P, Ninphanomchai S, Limohpasmanee W. Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress *Aedes aegypti* vector populations in semi-rural settings in Thailand. PLoS Negl Trop Dis. 2019;13:1–21.

66. van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, *et al*. Impact of *Wolbachia* on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector *Aedes aegypti*. PLoS Negl Trop Dis. 2012;6:e1892.

67. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, *et al*. A *Wolbachia* Symbiont in *Aedes aegypti* Limits Infection with dengue, chikungunya and *Plasmodium*. Cell. 2009;139:1268–78.

68. Ryan PA, Turley AP, Wilson G, Hurst TP, Retzki K, Brown-Kenyon J, *et al*. Establishment of *w*Mel *Wolbachia* in *Aedes aegypti* mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia. Gates Open Res. 2020;3:1547.

69. Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini M V., Golding N, *et al*. Establishment of *Wolbachia* Strain *w*AlbB in Malaysian Populations of *Aedes aegypti* for dengue Control. Curr Biol. 2019;29:4241-4248.e5.

70. Baldo L, Bordenstein S, Wernegreen JJ, Werren JH. Widespread Recombination Throughout *Wolbachia* Genomes. Mol Biol Evol. 2006;23:437–49.

71. Bordenstein SR, Wernegreen JJ. Bacteriophage flux in endosymbionts (*Wolbachia*): infection frequency, lateral transfer, and recombination rates. Mol Biol Evol. 2004;21:1981–91.

72. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc B Biol Sci. 1998;265:509–15.

73. O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci. 1992;89:2699.

74. Kaur R, Shropshire JD, Cross KL, Leigh B, Mansueto AJ, Stewart V, *et al*. Living in the endosymbiotic world of *Wolbachia*: A centennial review. Cell Host Microbe. 2021;29:879–93.

75. Baldo L, Hotopp JCD, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, *et al*. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol. 2006;72:7098–110.

76. Ramalho MDO, Kim Z, Wang S, Moreau CS. *Wolbachia* Across Social Insects: Patterns and Implications. Ann Entomol Soc Am. 2021;114:206–18.

77. Sicard M, Bonneau M, Weill M. *Wolbachia* prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes. Curr Opin Insect Sci. 2019; 34:12–20.

78. Inácio da Silva LM, Dezordi FZ, Paiva MHS, Wallau GL. Systematic Review of *Wolbachia* Symbiont Detection in Mosquitoes: An Entangled Topic about Methodological Power and True Symbiosis. Pathog. 2021;10:39.

79. Guo Y, Hoffmann AA, Xu XQ, Mo PW, Huang HJ, Gong JT, *et al*. Vertical transmission of *Wolbachia* is associated with host vitellogenin in *Laodelphax striatellus*. Front Microbiol. 2018;9:2016.

80. Tolley SJA, Nonacs P, Sapountzis P. *Wolbachia* horizontal transmission events in ants: What do we know and what can we learn? Front Microbiol. 2019;10:429888.

81. Chen H, Zhang M, Hochstrasser M. The Biochemistry of Cytoplasmic Incompatibility Caused by Endosymbiotic Bacteria. Genes (Basel). 2020;11:852.

82. Calvitti M, Marini F, Desiderio A, Puggioli A, Moretti R. *Wolbachia* density and cytoplasmic incompatibility in *Aedes albopictus*: Concerns with using artificial *Wolbachia* infection as a vector suppression tool. PLoS One. 2015;10:1–19.

83. Hochstrasser M. Molecular Biology of Cytoplasmic Incompatibility Caused by *Wolbachia* Endosymbionts. Annu Rev Microbiol. 2023;77:299–316.

84. Yen JH, Barr AR. The etiological agent of cytoplasmic incompatibility in *Culex pipiens*. J Invertebr Pathol. 1973;22:242–50.

85. Bonneau M, Caputo B, Ligier A, Caparros R, Unal S, Perriat-Sanguinet M, *et al*. Variation in *Wolbachia* cidB gene, but not cidA, is associated with cytoplasmic incompatibility mod phenotype diversity in *Culex pipiens*. Mol Ecol. 2019;28:4725–36.

86. Beckmann JF, Bonneau M, Chen H, Hochstrasser M, Poinsot D, Merçot H, *et al*. The Toxin-Antidote Model of Cytoplasmic Incompatibility: Genetics and Evolutionary Implications. Trends Genet. 2019;35:175.

87. Beckmann JF, Ronau JA, Hochstrasser M. A *Wolbachia* deubiquitylating enzyme induces cytoplasmic incompatibility. Nat Microbiol. 2017;2:1–7.

88. Namias A, Sicard M, Weill M, Charlat S. From *Wolbachia* genomics to phenotype: molecular models of cytoplasmic incompatibility must account for the multiplicity of compatibility types. Curr Opin insect Sci. 2022; 49:78–84.

89. Caragata EP, Dutra HLC, Moreira LA. Exploiting Intimate Relationships: Controlling Mosquito-Transmitted Disease with *Wolbachia*. Trends Parasitol. 2016;32:207–18.

90. Fricke LC, Lindsey ARI. Examining *Wolbachia*-Induced Parthenogenesis in Hymenoptera. Methods Mol Biol. 2024;2739:55.

91. Verhulst EC, Pannebakker BA, Geuverink E. Variation in sex determination mechanisms may constrain parthenogenesis-induction by endosymbionts in haplodiploid systems. Curr Opin insect Sci. 2023;56:101023.

92. Bordenstein SR, Reznikoff WS. Mobile DNA in obligate intracellular bacteria. Nat Rev Microbiol. 2005;3:688–99.

93. Li M, Zhou Y, Cheng J, Wang Y, Lan C, Shen Y. Response of the mosquito immune system and symbiotic bacteria to pathogen infection. Parasit Vectors. 2024;17:1–20.

94. Lewis J, Gallichotte EN, Randall J, Glass A, Foy BD, Ebel GD, *et al*. Intrinsic factors driving mosquito vector competence and viral evolution: a review. Front Cell Infect Microbiol. 2023;13:1330600.

95. Franz AWE, Kantor AM, Passarelli AL, Clem RJ. Tissue Barriers to Arbovirus Infection in Mosquitoes. Viruses. 2015;7:3741-67.

96. Sanchez-Vargas I, Olson KE, Black WC. The Genetic Basis for Salivary Gland Barriers to Arboviral Transmission. Insects. 2021;12:1–22.

97. Franz AWE, Sanchez-Vargas I, Raban RR, Black IV WC, James AA, Olson KE. Fitness Impact and Stability of a Transgene Conferring Resistance to Dengue-2 Virus following Introgression into a Genetically Diverse *Aedes aegypti* Strain. PLoS Negl Trop Dis. 2014;8:e2833.

98. Carpenter A, Clem RJ. Factors Affecting Arbovirus Midgut Escape in Mosquitoes. Pathog. 2023;12:220.

99. Romoser WS, Turell MJ, Lerdthusnee K, Neira M, Dohm D, Ludwig G, *et al*. Pathogenesis of Rift Valley fever virus in mosquitoes-tracheal conduits & the basal lamina as an extra-cellular barrier. Arch Virol Suppl. 2005;89–100.

100. Liang X, Liu J, Bian G, Xi Z. *Wolbachia* Inter-Strain Competition and Inhibition of Expression of Cytoplasmic Incompatibility in Mosquito. Front Microbiol. 2020;11:554720.

101. Pimentel AC, Cesar CS, Martins M, Cogni R. The Antiviral Effects of the Symbiont Bacteria *Wolbachia* in Insects. 2021;11:1–10.

102. Geoghegan V, Stainton K, Rainey SM, Ant TH, Dowle AA, Larson T, *et al*. Perturbed cholesterol and vesicular trafficking associated with dengue blocking in *Wolbachia*-infected *Aedes aegypti* cells. Nat Commun. 2017;8:526.

103. Edwards B, Ghedin E, Voronin D. *Wolbachia* interferes with Zika virus replication by hijacking cholesterol metabolism in mosquito cells. Microbiol Spectr. 2023;11:e0218023.

104. Lindsey ARI, Bhattacharya T, Newton ILG, Hardy RW. Conflict in the Intracellular Lives of Endosymbionts and Viruses: A Mechanistic Look at *Wolbachia*-Mediated Pathogen-blocking. Viruses. 2018;10:141.

105. Prince BC, Walsh E, Torres TZB, Rückert C. Recognition of Arboviruses by the Mosquito Immune System. Biomol. 2023;13:1159.

106. Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, *et al*. *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*. Proc Natl Acad Sci. 2012;109:E23-31.

107. Asad S, Parry R, Asgari S. Upregulation of *Aedes aegypti* Vago1 by *Wolbachia* and its effect on dengue virus replication. Insect Biochem Mol Biol. 2018;92:45–52.

108. Pan X, Pike A, Joshi D, Bian G, McFadden MJ, Lu P, *et al*. The bacterium *Wolbachia* exploits host innate immunity to establish a symbiotic relationship with the dengue vector mosquito *Aedes aegypti*. ISME J. 2017;12:277–88.

109. Zhang D, Wang Y, He K, Yang Q, Gong M, Ji M, *et al*. *Wolbachia* limits pathogen infections through induction of host innate immune responses. PLoS One. 2020;15:e0226736.

110. Molloy JC, Sinkins SP. *Wolbachia* Do Not Induce Reactive Oxygen Species-Dependent Immune Pathway Activation in *Aedes albopictus*. Viruses. 2015;7:4624–39.

111. Zug R, Hammerstein P. *Wolbachia* and the insect immune system: What reactive oxygen species can tell us about the mechanisms of *Wolbachia*-host interactions. Front Microbiol. 2015;6:159099.

112. Alomar AA, Pérez-Ramos DW, Kim D, Kendziorski NL, Eastmond BH, Alto BW, *et al*. Native *Wolbachia* infection and larval competition stress shape fitness and West Nile virus infection in *Culex quinquefasciatus* mosquitoes. Front Microbiol. 2023;14:1138476.

113. Tsai CH, Chen TH, Lin C, Shu PY, Su CL, Teng HJ. The impact of temperature and *Wolbachia* infection on vector competence of potential dengue vectors *Aedes aegypti* and *Aedes albopictus* in the transmission of dengue virus serotype 1 in southern Taiwan. Parasit and Vectors. 2017;10:1–11.

114. Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB. The Native *Wolbachia* Symbionts Limit Transmission of Dengue virus in *Aedes albopictus*. PLoS Negl Trop Dis. 2012;6:e1989.

115. Madhav M, Blasdell KR, Trewin B, Paradkar PN, López-Denman AJ. *Culex*-Transmitted Diseases: Mechanisms, Impact and Future Control Strategies using *Wolbachia*. Viruses. 2024; 16:1134.

116. Dumas E, Atyame CM, Milesi P, Fonseca DM, Shaikevich E V., Unal S, *et al*. Population structure of *Wolbachia* and cytoplasmic introgression in a complex of mosquito species. BMC Evol Biol. 2013;13:181.

117. Atyame C, Delsuc F, Pasteur N, Weill M, Duron O. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. Mol Biol Evol. 2011;28:2761–72.

118. Atyame C, Labbé P, Dumas E, Milesi P, Charlat S, Fort P, *et al*. *Wolbachia* divergence and the evolution of cytoplasmic incompatibility in *Culex pipiens*. PLoS One. 2014;9:21–6.

119. Lilja T, Lindström A, Hernández-Triana LM, Luca M Di, Lwande OW. European *Culex pipiens* Populations Carry Different Strains of *Wolbachia* pipientis. Insects. 2024;15:639.

120. Baldini F, Segata N, Pompon J, Marcenac P, Robert Shaw W, Dabiré RK, *et al*. Evidence of natural *Wolbachia* infections in field populations of *Anopheles gambiae*. Nat Commun. 2014;5:1–7.

121. Gomes FM, Hixson BL, Tyner MDW, Ramirez JL, Canepa GE, Alves e Silva TL, *et al*. Effect of naturally occurring *Wolbachia* in *Anopheles gambiae* s.l. mosquitoes from Mali on *Plasmodium falciparum* malaria transmission. Proc Natl Acad Sci. 2017;114:12566–71.

122. Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, Spence K, *et al*. Novel *Wolbachia* strains in *Anopheles* malaria vectors from Sub-Saharan Africa. Wellcome Open Res. 2018;3:113.

123. Baldini F, Rougé J, Kreppel K, Mkandawile G, Mapua SA, Sikulu-Lord M, *et al*. First report of natural *Wolbachia* infection in the malaria mosquito *Anopheles arabiensis* in Tanzania. Parasit and Vectors. 2018;11:1–7.

124. Niang EHA, Bassene H, Makoundou P, Fenollar F, Weill M, Mediannikov O. First report of natural *Wolbachia* infection in wild *Anopheles funestus* population in Senegal. Malar J. 2018; 17:408.

125. Ayala D, Akone-Ella O, Rahola N, Kengne P, Ngangue MF, Mezeme F, *et al*. Natural *Wolbachia* infections are common in the major malaria vectors in Central Africa. Evol Appl. 2019;12:1583.

126. Shaw WR, Marcenac P, Childs LM, Buckee CO, Baldini F, Sawadogo SP, *et al*. *Wolbachia* infections in natural *Anopheles* populations affect egg laying and negatively correlate with Plasmodium development. Nat Commun. 2016;7:11772.

127. Walker T, Quek S, Jeffries CL, Bandibabone J, Dhokiya V, Bamou R, *et al*. Stable high-density and maternally inherited *Wolbachia* infections in *Anopheles moucheti* and *Anopheles demeilloni* mosquitoes. Curr Biol. 2021;31:2310-2320.e5.

128. Sawadogo SP, Kabore DA, Tibiri EB, Hughes A, Gnankine O, Quek S, *et al*. Lack of robust evidence for a *Wolbachia* infection in *Anopheles gambiae* from Burkina Faso. Med Vet Entomol. 2022;36:301–8.

129. Näslund J, Ahlm C, Islam K, Evander M, Bucht G, Lwande OW. Emerging Mosquito-Borne Viruses Linked to *Aedes aegypti* and *Aedes albopictus*: Global Status and Preventive Strategies. Vector Borne Zoonotic Dis. 2021;21:731–46.

130. Das B, Satapathy T, Kar SK, Hazra RK. Genetic Structure and *Wolbachia* Genotyping in Naturally Occurring Populations of *Aedes albopictus* across Contiguous Landscapes of Orissa, India. PLoS One. 2014;9:e94094.

131. Melo T, Sousa CA, Delacour-Estrella S, Bravo-Barriga D, Seixas G. Characterization of the microbiome of *Aedes albopictus* populations in different habitats from Spain and São Tomé. Sci Rep. 2024;14:20545.

132. Kulkarni A, Yu W, Jiang J, Sanchez C, Karna AK, Martinez KJL, *et al*. *Wolbachia pipientis* occurs in *Aedes aegypti* populations in New Mexico and Florida, USA. Ecol Evol. 2019;9:6148–56.

133. Carvajal TM, Hashimoto K, Harnandika RK, Amalin DM, Watanabe K. Detection of *Wolbachia* in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. Parasit Vectors. 2019;12:361.

134. Reyes JIL, Suzuki T, Suzuki Y, Watanabe K. Detection and quantification of natural *Wolbachia* in *Aedes aegypti* in Metropolitan Manila, Philippines using locally designed primers. Front Cell Infect Microbiol. 2024;14:1360438.

135. Bennett KL, Gómez-Martínez C, Chin Y, Saltonstall K, McMillan WO, Rovira JR, *et al*. Dynamics and diversity of bacteria associated with the disease vectors *Aedes aegypti* and *Aedes albopictus*. Sci Rep. 2019;9:1–12.

136. Teo CHJ, Lim PKC, Voon K, Mak JW. Detection of dengue viruses and *Wolbachia* in *Aedes aegypti* and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur, Malaysia. Trop Biomed. 2017;34:583–97.

137. Balaji S, Jayachandran S, Prabagaran SR. Evidence for the natural occurrence of *Wolbachia* in *Aedes aegypti* mosquitoes. FEMS Microbiol Lett. 2019;366:fnz055.

138. Ross PA, Callahan AG, Yang Q, Jasper M, Arif MAK, Afizah AN, *et al*. An elusive endosymbiont: Does *Wolbachia* occur naturally in *Aedes aegypti*? Ecol Evol. 2020;10:1581–91.

139. Gloria-Soria A, Chiodo TG, Powell JR. Lack of Evidence for Natural *Wolbachia* Infections in *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. 2018;55:1354–6.

140. Xi Z, Dean JL, Khoo C, Dobson SL. Generation of a novel *Wolbachia* infection in *Aedes albopictus* (Asian tiger mosquito) via embryonic microinjection. Insect Biochem Mol Biol. 2005;35:903–10.

141. Xi Z, Khoo CCH, Dobson SL. *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. Science. 2005;310:326–8.

142. Fraser JE, O'Donnell TB, Duyvestyn JM, O'Neill SL, Simmons CP, Flores HA. Novel phenotype of *Wolbachia* strain *w*Pip in *Aedes aegypti* challenges assumptions on mechanisms of *Wolbachia*-mediated dengue virus inhibition. PLoS Pathog. 2020;16:1–21.

143. Laven H. Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. Nat.1967;216:383–4.

144. Beebe NW, Pagendam D, Trewin BJ, Boomer A, Bradford M, Ford A, *et al*. Releasing incompatible males drives strong suppression across populations of wild and *Wolbachia*-carrying *Aedes aegypti* in Australia. Proc Natl Acad Sci. 2021;118.

145. Lim JT, Bansal S, Chong CS, Dickens B, Ng Y, Deng L, *et al*. Efficacy of *Wolbachia*-mediated sterility to reduce the incidence of dengue: a synthetic control study in Singapore. The Lancet Microbe. 2024;5: e422–32.

146. Martín-Park A, Che-Mendoza A, Contreras-Perera Y, Pérez-Carrillo S, Puerta-Guardo H, Villegas-Chim J, *et al*. Pilot trial using mass field-releases of sterile males produced with the incompatible and sterile insect techniques as part of integrated *Aedes aegypti* control in Mexico. PLoS Negl Trop Dis. 2022;16:e0010324.

147. Zeng Q, She L, Yuan H, Luo Y, Wang R, Mao W, *et al*. A standalone incompatible insect technique enables mosquito suppression in the urban subtropics. Commun Biol. 2022;5:1419.

148. Mains JW, Brelsfoard CL, Rose RI, Dobson SL. Female Adult *Aedes albopictus* Suppression by *Wolbachia*-Infected Male Mosquitoes. Sci Reports. 2016;6:1–7.

149. Caputo B, Moretti R, Manica M, Serini P, Lampazzi E, Bonanni M, *et al*. A bacterium against the tiger: preliminary evidence of fertility reduction after release of *Aedes albopictus* males with manipulated *Wolbachia* infection in an Italian urban area. Pest Manag Sci. 2020;76:1324–32.

150. Pagendam DE, Trewin BJ, Snoad N, Ritchie SA, Hoffmann AA, Staunton KM, *et al*. Modelling the *Wolbachia* incompatible insect technique: strategies for effective mosquito population elimination. BMC Biol. 2020;18:161.

151. Ant TH, Herd CS, Geoghegan V, Hoffmann AA, Sinkins SP. The *Wolbachia* strain wAu provides highly efficient virus transmission blocking in *Aedes aegypti*. PLoS Pathog. 2018;14: e1006815.

152. Velez ID, Uribe A, Barajas J, Uribe S, Ángel S, Suaza-Vasco JD, *et al*. Large-scale releases and establishment of *w*Mel *Wolbachia* in *Aedes aegypti* mosquitoes throughout the Cities of Bello, Medellín and Itagüí, Colombia. PLoS Negl Trop Dis. 2023;17:e0011642.

153. Flores HA, de Bruyne JT, O'Donnell TB, Nhu VT, Giang NT, Trang HTX, *et al*. Multiple *Wolbachia* strains provide comparative levels of protection against dengue virus infection in *Aedes aegypti*. PLoS Pathog. 2020;16: e1008433.

154. Maciel-de-Freitas R, Sauer FG, Kliemke K, Garcia GA, Pavan MG, David MR, *et al*. *Wolbachia* strains *w*Mel and *w*AlbB differentially affect *Aedes aegypti* traits related to fecundity. Microbiol Spectr. 2024;12:e0012824.

155. Utarini A, Indriani C, Ahmad RA, Tantowijoyo W, Arguni E, Ansari MR, *et al*. Efficacy of *Wolbachia*-Infected Mosquito Deployments for the Control of Dengue. N Engl J Med. 2021;384:2177–86.

156. Frentiu FD, Zakir T, Walker T, Popovici J, Pyke AT, van den Hurk A, *et al*. Limited Dengue Virus Replication in Field-Collected *Aedes aegypti* Mosquitoes Infected with *Wolbachia*. PLoS Negl Trop Dis. 2014;8:1–10.

157. Blagrove MSC, Arias-Goeta C, Failloux AB, Sinkins SP. *Wolbachia* strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. Proc Natl Acad Sci U S A. 2012;109:255–60.

158. Blagrove MSC, Arias-Goeta C, Di Genua C, Failloux AB, Sinkins SP. A *Wolbachia* *w*Mel Transinfection in *Aedes albopictus* Is Not Detrimental to Host Fitness and Inhibits Chikungunya virus. PLoS Negl Trop Dis. 2013;7:e2152.

159. Mancini MV, Herd CS, Ant TH, Murdochy SM, Sinkins SP. *Wolbachia* strain *w*Au efficiently blocks arbovirus transmission in *Aedes albopictus*. PLoS Negl Trop Dis. 2020;14:e0007926.

160. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, *et al*. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science. 2013;340:748–51.

161. Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, Lu P, *et al*. The maternally inheritable *Wolbachia w*AlbB induces refractoriness to *Plasmodium berghei* in *Anopheles stephensi*. Front Microbiol. 2017;8:366.

CHAPTER 2: Prevalence and Genetic Diversity of *Wolbachia* in Mosquitoes (Diptera: Culicidae) from Cape Verde (article 1)

Screening of natural *Wolbachia* infection in mosquitoes (Diptera: Culicidae) from the Cape Verde islands.

Aires Januário Fernandes da Moura[1,2*], Vera Valadas[1], Silvania Da Veiga Leal[3], Eddyson Montalvo Sabino[1,4], Carla A. Sousa [1], João Pinto[1].

[1] Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisbon, Portugal.
[2] Unidade de Ciências da Natureza, da Vida e do Ambiente, Universidade Jean Piaget de Cabo Verde, Praia, Cape Verde.
[3] Laboratório de Entomologia Médica, Instituto Nacional de Saúde Pública, Praia, Cape Verde.
[4] Laboratório de Simulidos, Universidad Nacional Hermilio Valdizan, Huánuco, Peru.

Journal: Parasites & Vectors

Accepted: 17 March 2023

Published online: 25 April 2023

DOI: https://doi.org/10.1186/s13071-023-05745-w

*Correspondence:

Aires Januário Fernandes da Moura

a21001274@ihmt.unl.pt

Abstract

Background: *Wolbachia pipientis* is an endosymbiont bacterium that induces cytoplasmic incompatibility and inhibits arboviral replication in mosquitoes. This study aimed to assess *Wolbachia* prevalence and genetic diversity in different mosquito species from Cape Verde.

Methods: Mosquitoes were collected on six islands of Cape Verde and identified to species using morphological keys and PCR-based assays. *Wolbachia* was detected by amplifying a fragment of the surface protein gene (*wsp*). Multilocus sequence typing (MLST) was performed with five housekeeping genes (*coxA, gatB, ftsZ, hcpA, and fbpA*) and the *wsp* hypervariable region (HVR) for strain identification. Identification of *w*Pip groups (*w*Pip-I to *w*Pip-V) was performed using PCR–restriction fragment length polymorphism (RFLP) assay on the ankyrin domain gene *pk1*.

Results: Nine mosquito species were collected, including the major vectors *Aedes aegypti*, *Anopheles arabiensis*, *Culex pipiens sensu stricto,* and *Culex quinquefasciatu*s. *Wolbachia* was only detected in *Cx. pipiens s.s.* (100% prevalence), *Cx. quinquefasciatus* (98.3%), *Cx. pipiens/quinquefasciatus* hybrids (100%) and *Culex tigripes* (100%). Based on the results of MLST and *wsp* hypervariable region typing, *Wolbachia* from the *Cx. pipiens* complex was assigned to sequence type 9, *w*Pip clade and supergroup B. PCR/RFLP analysis revealed three *w*Pip groups in Cape Verde, namely *w*Pip-II, *w*Pip-III and *w*Pip-IV. *w*Pip-IV was the most prevalent, while *w*Pip-II and *w*Pip-III were found only on Maio and Fogo islands. *Wolbachia* detected in *Cx. tigripes* belongs to supergroup B, with no attributed MLST profile, indicating a new strain of *Wolbachia* in this mosquito species.

Conclusions: A high prevalence and diversity of *Wolbachia* was found in species from the *Cx. pipiens* complex. This diversity may be related to the mosquito's colonization history on the Cape Verde islands. To the best of our knowledge, this is the first study to detect *Wolbachia* in *Cx. tigripes*, which may provide an additional opportunity for biocontrol initiatives.

Keywords: *Wolbachia*, Genotyping, Mosquitoes, *Culex pipiens*, *Culex tigripes*, Cape Verde

2.1 Background

Wolbachia pipientis (Alphaproteobacteria, Rickettsiales) is an obligatory intracellular gram-negative bacterium and proteobacterial symbiont found in a variety of invertebrates, including insects, crustaceans, arachnids, and filarial nematodes [1]. Currently, the *Wolbachia* genus is subdivided into 17 supergroups (A–F; H–Q and S) and most species known belong to supergroups A and B [2].

Wolbachia is transmitted vertically through host eggs and can influence longevity and reproduction, including feminization, parthenogenesis, and incompatibility between the female and male sex cells [3]. The best-known phenotype induced by *Wolbachia* in arthropods is cytoplasmic incompatibility (CI). It occurs when males harboring *Wolbachia* are crossed with uninfected females or between individuals infected with incompatible strains [4,5]. The generally accepted model stipulates that cytoplasmic incompatibility results from a *Wolbachia* "modification" factor (mod; toxin) in the sperm that blocks early embryogenesis and a *Wolbachia* "rescue" factor (resc; antitoxin) produced in the oocyte that allows the diploid zygote to develop if the cross is compatible [6, 7].

Besides cytoplasmic incompatibility, *Wolbachia* can inhibit viral replication in mosquitoes, including Zika, dengue, West Nile, and chikungunya arboviruses in *Aedes aegypti* [8,9]. Other studies also suggest inhibition of pathogens such as *Plasmodium falciparum* in *Anopheles stephensi* and *Anopheles gambiae* and West Nile virus in *Cx. quinquefasciatus* [1,10,11]. These abilities make *Wolbachia* a promising tool against mosquito-borne diseases and possibly an alternative to conventional vector control programs using insecticides. In fact, the release of males harboring incompatible *Wolbachia* into target populations has successfully decreased reproduction by sterilization [12,13]. The release of *Ae. aegypti* transfected with the *Wolbachia w*Mel strain (derived from *Drosophila melanogaster*) led to the establishment of *Ae. aegypti* populations infected with *Wolbachia* and a proven decrease in dengue incidence in Australia [14] and Malaysia [15].

Cape Verde is threatened by several species of vector mosquitoes, including *Ae. aegypti*, *Anopheles arabiensis, Cx. quinquefasciatus* and *Culex pipiens sensu stricto* (*s.s.*) [16]. Integrated vector control strategies are mainly directed against *An. arabiensis* and *Ae.*

aegypti, using chemical insecticides, diesel, and biological control with *Gambusia sp*. fish [17]. However, despite control efforts, the country had its first dengue epidemic in 2009, followed by an outbreak of Zika in 2015–2016 [18] and a malaria outbreak in 2017 [19].

There is no data on the genetic diversity of *Wolbachia* infecting mosquitoes (Diptera: Culicidae) from the Cape Verde islands. This knowledge would be a first step for the design and implementation of programs to suppress mosquito populations through cytoplasmic incompatibility. In this context, the present study aims to detect and genetically characterize *Wolbachia* in populations of Culicidae from Cape Verde.

2.2 Methods

Study area and sample collection.

An entomological survey was carried out in Cape Verde between February and June 2021. Larval and adult mosquito samples were collected on six islands (Santiago, Brava, Fogo, Maio, Santo Antão and Boavista; Fig.1) using BG-Sentinel and Centers for Disease Control and Prevention (CDC) light traps, dorsal aspirators, dippers and pipettes. All collection sites were geo-referenced with a portable global positioning system (GPS) device (Garmin eTrex 10).

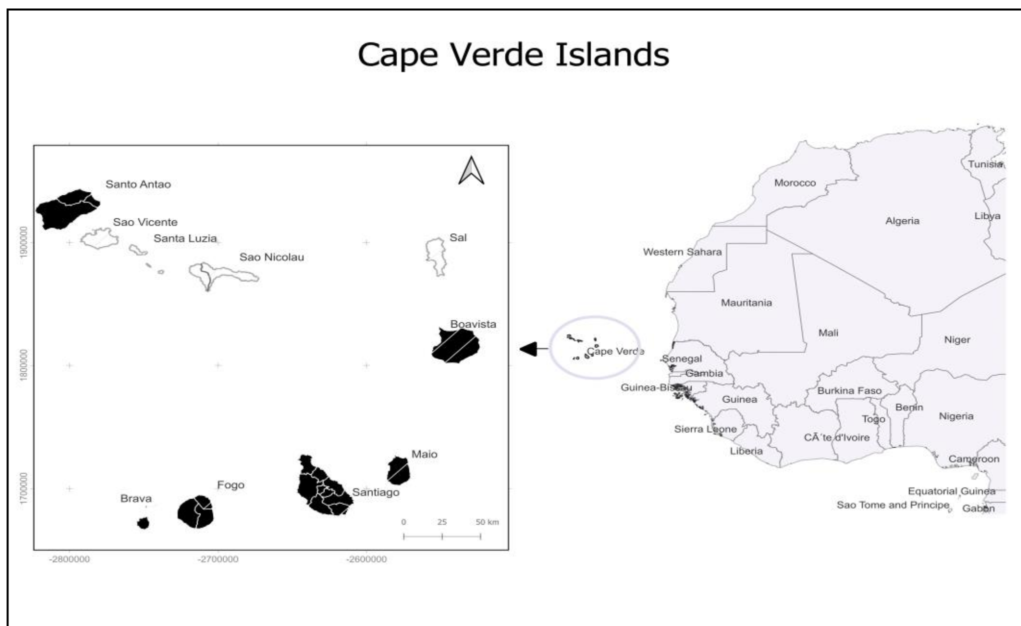

Fig. 1 Map of the North Atlantic region showing the geographic location of the Cape Verde islands. Mosquito samples were collected on the islands of Santo Antão, Boavista, Maio, Santiago, Fogo and Brava (highlighted in black).

Mosquitoes were identified to species/complex using the Ribeiro *et al.* [20] identification key and stored individually in microtubes containing silica gel (for adults) or 80% ethanol (for larvae). For genetic analysis, DNA was extracted from single specimens using cetrimonium bromide (CTAB) 2% and proteinase K, according to Weeks *et al.* [21].

Species of the *An. gambiae* complex were identified by polymerase chain reaction (PCR) according to Scott *et al.* [22] using primer sequences described in Table S1 (Additional file 1: Table S1). PCR was performed using 12.5 µl of Xpert Taq^{PLUS} Mastermix (GriSP), 0.1 µM of ME and UN primers, 0.05 µM of GA primer, and 0.15 µM of AR primer, plus 1 µl of DNA template and water to a final volume of 25 µl. Cycling conditions were as follows: one cycle at 95 °C for 5 min, 30 cycles at 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; and a final cycle of 72 °C for 5 min.

For the *Cx. pipiens* complex, specimens were identified to species by PCR amplification of acetylcholinesterase-2 (*ace-2*) gene sequences using primers described by Smith & Fonseca [23] (Additional file 1: Table S1). PCR was performed using 12.5 µl of Xpert Taq^{PLUS} Mastermix (GriSP), 0.4 µM of ACEquin and B1246 primer, 0.2 µM of ACEpip primer, 1 µl of DNA template and water to a final volume of 25 µl. Cycling conditions were performed as follows: one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and one cycle at 72 °C for 5 min.

Whenever necessary, morphological identification of species other than the above was supported with the sequencing of a 710-base-pair (bp) fragment of cytochrome c oxidase subunit 1 mitochondrial gene (*COI*) with primers LCOI1490_F1 and HCOI2198_R1 (Additional file 1: Table S1) according to Folmer *et al.* [24]. PCR was performed using 1X PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, 1 U Taq polymerase (Robust HotStart PCR Kit, Roche/Kapa Biosystems), 0.5 µM of each primer, 2 µl of DNA template and water to a final volume of 20 µl. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 50 s; annealing at 45 °C during 30 s and 72 °C for 1 min; and final elongation at 72 °C for 5 min.

Screening of *Wolbachia*

Wolbachia detection in mosquito samples was performed by amplifying a 610-bp region of the *Wolbachia* surface protein gene (*wsp*) using primers 81F and 691R (Additional file 1: Table S2) described by Zhou *et al.* [25]. The amplification reaction comprised 12.5 µl

of Xpert Taq^{PLUS} Mastermix (GriSP), 0.4 µM of each primer, 1 µl of DNA template, and water to a final volume of 25 µl. Cycling conditions were as follows: one cycle at 95 °C for 3 min, 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and one cycle at 72 °C for 10 min.

All PCR products from the assays described above were analyzed by electrophoresis on a 1.5% agarose gel stained with GreenSafe Premium (NZYTech).

***Wolbachia* multilocus sequence typing (MLST) and *wsp* typing.**

Wolbachia genotyping was performed through amplification and sequencing of five MLST loci (*gatB, coxA, hcpA, ftsZ, fbpA*) and the *wsp* hypervariable region [26,27]. The primer pairs for each locus and the size of amplified products are shown in supplemental materials (Additional file 1: Table S3).

PCR for each locus was performed using 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 0.5U Taq polymerase (Robust HotStart PCR Kit, Roche/Kapa Biosystems), 1 µM of each primer, 2 µl of DNA template and water to a final volume of 40 µl. Cycling conditions were as follows: initial denaturation at 94 °C for 2 min; 37 cycles at 94 °C for 30 s, annealing at 54 °C (for *hcpA, gatB, ftsZ, and coxA*) and 59 °C (*fbpA* and *wsp*) for 45 s and 72 °C for 90 s; and final elongation at 72 °C for 10 min.

Five microliters of PCR product from each locus were used in electrophoresis to confirm amplification. The remaining 35 µl was purified using an Exo/SAP Go PCR purification kit (GriSP) and sent for direct DNA sequencing at STAB Vida (Oeiras, Portugal) using forward and reverse primers.

Wolbachia MLST and hypervariable *wsp* sequences were edited and aligned using BioEdit (version 7.0.9.0). Consensus and concatenated sequences (*gatB, coxA, fbpA, ftsZ, hcpA,* and *wsp* hypervariable region [HVR]) were queried in the *Wolbachia* MLST database (https://pubmlst.org/bigsdb?db=pubmlst_wolbachia_seqdef) for strain characterization. Sequences were also subjected to the nucleotide Basic Local Alignment Search Tool (BLAST) to verify the similarity with deposited sequences in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was conducted using the gamma-distributed Tamura 3-parameter nucleotide substitution model and a neighbor-

joining tree was generated employing 1000 bootstraps in Molecular Evolutionary Genetics Analysis version 11 (MEGA11) [28].

Identification of *w*Pip groups by PCR–RFLP

Identification of *w*Pip groups (*w*Pip-I to *w*Pip-V) was performed using a PCR-restriction fragment length polymorphism (RFLP) assay based on the ankyrin (ANK) *Wolbachia* marker *pk1* [29–31]. A PCR that amplifies a 1300-bp fragment of the ANK domain gene (*pk1*) was performed with primers pk1_For and pk1_Rev (Additional file 1: Table S2) [32]. The reaction components included 10 µl of Xpert Taq^{PLUS} Mastermix (GriSP), 0.4 µM of each primer, 2 µl of DNA template, and water to a final volume of 20 µl. Cycling conditions were as follows: one cycle at 94ºC for 5 min; 35 cycles at 94 °C for 30 s, 52ºC for 30 s and 72 °C for 90 s; and a final cycle of 72 °C for 5 min. PCR product was analyzed by electrophoresis on a 2% agarose gel stained with GreenSafe Premium (NZYTech).

The *pk1* PCR product was digested with restriction enzymes *Taqα*I and *Pst*I to identify different *w*Pip groups [29]. Digestion with *Taqα*I was performed with the following reaction mixture: 2 µl of Buffer C (NZYTech), 10 µl of the PCR product, 18 µl of water, and 2 µl TaqαI enzyme (NZYTech) at 10U/µl. The mixture was placed in a thermal cycler at 65ºC for 90 min. The reaction was stopped by adding 0.02 mM of ethylenediaminetetraacetic acid (EDTA) (pH = 8) to each tube and the digestion product was visualized by electrophoresis on a 2% agarose gel. Each allele (*w*Pip group) was detected according to the size of the resulting fragments: allele “a” or “e” (*w*Pip-I or *w*Pip-V; 991, 251, 107 bp); “b” (*w*Pip-III; 669, 665 bp); “c” (*w*Pip-II; 851, 498 bp); “d” (*w*Pip-IV; 497,251, 107 bp) [29].

If alleles "a" or "e" (*w*Pip-I or *w*Pip-V) were present, the two were differentiated by digesting the *pk1* PCR product with the *Pst*I restriction enzyme. For this purpose, a reaction mixture was prepared with 2 µl of Buffer A (NZYTech), 12 µl *pk1* PCR product, 1 µl PstI enzyme (NZYTech) at 10U/µl and 5 µl of water. The mixture was incubated at 37 °C for 1 h and the reaction stopped by incubating at 80 °C for 20 min. Digested DNA fragments were separated by electrophoresis on a 2% agarose gel. *w*Pip alleles resulting from *Pst*I digestion include “a” (*w*Pip- I; 903, 303, 141 bp) and “e” (*w*Pip-V; 903, 430 bp) [29, 30].

Sequencing of *pk1* PCR products was performed to confirm the RFLP profile. For this purpose, the *pk1* PCR product was purified as described above for the MLST and sent for direct sequencing using reverse and forward primers. Sequences were subjected to the nucleotide BLAST and phylogenetic analysis was performed using the gamma-distributed Tamura 3-parameter nucleotide substitution model and a Neighbor-joining tree was generated using 1000 bootstraps in MEGA software version 11.0.11.

2.3 Results

Mosquito species identification

A total of 1648 mosquitoes (303 larvae and 1345 adults) were collected (Additional file 2: Table S4 for details). Species identification by morphological characters revealed the presence of *Ae. aegypti* (n = 663, 40.2%), *Aedes caspius* (n = 39, 2.4%), *An. gambiae sensu lato (s.l.)* (n = 49, 3.0%), *Anopheles pretoriensis* (n = 275, 16.7%), *Cx. pipiens s.l.* (n = 584, 35.4%), *Culex thalassius* (n = 7, 0.4%), *Culex tigripes* (n = 3, 0.2%) and *Culiseta longiareolata* (n = 28, 1.7%).

Ribosomal DNA PCR for identifying species of the *An. gambiae* complex revealed that all collected specimens from this complex belonged to *An. arabiensis.* For the *Cx. pipiens* complex, specimens were identified by *ace-2* PCR as *Cx. pipiens s.s.* (n = 10, 1.7%), *Cx. quinquefasciatus* (n = 545, 93.3%) and *Cx. pipiens/Cx. quinquefasciatus* hybrids (n = 29, 5.0%).

Screening of *Wolbachia*

The *wsp* fragment was amplified only in *Cx. pipiens s.s.* (10/10 = 100% prevalence), *Cx. quinquefasciatus* (536/545 = 98.3%), *Cx. pipiens/Cx. quinquefasciatus* hybrids (29/29 = 100%) and *Cx. tigripes* (3/3 = 100%). The remaining species were negative for *Wolbachia*.

***Wolbachia* MLST and *wsp* typing**

We analyzed 80 mosquitoes that were positive for *wsp* for *Wolbachia* MLST and *wsp* typing. Allelic profiles resulting from MLST loci and the *wsp* hypervariable region sequencing revealed that *Wolbachia* from *Cx. pipiens s.s.*, *Cx. quinquefasciatus* and *Cx. pipiens/Cx. quinquefasciatus* hybrids belong to sequence type 9, *w*Pip clade and

Supergroup B *Wolbachia* (Table 1). The same result was obtained from phylogenetic analysis using concatenated sequences of MLST loci (coxA, gatB, ftsZ, *fbpA, hcpA*) and the *wsp* hypervariable region (Fig. 2).

Fig. 2 Phylogenetic tree generated from concatenated sequences of MLST loci (coxA, gatB, ftsZ, fbpA, hcpA) and the *wsp* hypervariable region. Numbers on branches indicate percentage bootstrap support (1000 replicates). Reference sequences were obtained from the *Wolbachia* MLST database and are marked by full circles. Each *Wolbachia* supergroup is marked with a different color: yellow, supergroup B; black, supergroup A; red, supergroup D; and green, supergroup F. The scale bar indicates the number of substitutions.

Table 1- Allelic profile of MLST genes and wsp hypervariable region for different species of Culicidae collected in Cape Verde islands

Host species	**Island (n)**	***gatB***	***coxA***	***hcpA***	***ftsZ***	***fbpA***	***wsp***	***HVR1***	***HVR2***	***HVR3***	***HVR4***	***ST***
Cx. quinquefasciatus	Santiago (n=15) Brava (n=15) Boavista (n=12) Maio (n= 7) Fogo (n=1) S. Antão (n =10)	4	3	3	22	4	10	10	8	10	8	**9**
Cx. pipiens s.s.	Maio (n=2) S. Antão (n= 2)	4	3	3	22	4	10	10	8	10	8	**9**
Hybrids pip/qui	Maio (n=1) Fogo (n=2) S. Antão (n=10)	4	3	3	22	4	10	10	8	10	8	**9**
Cx. tigripes	Santiago (n=3)	9	182[b]	12	117	203[b]	NA[a]	NA[a]	232	222	84	NA[a]

[a]NA represents allelic profile or sequence type not available in the *Wolbachia* MLST database.
[b]Sequences with partial match in the *Wolbachia* MLST database

***w*Pip groups and their distribution in the archipelago**

Results from pk1 PCR–RFLP showed the occurrence of three different *w*Pip groups in Cape Verde, namely *w*Pip-IV (88.9%), *w*Pip-II (7.4%) and *w*Pip-III (3.7%) (Table 2).

Table 2- *w*Pip groups detected in *Cx. pipiens s.l.* from Cape Verde islands according to pk1 PCR–RFLP

Islands	Species	n	*w*Pip group
Santiago	*Culex quinquefasciatus*	15	*w*Pip- IV
Brava	*Culex quinquefasciatus*	15	*w*Pip- IV
Santo Antão	*Culex pipiens*	2	*w*Pip- IV
	Culex quinquefasciatus	10	*w*Pip- IV
	Hybrids pipiens/ quinquefasciatus	10	*w*Pip- IV
Maio	*Culex pipiens*	6	*w*Pip- II
	Culex quinquefasciatus	7	*w*Pip- IV
	Hybrids pipiens/ quinquefasciatus	1	*w*Pip- IV
Fogo	*Culex quinquefasciatus*	1	*w*Pip- III
	Hybrids pipiens/quinquefasciatus	2	*w*Pip- III
Boavista	*Culex quinquefasciatus*	12	*w*Pip- IV

The *w*Pip-IV group was detected in *Cx. quinquefasciatus* from five islands (Santiago, Brava, Santo Antão, Maio and Boavista) and in *Cx. pipiens s.s.* from Santo Antão. The *w*Pip-II group was detected only in *Cx. pipiens s.s.* from Maio, while *w*Pip-III was found exclusively in *Cx. quinquefasciatus* and *Cx. pipiens/quinquefasciatus* hybrids from the island of Fogo (Table 2). Sequencing of *pk1* PCR products confirmed the observed RFLP profiles and similarity with *pk1* sequences deposited in GenBank (Fig. 3).

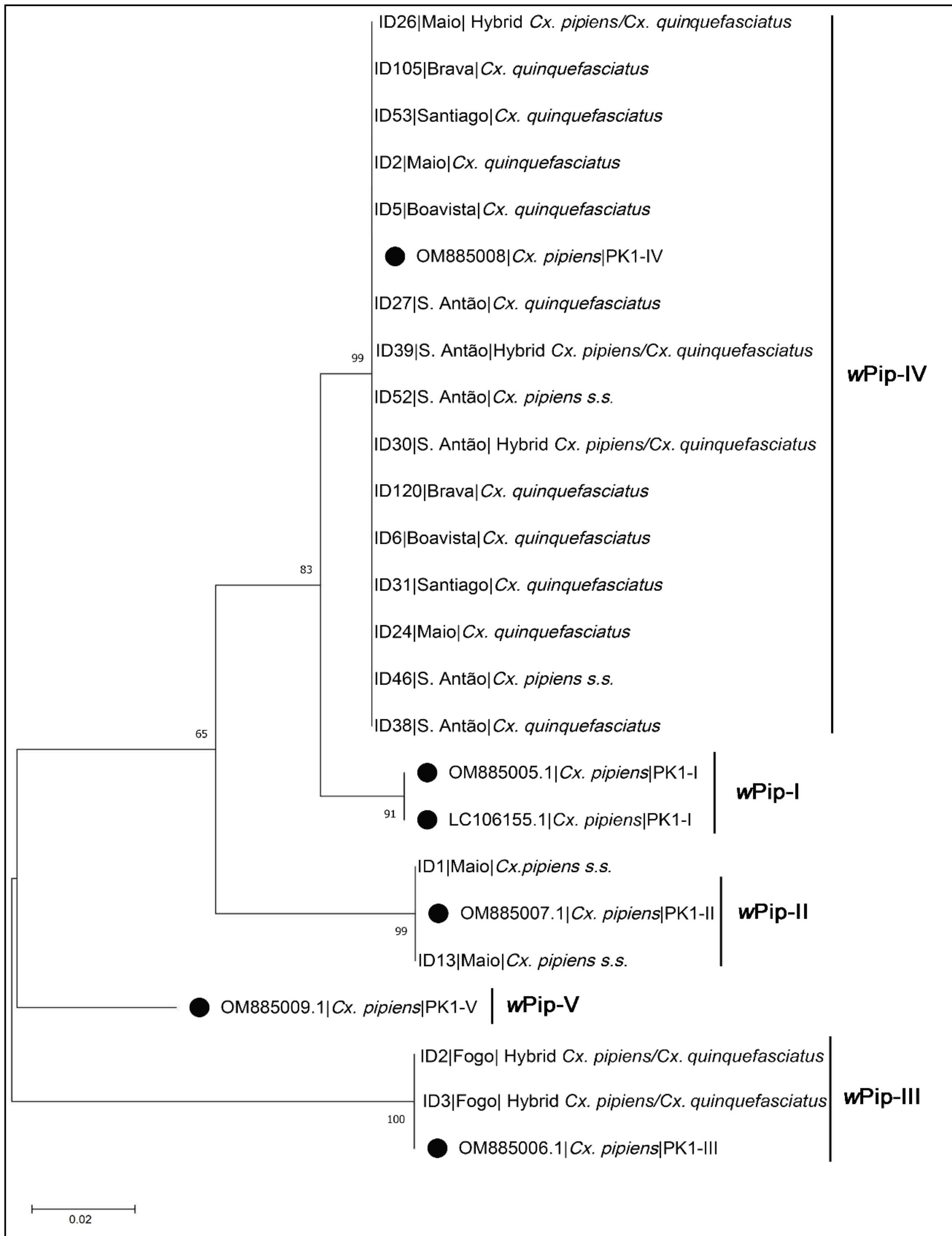

Fig. 3 Phylogenetic tree generated from *pk1* sequences by Bayesian analysis. Known *w*Pip group *pk1* sequences are marked by full circles. Numbers on branches indicate percentage bootstrap support (1000 replicates). The scale bar indicates the number of substitutions.

2.4 Discussion

Wolbachia has garnered substantial attention for its ability to control diseases transmitted by mosquitoes. This study represents the first assessment of *Wolbachia*'s prevalence and genetic diversity in mosquitoes from Cape Verde. Our objective is to expand knowledge of this bacterium through our findings and illustrate its potential for controlling mosquito-borne diseases in the archipelago.

The MLST and *wsp* typing results revealed that *Wolbachia* from *Cx. pipiens* and *Cx. quinquefasciatus* and their hybrids belong to the *w*Pip clade and share a monophyletic origin within *Wolbachia* supergroup B. The same results were obtained by Atyame *et al.* [33] and Dumas *et al.* [30] when studying *Wolbachia* genetic diversity from *Cx. pipiens s.l.* populations originating from different regions of the world. According to the authors, these findings suggest that *w*Pip strains comprise a recent clade of the *Wolbachia* supergroup B [30,33].

The analysis of the fast-evolving *pk1* gene revealed further variation within the *w*Pip strain, indicating the presence of *w*Pip-II, *w*Pip-III, and *w*Pip-IV groups in Cape Verde. The occurrence of different *w*Pip groups suggests multiple introduction events into the archipelago. In the past, Cape Verde was a maritime hub between Europe and mainland Africa and the intense movement of ships may explain the diversity of *w*Pip found on the islands. This result contrasts with that of the southwestern Indian Ocean islands, in which *Wolbachia* infecting *Cx. quinquefasciatus* all belonged to the wPip-I group [13].

It is noteworthy that *w*Pip-I was the only group found in mainland Sub-Saharan Africa, South America, and Southeast Asia, whereas only *w*Pip-III was detected in North America [30,33]. Europe shows the highest diversity, with all five groups of the *w*Pip clade being found in this continent [30]. The presence of *w*Pip-II, *w*Pip-III, and *w*Pip-IV groups in Cape Verde islands suggests at least three introduction events of *Wolbachia* possibly originating from Europe. However, a North American origin for the *w*Pip-III group in Fogo Island cannot be excluded. Interestingly, the differences found in the genetic composition of the *w*Pip clade among islands agree with the genetic structure of the *Cx. pipiens* complex in Cape Verde. Previous microsatellite-based analysis suggested that *Cx. quinquefasciatus* from Fogo Island may comprise a genetic ancestry cluster distinct from the other islands [34]. What was previously considered an admixed *Cx. quinquefasciatus*

population in Fogo Island [34] may, in fact, represent a genetically differentiated population originating from a *w*Pip-III group source population.

The absence of the African *w*Pip-I group from Cape Verde *Cx. quinquefasciatus* is not easily explained. Mainland Africa would be the natural candidate for a source population of *w*Pip-I *Cx. quinquefasciatus* that would have colonized the Cape Verdean islands, as suggested for the southwestern Indian Ocean islands [30]. However, *Cx. quinquefasciatus* was predominantly infected by the *w*Pip-IV group. This result may suggest that *Cx. quinquefasciatus* from Cape Verde may have derived from a yet to be sampled *w*Pip-IV population of mainland Africa. Another explanation would involve the cytoplasmic transfer of *w*Pip-IV from European *Cx. pipiens s.s.* to *w*Pip-I *Cx. quinquefasciatus* via hybridization, followed by the latter's replacement through cytoplasmic incompatibility (CI). High levels of CI have been reported in crosses between *w*Pip-II and *w*Pip-IV, as well as between *w*Pip-III- and *w*Pip-IV-infected mosquitoes [7, 29]. Studies involving experimental crosses would be required to assess CI between *w*Pip-I and *w*Pip-IV and whether this CI would confer an adaptive advantage to *w*Pip-IV-infected mosquitoes.

Wolbachia was not detected in *Ae. aegypti* from Cape Verde islands, which is consistent with most surveys on this species where no evidence of *Wolbachia* natural infection was found [35–38]. The presence of *Wolbachia* in *Ae. aegypti* has been reported on only a few occasions, including those from New Mexico, the USA [39], and Kuala Lumpur, Malaysia [40]. However, the possibility of *Wolbachia* detection in *Ae. aegypti* being the result of an infection with a *Wolbachia*-carrying nematode or of environmental contamination during field collections could not be excluded [36]. *Wolbachia* was also not detected in *An. arabiensis* and *An. pretoriensis* from Cape Verde. While this result is in line with most studies that screened for *Wolbachia* in *Anopheles* species [41,42], there have been a few reports on the presence of the endosymbiont in *An. gambiae* and *An. coluzzii* from Mali [43], *An. gambiae* from the Democratic Republic of Congo and *An. coluzzii* in Ghana [44]. Shaw *et al.* [45] concluded that *Wolbachia* natural *Anopheles* infections do not induce cytoplasmic incompatibility or sex ratio distortion but show a negative correlation with *Plasmodium* infection, suggesting that *Wolbachia* may interfere with malaria transmission.

This study reports for the first time the presence of *Wolbachia* in *Cx. tigripes*. Phylogenetic analyses indicate that *Wolbachia* isolated from this mosquito belongs to supergroup B, with no attributed MLST profile. This result suggests the presence of a new strain of *Wolbachia* infecting *Cx. tigripes* in Santiago Island. *Culex tigripes* is the only predatory mosquito in Cape Verde [46] and on the island of Santiago, its larvae are often found in breeding sites associated with *Cx. pipiens s.l.* species [47]. Our results exclude environmental contamination by *Cx. pipiens s.l. Wolbachia* since we detected *Wolbachia* in both larvae and an adult male of *Cx. tigripes* (Additional file 2: Table S4). More importantly, the concatenated sequences of the MLST loci and the *wsp* HVR region clearly showed that the strain detected in *Cx. tigripes* forms a monophyletic group separate from the *w*Pip clade. Our phylogenetic analyses also exclude contamination with *Wolbachia* from supergroups D and F, which are generally found in nematodes [2, 48].

The use of *Wolbachia*-based methods in vector management holds significant promise. The newly detected *Wolbachia* strain in *Cx. tigripes* from Cape Verde encourages further research to assess their ability to be firmly established in major vector trans-infected lines, induce cytoplasmic incompatibility, or reduce the ability to transmit pathogens. Proof of these abilities may offer an additional opportunity for biocontrol initiatives.

The incompatible insect technique (IIT), a variation of the sterile insect technique (SIT), can be performed by taking advantage of the *w*Pip-induced cytoplasmic incompatibility. Studies have indicated that *Cx. pipiens s.l.* mosquitoes infected with identical *Wolbachia* *w*Pip groups tend to exhibit cytoplasmic compatibility, while crossing between mosquitoes carrying different *w*Pip groups is often incompatible [13, 31]. As a result, our findings regarding the natural occurrence of *w*Pip groups in Cape Verde can provide valuable insights for implementing control programs for *Cx. pipiens s.l.* in the archipelago.

Experiments conducted in semi-field conditions on La Réunion showed that the mating between local *Cx. quinquefasciatus* *w*Pip-I females and non-native males carrying the *w*Pip-IV (Istanbul strain) resulted in 100% embryonic mortality [13]. Altinli *et al.* [29] demonstrated naturally occurring CI patterns between *w*Pip-IV-harboring males and *w*Pip-I- or *w*Pip-II-harboring females in *Cx. pipiens s.l.* populations from Turkey. These observations reveal that IIT based on *w*Pip-inducing IC could be employed to control *Cx.*

pipiens populations. The same methodology can be implemented in Cape Verde considering the data we gathered on the prevalence and distribution of *w*Pip groups in the archipelago. It would be worthwhile to analyze the pattern of cytoplasmic incompatibility among the different *w*Pip groups in Cape Verde and determine whether an island-specific *w*Pip group could be used to regulate *Cx. pipiens s.l.* populations on another island. As an alternative, male *Cx. pipiens* from other regions of the world carrying *w*Pip groups not present in Cape Verde, could be introduced into the archipelago to sterilize local females. It is noteworthy that IIT based on *w*Pip-inducing IC could be a favorable alternative to the costly radiation and genetic manipulation methods and its implementation would provide a more advantageous solution for low-income nations.

2.5 Conclusion

Our study revealed that *Wolbachia* is widespread in *Cx. pipiens s.l.* from the Cape Verde islands but absent from other mosquito species except for *Cx. tigripes*, where a novel *Wolbachia* strain was unveiled. The three distinct *w*Pip groups circulating in *Cx. pipiens s.l.* suggest multiple introduction events in the archipelago, possibly of non-African origin. The finding of a novel *Wolbachia* strain in *Cx. tigripes* may provide an additional candidate to be used in biocontrol approaches. Further studies would be required to isolate this new *Wolbachia* strain to be used in transfection studies with major mosquito vectors in order to assess its potential impact on mosquito fitness and vector competence.

Abbreviations

CI: Cytoplasmic incompatibility

MLST: Multilocus sequence typing

RFLP: Restriction fragment length polymorphism

Wsp: *Wolbachia* surface protein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-023-05745-w.

Additional file 1: Table S1. Primer sequences used for molecular identification of mosquito species collected in Cape Verde islands. **Table S2.** Primers used for PCR detection of *Wolbachia* and genotyping of *w*Pip I–V groups by PCR-RFLP. **Table S3.** Primers used for *Wolbachia* MLST loci and *wsp* hypervariable region amplification and sequence analysis.

Additional file 2: Table S4. Mosquito species collected on each island and tested for *Wolbachia* using *wsp.*

Acknowledgments

We are grateful to the National Institute of Public Health for the laboratory support in Cape Verde and to technicians from the Ministry of Health for their assistance in field work. We would like to thank Prof. Paulo Almeida for providing DNA controls of *Cx. pipiens* and *Cx. quinquefasciatus* used in the species identification PCR assay.

Author contributions

AJFM, CAS and JP designed the study. AJFM, EMS and SLV performed field work and specimen identification. AJFM and VV performed the molecular laboratory work. AJFM, SLV, CAS and JP drafted the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by national funds through FCT—Fundação para a Ciência e Tecnologia, I.P., within the framework of the project ARBOMONITOR (PTDC/BIA-OUT/29477/2017. Aires da Moura was funded by the Ph.D. fellowship program of Camões I.P.

Availability of data and materials

Sequences generated in this study are available in the GenBank database: *pk1* sequences (OQ223307-OQ223325); *ftsZ* (OQ223326-OQ223348); *hcpA* (OQ223349-OQ223371); *fbpA* (OQ223372-OQ223394); *coxA* (OQ225016-OQ225038); *gatB* (OQ225039-OQ225061); and *wsp* (OQ236526-OQ236548). All reference sequence accession numbers (GenBank) and MLST database IDs are included in the article.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

2.6 References

1. Glaser RL, Meola MA. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One. 2010; 5: e11977.

2. Kaur R, Shropshire JD, Cross KL, Leigh B, Mansueto AJ, Stewart V, *et al*. Living in the endosymbiotic world of *Wolbachia*: A centennial review. Cell Host Microbe. 2021; 29:879-893.

3. Werren JH, Baldo L, Clark ME. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol. 2008; 6:741–51.

4. Flores HA, O'Neill SL. Controlling vector-borne diseases by releasing modified

mosquitoes. Nat Rev Microbiol. 2018; 16:508–18.

5. Duron O, Bernard C, Unal S, Berthomieu A, Berticat C, Weill M. Tracking factors modulating cytoplasmic incompatibilities in the mosquito *Culex pipiens*. Mol Ecol. 2006; 15:3061–71.

6. Bonneau M, Atyame C, Beji M, Justy F, Cohen-Gonsaud M, Sicard M, *et al*. *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. Nat Commun. 2018; 9:1491.

7. Atyame C, Labbé P, Dumas E, Milesi P, Charlat S, Fort P, *et al*. *Wolbachia* divergence and the evolution of cytoplasmic incompatibility in *Culex pipiens*. PLoS One. 2014; 9: e87336.

8. Kittayapong P, Chansang C, Chansang U, Mongkalangoon P, Ninphanomchai S, Limohpasmanee W. Combined sterile insect technique and incompatible insect technique: The first proof-of-concept to suppress *Aedes aegypti* vector populations in semi-rural settings in Thailand. PLoS Negl Trop Dis. 2019;13: e0007771.

9. van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, *et al*. Impact of *Wolbachia* on Infection with Chikungunya and Yellow Fever Viruses in the Mosquito Vector *Aedes aegypti*. PLoS Negl Trop Dis. 2012;6: e1892.

10. Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL. *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. PLoS Pathog. 2011;7: e1002043.

11. Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, *et al*. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to *Plasmodium* infection. Science. 2013; 340:748–51.

12. Atyame CM, Pasteur N, Dumas E, Tortosa P, Tantely ML, Pocquet N, *et al*. Cytoplasmic incompatibility as a means of controlling *Culex pipiens quinquefasciatus* mosquito in the islands of the south-western Indian ocean. PLoS Negl Trop Dis. 2011;5: e1440.

13. Atyame CM, Cattel J, Lebon C, Flores O, Dehecq JS, Weill M, *et al*. *Wolbachia*-based population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient

under semi-field conditions. PLoS One. 2015;10: e0119288.

14. Ryan PA, Turley AP, Wilson G, Hurst TP, Retzki K, Brown-Kenyon J, *et al*. Establishment of *w*Mel *Wolbachia* in *Aedes aegypti* mosquitoes and reduction of local dengue transmission in Cairns and surrounding locations in northern Queensland, Australia. Gates Open Res. 2020; 3:1547.

15. Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini M V., Golding N, *et al*. Establishment of *Wolbachia* Strain *w*AlbB in Malaysian Populations of *Aedes aegypti* for dengue Control. Curr Biol. 2019; 29:4241-4248.e5.

16. Alves J, Gomes B, Rodrigues R, Silva J, Arez AP, Pinto J, *et al*. Mosquito fauna on the Cape Verde islands (West Africa): An update on species distribution and a new finding. J Vector Ecol. 2010; 35:307–12.

17. Salgueiro P, Serrano C, Gomes B, Alves J, Sousa CA, Abecasis A, *et al*. Phylogeography and invasion history of *Aedes aegypti*, the dengue and Zika mosquito vector in Cape Verde islands (West Africa). Evol Appl. 2019; 12:1797–811.

18. Faye O, de Lourdes Monteiro M, Vrancken B, Prot M, Lequime S, Diarra M, *et al.* Genomic epidemiology of 2015-2016 Zika virus outbreak in Cape Verde. Emerg Infect Dis. 2020; 26:1084-1090.

19. Depina AJ, Dia AK, De Ascenção Soares Martins A, Ferreira MC, Moreira AL, Leal SV, *et al*. Knowledge, attitudes and practices about malaria in Cabo Verde: a country in the pre-elimination context. BMC Public Health. 2019; 19:850.

20. Ribeiro H, Ramos HC. Guia ilustrado para a identificação dos mosquitos de Angola. 4th ed. Lisboa: Soc. Portuguesa Entomologia; 1995.

21. Weeks AR, van Opijnen T, Breeuwer JAJ. AFLP fingerprinting for assessing intraspecific variation and genome mapping in mites. Exp. Appl. Acarol. 2000; 24:775-93.

22. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg. 1993; 49:520-9.

23. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex (Culex) pipiens* complex, their hybrids and other sibling species (Diptera: Culicidae). Am J Trop Med Hyg. 2004; 70:339-45.

24. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994; 3:294–9.

25. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc B Biol Sci. 1998; 265:509–15.

26. Baldo L, Hotopp JCD, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, *et al.* Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol. 2006; 72:7098–110.

27. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Welcome Open Res. 2018; 3:1–20.

28. Tamura K, Stecher G, Kumar S. MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol. 2021; 38:3022–7.

29. Altinli M, Gunay F, Alten B, Weill M, Sicard M. *Wolbachia* diversity and cytoplasmic incompatibility patterns in *Culex pipiens* populations in Turkey. Parasit Vectors; 2018; 11:1–9.

30. Dumas E, Atyame CM, Milesi P, Fonseca DM, Shaikevich E V., Unal S, *et al*. Population structure of *Wolbachia* and cytoplasmic introgression in a complex of mosquito species. BMC Evol Biol. 2013; 13:181.

31. Sicard M, Namias A, Perriat-Sanguinet M, Carron E, Unal S, Altinli M, *et al*. Cytoplasmic incompatibility variations in relation with *Wolbachia cid* genes divergence in *Culex pipiens*. mBio. 2021;12: e02797-20.

32. Duron O, Boureux A, Echaubard P, Berthomieu A, Berticat C, Fort P, *et al*. Variability and expression of ankyrin domain genes in *Wolbachia* variants infecting the mosquito *Culex pipiens*. J Bacteriol. 2007; 189:4442-8.

33. Atyame C, Delsuc F, Pasteur N, Weill M, Duron O. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. Mol Biol Evol. 2011; 28:2761–72.

34. Gomes B, Alves J, Sousa CA, Santa-Ana M, Vieira I, Silva TL, *et al*. Hybridization and population structure of the *Culex pipiens* complex in the islands of Macaronesia. Ecol Evol. 2012; 2:1889-902.

35. Goindin D, Cannet A, Delannay C, Ramdini C, Gustave J, Atyame C, *et al*. Screening of natural *Wolbachia* infection in *Aedes aegypti*, *Aedes taeniorhynchus* and *Culex quinquefasciatus* from Guadeloupe (French West Indies). Acta Trop. 2018; 185:314–317.

36. Ross PA, Callahan AG, Yang Q, Jasper M, Arif MAK, Afizah AN, *et al*. An elusive endosymbiont: does *Wolbachia* occur naturally in *Aedes aegypti*? Ecol Evol. 2020; 10:1581–91.

37. Gloria-Soria A, Chiodo TG, Powell JR. Lack of evidence for natural *Wolbachia* infections in *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. 2018; 55:1354–6.

38. Torres R, Hernandez E, Flores V, Ramirez JL, Joyce AL. *Wolbachia* in mosquitoes from the Central Valley of California, USA. Parasit Vectors. 2020;13(1):558.

39. Kulkarni A, Yu W, Jiang J, Sanchez C, Karna AK, Martinez KJL, *et al*. *Wolbachia* pipientis occurs in *Aedes aegypti* populations in New Mexico and Florida, USA. Ecol Evol. 2019; 9:6148–56.

40. Teo CHJ, Lim PKC, Voon K, Mak JW. Detection of dengue viruses and *Wolbachia* in *Aedes aegypti* and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur, Malaysia. Trop Biomed. 2017; 34:583–97.

41. Chrostek E, Gerth M. Is *Anopheles gambiae* a natural host of *Wolbachia*? MBio. 2019; 10:1–10.

42. Sawadogo SP, Kabore DA, Tibiri EB, Hughes A, Gnankine O, Quek S, *et al*. Lack of robust evidence for a *Wolbachia* infection in *Anopheles gambiae* from Burkina Faso. Med Vet Entomol. 2022; 36:301–8.

43. Gomes FM, Hixson BL, Tyner MDW, Ramirez JL, Canepa GE, Alves e Silva TL, *et al*. Effect of naturally occurring *Wolbachia* in *Anopheles gambiae s.l.* mosquitoes from

Mali on *Plasmodium falciparum* malaria transmission. Proc Natl Acad Sci U S A. 2017; 114:12566–71.

44. Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, Spence K, *et al*. Novel *Wolbachia* strains in *Anopheles* malaria vectors from Sub-Saharan Africa. Wellcome Open Res. 2018; 3:113.

45. Shaw WR, Marcenac P, Childs LM, Buckee CO, Baldini F, Sawadogo SP, *et al*. *Wolbachia* infections in natural *Anopheles* populations affect egg laying and negatively correlate with *Plasmodium* development. Nat Commun. 2016; 7:11772.

46. Duarte EH. The haematophagous arthropods (Animalia: Arthropoda) of the Cape Verde Islands: a review. Zool Caboverdiana. 2014; 4:31–42.

47. Correia W, Varela I, Spencer H, Alves J, Heleno Duarte E. Characterization of mosquito breeding sites in the Cape Verde islands with emphasis on major vectors. Int J Mosq Res. 2015; 2:192–9.

48. Bandi C, Anderson TJC, Genchi C, Blaxter ML. Phylogeny of *Wolbachia* in filarial nematodes. Proc R Soc B Biol Sci. 1998; 265:2407-13.

2.7 Supplementary Information

Additional file 1

Table S1- Primer sequences used for molecular identification of mosquito species collected in Cape Verde islands.

Species	Primers	Sequences (5'- 3')	References
An. gambiae complex	AR (*An. arabiensis*)	AAGTGTCCTTCTCCATCCTA	[1]
	ME (*An. melas*)	TGACCAACCCACTCCCTTGA	
	GA (*An. gambiae*)	CTGGTTTGGTCGGCACGTTT	
	UN (*Universal*)	GTGTGCCCCTTCCTCGATGT	
Culex pipiens complex	ACEquin	CCTTCTTGAATGGCTGTGGCA	[2]
	ACEpip	GGAAACAACGACGTATGTACT	
	B1246s	TGGAGCCTCCTCTTCACGG	
Other species (COI)	LCOI1490_F1	GGTCAACAAATCATAAAGATATTG	[3]
	HCOI2198_R1	TAAACTTCAGGGTGACCAAAAAATCA	

Table S2- Primers used for PCR detection of *Wolbachia* and genotyping of wPip I-V groups by PCR-RFLP.

Target	Primers sequences (5'-3')	Size (bp)	References
wsp	81F - TGGTCCAATAAGTGATGAAGAAA 691R - AAAAATTAAACGCTACTCCA	610	[4]
Pk1	pk1 For - CCACTACATTGCGCTATAGA pk1 Rev - ACAGTAGAACTACACTCCTCCA	1300	[5]

Table S3- Primers used for *Wolbachia* MLST loci and wsp hypervariable region amplification and sequence analysis.

Target	Primers sequences (5'-3')	Size (bp)	References
wsp HVR	wsp_F1: GTCCAATARSTGATGARGAAAC wsp_R1: CYGCACCAAYAGYRCTRTAAA	603	[6,7]
gatB	gatB_F1: GAKTTAAAYCGYGCAGGBGTT gatB_R1: TGGYAAYTCRGGYAAAGATGA	471	
coxA	coxA_F1: TTGGRGCRATYAACTTTATAG coxA_R1: CTAAAGACTTTKACRCCAGT	487	
hcpA	hcpA_F1: GAAATARCAGTTGCTGCAAA hcpA_R1: GAAAGTYRAGCAAGYTCTG	515	
ftsZ	ftsZ_F1: ATYATGGARCATATAAARGATAG ftsZ_R1: TCRAGYAATGGATTRGATAT	524	
fbpA	fbpA_F1: GCTGCTCCRCTTGGYWTGAT fbpA_R1: CCRCCAGARAAAAYYACTATTC	509	

References:

1. Scott JA, Brogdon WG, Collins FH. Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. Am J Trop Med Hyg. 1993; 49:520–9.

2. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids and other sibling species (Diptera: Culicidae). Am J Trop Med Hyg. 2004; 70:339-45.

3. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994; 3:294–9.

4. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proc R Soc B Biol Sci. 1998; 265:509–15.

5. Duron O, Boureux A, Echaubard P, Berthomieu A, Berticat C, Fort P, *et al.* Variability and expression of ankyrin domain genes in *Wolbachia* variants infecting the Mosquito *Culex* pipiens. J Bacteriol. 2007; 189:4442.

6. Baldo L, Hotopp JCD, Jolley KA, Bordenstein SR, Biber SA, Choudhury RR, *et al*. Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. Appl Environ Microbiol. 2006; 72:7098–110.

7. Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. Wellcome Open Res. 2018; 3:1–20.

Additional file 2

Table S4- Mosquito species collected on each island and tested for *Wolbachia* using *wsp*.

	Boavista		**Brava**		**Fogo**		**Maio**		**Santiago**		**Santo Antão**		**Total capture (%)**	***Wolbachia* prevalence**[a]
	Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae	Adults	Larvae		
Ae. aegypti	6	1	21	11	100	15	66	20	314	67	37	5	663 (40,2%)	0/663 (0,0%)
Ae. caspius	-	-	-	-	-	-	28	11	-	-	-	-	39 (2,4%)	0/39 (0,0%)
An. arabiensis	-	-	-	-	-	-	-	-	49	-	-	-	49 (3,0%)	0/49 (0%)
An. pretoriensis	-	-	40	8	39	5	-	-	82	9	63	29	275 (16,7%)	0/275 (0,0%)
C. longioreolata	-	-	6	8	-	-	-	-	-	-	7	7	28 (1,7%)	0/28 (0,0%)
Cx. pipiens s.s.	-	-	-	-	-	-	6	-	-	-	4	-	10 (0,6%)	10/10 (100%)
Cx. quinquefasciatus	54	-	115	41	1	-	15	5	247	32	20	15	545 (33,1%)	536/545 (98,3%)
Cx. thalassius	-	-	-	-	-	-	-	-	3	4	-	-	7 (0,4%)	0/7 (0,0%)
Cx. tigripes	-	-	-	-	-	-	-	-	1	2	-	-	3 (0,2%)	3/3 (100%)
Hybrids pip/qui	-	-	-	-	2	-	1	-	-	-	18	8	29 (1,8%)	29/29 (100%)

[a] *Wolbachia* prevalence = number of positive mosquitoes for *wsp*/number tested

CHAPTER 3: The Role of Native *Wolbachia* on Vector Competence (article 2)

Vector Competence of *Culex quinquefasciatus* from Santiago Island, Cape Verde, to West Nile Virus: exploring the potential effect of the vector native *Wolbachia*

Aires Januário Fernandes da Moura[1,2*], Filipe Tomaz[1], Tiago Melo[1], Gonçalo Seixas[1], Carla A. Sousa[1], João Pinto[1].

[1] Global Health and Tropical Medicine, GHTM, Associate Laboratory in Translation and Innovation Towards Global Health, LA-REAL, Instituto de Higiene e Medicina Tropical, IHMT, Universidade NOVA de Lisboa, UNL, Rua da Junqueira 100, 1349-008 Lisboa, Portugal.
[2] Unidade de Ciências da Natureza, da Vida e do Ambiente, Universidade Jean Piaget de Cabo Verde, Praia, Cape Verde.

Journal: Parasites & Vectors

Accepted: 28 November 2024

Published online: 23 December 2024

DOI: https://doi.org/10.1186/s13071-024-06609-7

*Correspondence:

Aires Januário Fernandes da Moura

a21001274@ihmt.unl.pt

Abstract

Background: *Culex quinquefasciatus* plays a crucial role as a vector of West Nile virus (WNV). This mosquito species is widely distributed in Cape Verde, being found in all inhabited islands of the archipelago. However, no data are currently available on the susceptibility of the local mosquito population to WNV. This study aimed to assess the vector competence of *Cx. quinquefasciatus* mosquitoes from Santiago Island, Cape Verde, for WNV and to explore the potential impact of its native *Wolbachia* on virus transmission.

Methods: *Wolbachia*-infected and uninfected *Cx. quinquefasciatus* female mosquitoes were exposed to WNV lineage 1 PT6.39 strain using a Hemotek membrane feeding system. Mosquito samples, including the body, legs, wings, and saliva, were collected at days 7, 14, and 21 post-infection (dpi) to assess WNV infection through one-step quantitative real-time PCR (RT-qPCR).

Results: *Culex quinquefasciatus* from Cape Verde exhibited high susceptibility to the tested strain of WNV. Also, treated females without their native *Wolbachia* exhibited significantly higher WNV load in their bodies and greater dissemination rate at 7 dpi than their wild-type counterparts carrying *Wolbachia*.

Conclusions: The high susceptibility to WNV of *Cx. quinquefasciatus* from Cape Verde poses a potential risk for virus transmission in the archipelago. However, *Wolbachia* infection in this mosquito species seems to confer protection against WNV dissemination in the early stages of viral infection. Additional research is required to uncover the mechanisms driving this protection and its potential impact on WNV transmission.

Keywords: *Culex quinquefasciatus*, West Nile virus, *Wolbachia*, Vector competence, Cape Verde.

3.1 Background

West Nile virus (WNV) is a positive-sense RNA flavivirus belonging to the Flaviviridae family [1]. It was initially documented in Uganda in 1937 and since then, it has spread across regions in Africa, Europe, Asia, North America, Australia, and the Middle East, with multiple outbreaks [2]. Approximately 80% of individuals infected with WNV experience no symptoms, while the remaining 20% may display a broad spectrum of clinical manifestations, ranging from influenza-like symptoms to severe neurological complications and even death [3]. Severe forms of illness, which occur in up to 1% of infections, are primarily associated with older or immunocompromised individuals [4].

WNV is transmitted through the bites of mosquitoes, and it persists in the natural environment through a cycle involving vectors and birds [5]. Humans, horses, and other mammalian hosts are designated as WNV dead-end hosts, as they can become infected but do not generate the virus loads necessary for the infection of a naïve mosquito [6].

Transmission of WNV has been associated with mosquitoes of different genera, including *Anopheles*, *Aedes*, *Culex*, *Culiseta*, *Mansonia,* and *Ochlerotatus*. However, species of the *Culex pipiens* complex stand out as primary vectors of WNV due to their remarkable efficiency in transmitting the virus, widespread distribution, tendency to feed on birds and strong preference for domestic settings [7–9]. Among the species in this complex, *Cx. quinquefasciatus* is typically found in low-altitude regions between latitudes of 30° N and 30° S [10]. *Culex quinquefasciatus* is an important vector for WNV across the Americas, Africa, and Asia, being accountable for the transmission of the virus in both rural and urban areas [11, 12].

Culex quinquefasciatus is naturally infected by *Wolbachia*, an obligate endosymbiotic bacterium found in approximately 66% of all insect species [13]. Although there is sufficient evidence that mosquitoes hosting transinfected *Wolbachia* strains exhibit reduced competence for arboviruses [14–16], there is limited information on the potential influence of *Wolbachia* infections on the transmission of arboviruses by their natural mosquito hosts. Studies on vector competence using *Cx. quinquefasciatus* mosquitoes treated to remove their native *Wolbachia* suggest that the endosymbiont can interfere with WNV dynamics in mosquito tissues [17, 18].

In Cape Verde, *Cx. quinquefasciatus* has a wide distribution, being present in all inhabited of the archipelago [19]. A previous study showed that *Cx. quinquefasciatus* populations from the islands harbor *Wolbachia* from the *w*Pip-III and *w*Pip-IV groups, with a total prevalence of 98.3% [20]. Although there are no recent records of WNV occurrence in Cape Verde, seroepidemiological surveys from the 1980s detected the circulation of the virus in humans, possibly introduced into the country by migratory birds [21]. Nevertheless, to date, there has been no investigation aimed at determining the vector competence of Cape Verdian *Cx. quinquefasciatus* for WNV or the influence of circulating *Wolbachia* strains on the outcome of WNV infection.

The aim of the study reported here was to fill these gaps in current knowledge by testing the *Cx. quinquefasciatus* population from Santiago Island.

3.2 Methods

***Culex quinquefasciatus* field collection and colony establishment**

Immature mosquitoes were collected in all Santiago Island municipalities, Cape Verde (15°07′48.0″ N, 23°31′48.0″ W) between August and October 2022. *Culex sp*. larvae were collected using standard sampling techniques with dippers and pipettes [22]. A subsample of collected larvae were morphologically identified to species complex using the identification key of Ribeiro and Ramos [23]; the remaining larvae were reared to adults in trays filled with dechlorinated tap water and fed with fish food (TetraMin; Tetra Werke, Melle, Germany).

Adult mosquitoes were maintained at 28 °C and 70–80% relative humidity under a 12:12-h light:dark cycle, with access to a sucrose 10% solution ad libitum. One-week-old females were blood-fed on human blood using an artificial blood-feeding protocol described in Siria *et al.* [24] and following the Standard Operational Procedures adopted by the Instituto Nacional de Saúde Pública of Cape Verde. An oviposition container with water was placed inside the cage 4 to 5 days after blood-feeding.

The resulting F1 generation egg rafts were transported to the In Vivo Arthropod Security Facility (VIASEF) of the Institute of Hygiene and Tropical Medicine (IHMT), Portugal,

where all experiments with WNV were conducted under biosafety level 3 (BSL3) conditions.

***Wolbachia* detection and treatment with tetracycline**

At VIASEF, *Cx. quinquefasciatus* egg rafts were reared to adults as described in the previous section. The resulting adult females were blood-fed with human blood on an artificial membrane feeder (Hemotek®, Blackburn, UK) and allowed to oviposit eggs to produce an F2 generation.

A subsample of 200 non-blood-fed F1 females was used for molecular confirmation of species and screening of *Wolbachia*. DNA was extracted individually from each specimen using 2% cetyltrimethylammonium bromide (CTAB), as previously described by Weeks *et al.* [25] and molecular identification was performed by amplifying species-specific gene fragments from the acetylcholinesterase-2 gene [26] and cytochrome c oxidase subunit 1 (COI) gene [27], using primers described in Additional file 1 Table S1. The acetylcholinesterase-2 PCR product was analyzed by gel electrophoresis and the COI product underwent purification and DNA sequencing. The resulting sequences were queried in the Barcode of Life Data Systems (BOLD Systems) (https://www.boldsystems.org). A sequence was assigned to a species when it showed at least 98% similarity with the reference sequence in BOLD. *Wolbachia* infection and prevalence were assessed in the same F1 individuals by conventional PCR using the 81F and 691R primers [28], which amplify a 610-bp segment of the *Wolbachia* surface protein gene (*wsp*). Amplification conditions were performed as previously described by da Moura *et al*. [20].

A subset of F2 mosquitoes was treated to remove *Wolbachia*. The treatment was performed using tetracycline hydrochloride (Selleck Biotechnology GmbH, Cologne, Germany) following the protocol of Shemshadian *et al*. [29] with modifications. Briefly, *Cx. quinquefasciatus* stage 1 larvae (L1) were placed in an aqueous tetracycline solution (50 µg/ml) until pupation. The resulting adults were placed in a cage and fed on a solution containing 10% sucrose and 50 µg/ml of tetracycline hydrochloride. Adults were blood-fed on an artificial membrane feeder (Hemotek®) and allowed to oviposit eggs. The treatment was repeated for one more generation.

A real-time PCR (RT-PCR) assay was developed to improve the sensitivity of *Wolbachia* detection and evaluate the effectiveness of the tetracycline treatment. The primers were designed using multiple alignments of *wsp* gene sequences available in the National Center for Biotechnology Information (NCBI) database (Additional file: Table S2). The specificity of the amplified region was evaluated with Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast) to confirm that the target of interest was unique. The RT-PCR reaction was performed in a reaction volume containing 10 µl iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.1 µM of forward and reverse primers, 2 µl of DNA and water to a final volume of 20 µl. The thermocycling conditions consisted of one cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C for 5 s and 60 °C for 15 s. Melting curve analysis was performed at 65–95 °C, with 0.5 °C increments with 2–5 s for each step. The quantitative PCR (qPCR) was conducted using a CFX96 real-time PCR detection system (Bio-Rad Laboratories).

After *Wolbachia* clearance, the treatment was stopped and the treated and untreated mosquitoes (henceforth referred to as wPip^{-} and wPip^{+}, respectively) were reared for four generations (until F7) in separated cages and trays but in the same room, using the same water and food, under a consistent nutritional regimen to ensure uniformity of the microbiome between the two lines. The F7 generation from both lines was used in experimental infections with WNV.

Since *Wolbachia* density may change under laboratory settings, which may account for variation observed in antiviral protection [30, 31], we used the RT-PCR assay described above to evaluate whether there were significant changes in *Wolbachia* density between the wild-type F1 generation and the non-treated F7 generation (wPip^{+}) used in artificial infections. For this purpose, DNA was extracted from both generations of mosquitoes using three biological replicates, each containing a pool of five whole-body females (collected 5 days post pupal eclosion). Total *Wolbachia* density was analyzed by relative quantification of the *Wolbachia* surface protein (*wsp*) against the 18S ribosomal RNA (rRNA) reference gene of *Cx. quinquefasciatus*. Primers for the 18S rRNA gene were designed using *Cx. quinquefasciatus* reference sequences from VectorBase and the Primer-Blast tool (Additional file 1: Table S2). The PCR reaction was conducted in a reaction volume containing 10 µl iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories), 0.1 µM of *wsp* primers, 0.5 µM of 18S ribosomal primers, 2 µl of DNA

template, and water to reach a final volume of 20 µl. The thermocycling parameters included one cycle at 95 °C for 5 min, followed by 35 cycles at 95 °C for 5 s and 60 °C for 15 s. Melting curve was performed at 65–95 °C, with 0.5 °C increments with 2–5 s for each step.

Production and quantification of viral stocks

Vector competence assays were conducted using the WNV PT6.39 strain (GeneBank accession number: AJ965630.2), which is a lineage 1 WNV strain isolated in Portugal in 2004 [32]. WNV was propagated using African Green monkey kidney cells (Vero E6) maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM l-glutamine, 1% penicillin/streptomycin (Sigma-Aldrich) and amphotericin B (Selleck Biotechnology GmbH), in a multiplicity of infection of 0.1. The cells were incubated at 37 °C with 5% CO2 for 2–3 days and checked daily until a 50% cytopathic effect was observed. Portions of the supernatant were employed to infect a fresh batch of Vero cells. After two consecutive passages, the supernatant was transferred to 1-ml cryotubes and stored in a freezer at − 80 °C until further use.

WNV particles in viral stocks were quantified using a plaque assay following the protocols of Brien *et al.* [33] and McAuley and Beasley [34] with modifications. In summary, 12-well tissue culture plates were seeded with 1.5×10^5 Vero cells/ml on the day preceding the assay using DMEM medium containing 10% FBS, 2 mM l-glutamine, 1% penicillin/streptomycin and amphotericin B. On the day of the assay, the wells were washed with 1× phosphate-buffered saline (PBS), then inoculated with 200 µl of serially diluted viral stock and incubated for 1 h. Following the incubation, 1 ml of the semi-solid medium was added to the well, comprising a 1:1 mixture of 1.5% high-viscosity carboxymethyl cellulose (CMC; Sigma-Aldrich) and 2× DMEM with 4% FBS. The plate was then incubated for 3 days at 37 °C with 5% CO2. Subsequently, the plate was fixed using 10% buffered formaldehyde for 1 h, washed twice with a 1× PBS solution and stained with 1% crystal violet to visualize the plaques.

The viral titer of the stocks was 7.6 $\log_{10}$ plaque-forming units (PFU)/ml.

Culex quinquefasciatus artificial infection with WNV

The infective blood meal was prepared by mixing blood and viral stock produced in the previous step to a final titer of 7.0 log_{10} PFU/ml. An aliquot of the infectious blood meal was stored at − 80 °C for subsequent backtitration by plaque assay.

Mosquito females between 7 and 10 days old from both the wPip− and wPip+ colonies were kept in groups of 100 individuals in paper cups and exposed to the infective blood meal using a Hemotek® membrane feeding system for 1 h. After feeding, engorged females were selected following sedation with CO2, transferred to new paper cups and maintained inside a climate chamber (Percival Scientific, Inc., Perry, IA, USA) at 28 °C and 70% relative humidity RH, under 12:12-h light:dark cycle, with access to a 10% sucrose solution.

At days 7, 14, and 21 post-infection (dpi), 30 mosquitoes per group were anesthetized using CO_2; the legs and wings were then removed, and saliva was collected by inserting the mosquito proboscis into pipette tips filled with 5 µl of DMEM with 10% FBS for 60 min. The collected saliva was then transferred to a microtube containing 200 µl of DMEM supplemented with 10% FBS. The dissected body and legs plus wings were stored separately in microtubes containing 300 µl of complete DMEM with 10% FBS, 1% amphotericin B, and three 2-mm-diameter glass beads for subsequent homogenization during the RNA extraction. All samples were kept at − 80 °C until further use in the RNA extraction.

RNA extraction and detection of WNV

For RNA extraction, the body, legs, and wings were homogenized twice at 7500 rpm for 10 s in a Precellys Evolution Homogenizer (Bertin Technologies, Paris Region, France) and then centrifuged at 3000g for 15 min. The supernatant was collected and used in the RNA extraction. RNA was extracted from 100 µl of mosquito saliva or tissue supernatants using NZYol, according to the manufacturer's protocol (Nzytech, Lisbon, Portugal). The RNA was further treated with Turbo DNase (Ambion, Austin, TX, USA) to remove genomic DNA and then stored at − 80 °C until further use.

WNV in each sample was detected and quantified by one-step RT-qPCR. The RT-qPCR was performed using the primers and probes described by Lanciotti *et al.* [35], which amplify a 70-bp fragment of the virus envelope gene (Additional file 1: Table S3). The

RT-qPCR was performed in a reaction volume containing 10 µl of Xpert One-Step Fast Probe (GRiSP, Porto, Portugal), 0.4 µM of each primer, 0.1 µM of probe, 0.8 µl of RTase mix, 5 µl of RNA template (unit mass normalized) and water to a final volume of 20 µl. The thermocycling conditions consisted of complementary DNA (cDNA) synthesis at 50 °C for 20 min, one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. WNV copies per microliter were extrapolated from a standard curve generated in each reaction by serial dilutions of a synthetic oligonucleotide (gBlocks®; IDT Inc., Coralville, IA, USA) encompassing the 70-bp target region. The number of copies of WNV per milliliter of homogenized tissue and saliva was calculated by considering the RNA elution volume (30 µl) and the volume used for RNA extraction (100 µl).

Aliquots of saliva that tested positive for WNV in the RT-qPCR analysis were titrated on Vero cells to validate the infectivity of viral particles.

Data analysis

The RT-qPCR results from each mosquito tissue and saliva were used to calculate the infection rate (percentage of WNV-positive bodies out of the total exposed mosquitoes), dissemination rate (percentage of virus-positive legs + wings out of the total number of positive mosquito bodies), and transmission efficiency (proportion of mosquitoes with positive saliva among the total number of fed mosquitoes). To detect potential differences in salivary gland infection barriers between $wPip^+$ and $wPip^-$ lines, we also calculated the transmission rate, which is defined as the percentage of mosquitoes with infected saliva out of those with disseminated infection.

Fisher's exact test was used to test for significant differences between $wPip^+$ and $wPip^-$ vector competence indices at each day post-infection. After assessing the normality and homogeneity of the data variances, the non-parametric Mann–Whitney U-test was applied to compare the viral loads between the two groups.

Statistical analyses were performed using GraphPad Prism for Windows, version 10.1.2 (GraphPad Software, Boston, MA, USA) and IBM SPSS Statistics for Windows, version 29.0.1.0 (SPSS IBM Corp, Armonk, NY, USA).

3.3 Results

Wolbachia **screening and efficacy of tetracycline treatment**

There was a 100% prevalence of *Wolbachia* in the F1 generation of *Cx. quinquefasciatus*. Following tetracycline treatment, both conventional and Real-Time PCR results confirmed the successful elimination of *Wolbachia* in the treated mosquito group (wPip^{-}) (Fig. 1).

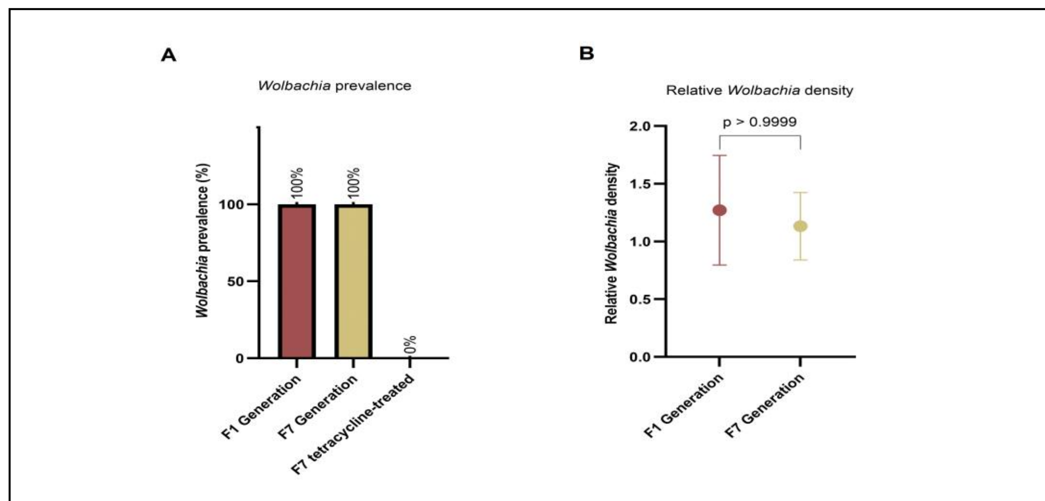

Fig 1. *Wolbachia* detection in *Culex quinquefasciatus* from Santiago Island, Cape Verde, based on the *wsp* gene. (A) *Wolbachia* prevalence in F1 and F7 generations of wild-type mosquitoes, as well as in the F7 generation group treated with tetracycline (n=200 per group), analyzed using conventional and real-time PCR. (B) *Wolbachia* density on a linear scale relative to the *Cx. quinquefasciatus* 18S ribosomal gene, as estimated by quantitative real-time PCR. The difference in *Wolbachia* density between the F1 and F7 generations was assessed using the Mann-Whitney test. Error bars represent 95% confidence intervals from three biological replicates, each containing DNA from a pool of five females.

Among the untreated group (wPip^{+}), the evaluation of *Wolbachia* density by qPCR revealed no statistically significant differences between generations F1 and F7 (Mann-Whitney U-test, $U_{(6)} = 4$, $Z = -0.22$, $P > 0.9999$) (Fig. 1).

Vector competence

The WNV infection rates of *Wolbachia*-uninfected mosquitoes (wPip^{-}) were 86.7% at 7 dpi, 83.3% at 14 dpi, and 90.0% at 21 dpi. For mosquitoes harboring *Wolbachia* (wPip+), the infection rates were 76.7% at 7 dpi, 63.3% at 14 dpi, and 93.3% at 21 dpi (Table 1). Although the infection rates at 7 and 14 dpi were higher for mosquitoes without *Wolbachia*, these differences were not significant (Fisher's exact tests, $P = 0.506$, OR = 1.98, 95% CI = 0.51–7.64 and $P = 0.143$, OR = 2.89, 95% CI = 0.86–9.74, respectively).

Table 1- Infection, dissemination, transmission rates and transmission efficiency for the population of *Culex quinquefasciatus* from Santiago Island exposed to WNV PT6.39 strain.

dpi	Infection Rate [a] (%) (95% CI)			Dissemination Rate [b] (%) (95% CI)			Transmission Rate [c] (%) (95% CI)			Transmission Efficiency [d] (%) (95% CI)		
	wPip $^{-}$	wPip $^{+}$	*P value* [e]	wPip $^{-}$	wPip $^{+}$	*P value* [e]	wPip $^{-}$	wPip $^{+}$	*P value* [e]	wPip $^{-}$	wPip $^{+}$	*P value* [e]
7	26/30 (86.7) (69.7-95.3)	23/30 (76.7) (58.8-88.5)	0.506	13/26 (50.0) (32.1-67.9)	3/23 (13.0) (3.7-33.0)	**0.007***	3/13 (23.1) (7.5-50.9)	0/3 (0.0) (0.0-61.8)	-	3/30 (10.0) (2.7-26.4)	0/30 (0.0) (0.0-13.5)	-
14	25/30 (83.3) (66.0-93.1)	19/30 (63.3) (45.5-79.2)	0.143	25/25 (100.0) (84.2-100.0)	16/19 (84.2) (61.6-95.3)	0.073	13/25 (52.0) (33.5-70.0)	7/16 (43.8) (23.1-66.9)	0.751	13/30 (43.3) (27.4-60.8)	7/30 (23.3) (11.5-41.2)	0.170
21	27/30 (90.0) (73.6-97.3)	28/30 (93.3) (77.6-99.2)	1.000	26/27 (96.3) (80.2-99.9)	27/28 (96.4) (80.8-99.9)	1.000	23/26 (88.5) (70.2-96.8)	18/27 (66.7) (47.7-81.5)	0.099	23/30 (76.7) (58.8-88.5)	18/30 (60.0) (42.3-75.4)	0.267

CI, Confidence interval (computed by the modified Wald method).
*Statistically significant difference between wPip $^{-}$ mosquitoes (those treated with tetracycline hydrochloride to clear *Wolbachia*) and wPip $^{+}$ mosquitoes (untreated mosquitoes).
[a] Infection rate is the percentage of West Nile virus-positive bodies out of the total number of exposed mosquitoes.
[b] Dissemination rate is the percentage of virus-positive legs + wings out of the total number of positive mosquito bodies.
[c] Transmission rate is the percentage of mosquitoes with infected saliva out of the number with disseminated infection.
[d] Transmission efficiency is the proportion of mosquitoes with positive saliva among the total number of fed mosquitoes.
[e] P-value represents Fisher’s exact test to verify significant differences between wPip $^{-}$ and wPip $^{+}$ rates at each day post-infection.

The viral load in the mosquitoes' bodies remained consistently elevated throughout the experiment (> 8 $\log_{10}$ copies/ml), regardless of the presence or absence of *Wolbachia*. However, at 7 dpi, we found that the number of WNV copies in mosquitoes lacking *Wolbachia* was significantly higher compared to those harboring *Wolbachia* (Mann-Whitney U-test, $U_{(49)} = 179$, $Z = -2.41$, $P = 0.0154$) (Fig. 2a).

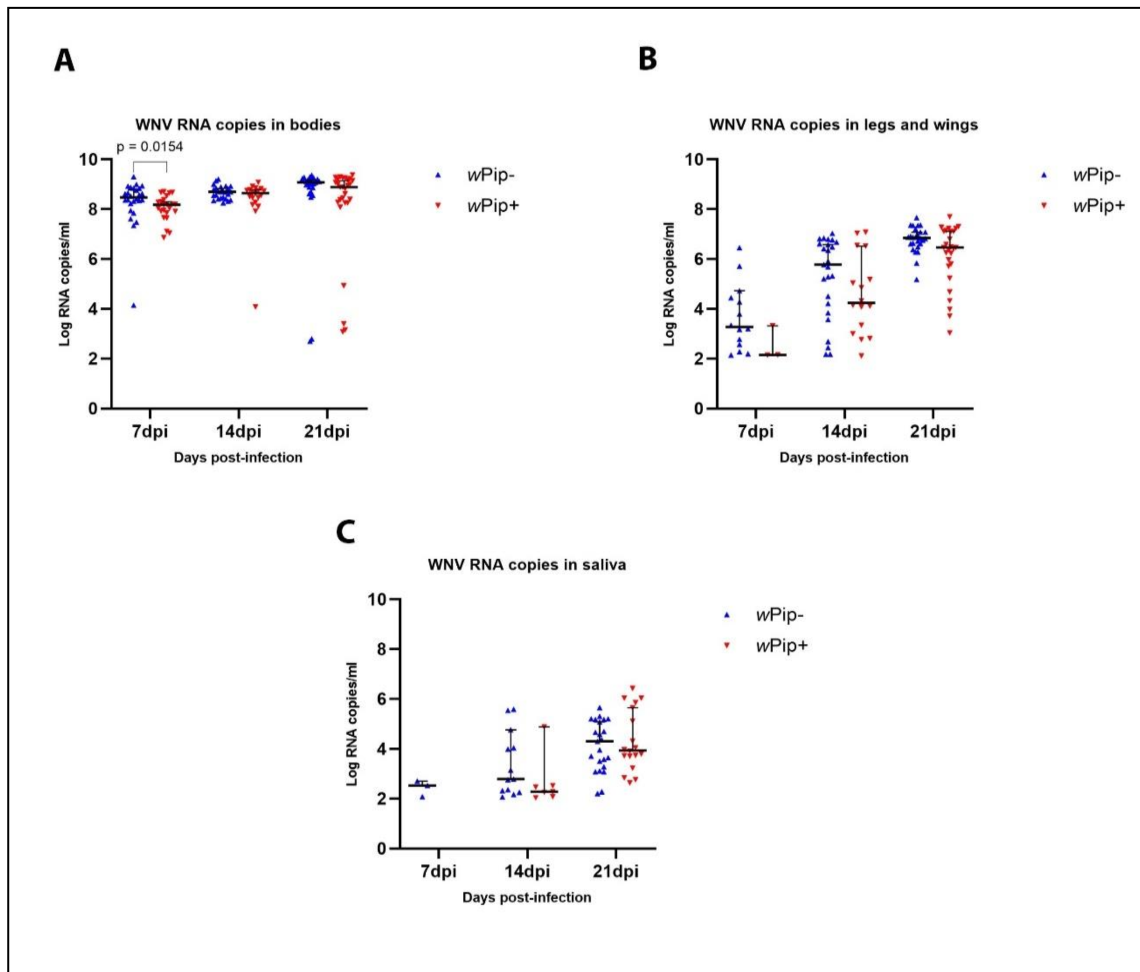

Fig 2- WNV RNA copies in *Cx. quinquefasciatus* body (A), legs plus wings (B), and saliva samples (C) at different dpi for $wPip^+$ and $wPip^-$ colonies. At each time point, 30 mosquitoes were screened for WNV. Error bars represent the median with 95% confidence intervals. Statistical significance at $P < 0.05$, was determined by the Mann-Whitney U test at each day post-infection, dpi. dpi, Day post-infection; WNV, West Nile virus; $wPip^-$/$wPip^+$, mosquitoes treated with tetracycline hydrochloride to clear *Wolbachia*/untreated mosquitoes.

WNV dissemination rates in mosquitoes lacking *Wolbachia* surpassed those observed in mosquitoes harboring the endosymbiont (Table 1). Among mosquitoes carrying *Wolbachia*, rates of 13.0% at 7 dpi, 84.2% at 14 dpi, and 96.4% at 21 dpi were recorded;

in comparison, mosquitoes lacking *Wolbachia* exhibited dissemination rates of 50% at 7 dpi, 100% at 14 dpi and 96.3% at 21 dpi. However, differences between the two groups were significant only at 7 dpi (Fisher's exact test, $P = 0.007$, OR = 6.67, 95% CI = 1.59–28.04) (Table 1).

The quantity of viral RNA copies in legs plus wings increased in both groups as the extrinsic incubation period of the virus advanced. In these tissues, mosquitoes lacking *Wolbachia* exhibited higher titers than mosquitoes harboring *Wolbachia*, but the differences were not statistically significant (Mann–Whitney U-tests, $U_{(17)} = 8.50$, $Z = -1.58$, $P = 0.121$ at 7 dpi; $U_{(41)} = 149$, $Z = -1.36$, $P = 0.179$ for 14 dpi; and $U_{(53)} = 241$, $Z = -1.97$, $P = 0.05$ at 21 dpi) (Fig. 2B).

Regarding transmission rates at 7 dpi, WNV was exclusively detected in the saliva of *Wolbachia*-free mosquitoes, with a transmission rate of 21.4%. At 14 dpi, mosquitoes harboring *Wolbachia* exhibited a transmission rate of 43.8%, compared to 52.0% recorded in mosquitoes lacking *Wolbachia*. At 21 dpi, the transmission rate for the *Wolbachia*-positive and *Wolbachia*-negative mosquitoes was 66.7% and 88.5%, respectively (Table 1). However, the differences in transmission rate between the two groups at 14 and 21 dpi were not significant (Fisher's exact tests, $P = 0.751$, OR = 1.39, 95% CI = 0.39–4.92 and $P = 0.099$, OR = 3.83, 95% CI = 0.90–16.26, respectively).

The transmission efficiency for mosquitoes lacking *Wolbachia* was 10% at 7 dpi and no viral transmission occurred in the $wPip^+$ group during this incubation period, as previously noted. By 14 dpi, transmission efficiencies of 43.3% and 23.3% were recorded for the $wPip^-$ and $wPip^+$ groups, respectively. At 21 dpi, peak transmission efficiencies were observed in both groups, reaching 76.7% for the $wPip^-$ group and 60% for the $wPip^+$ (Table 1). Although higher transmission efficiencies were noted at 14 and 21 dpi for mosquitoes lacking *Wolbachia*, these differences were not significant (Fisher's exact tests, $P = 0.170$, OR = 2.51, 95% CI = 0.83–7.64 for 14 dpi and $P = 0.267$, OR = 2.19, 95% CI = 0.72–6.70 at 21dpi).

WNV RNA copies in saliva increased with the extrinsic incubation period of the virus. At 14 and 21 dpi, mosquitoes without *Wolbachia* exhibited higher titers, although the differences were not statistically significant (Mann–Whitney U-tests, $U_{(20)} = 27.5$, $Z = -1.43$, P = 0.157 and $U_{(41)} = 225$, $Z = 0.47$, $P = 0.636$, respectively) (Fig. 2c).

3.4 Discussion

The results of this study showed that *Cx. quinquefasciatus* mosquitoes from Santiago are susceptible to the WNV strain tested during our investigation. To the best of our knowledge, this is the first assessment of the vector competence of a *Cx. quinquefasciatus* population from Cape Verde to WNV. The high transmission efficiency observed for the tested lineage 1 of WNV holds significant epidemiological importance for the archipelago, as it is the most prevalent lineage on the African continent, where it co-circulates concomitantly with lineages 2 and 8 [36].

Lutomiah *et al.* [37] tested the vector competence of *Cx. quinquefasciatus* from Kenya for WNV and found a transmission efficiency of 46% at 21 dpi. In Senegal, Fall *et al.* [11] documented a maximum transmission rate of 25% at 15 dpi for the local population of *Cx. quinquefasciatus*. In both of these African countries, WNV-infected *Cx. quinquefasciatus* has been detected in the field [36, 38], alongside the detection of the virus in humans, birds, and horses [3, 39]. These observations highlight the potential of *Cx. quinquefasciatus* for sustaining WNV transmission in Africa and the implications of our findings for the risk of WNV transmission in Cape Verde.

In other regions of the world, studies on the vector competence of *Cx. quinquefasciatus* for WNV have shown variable transmission rates. In Florida (USA), Richards *et al.* [40] documented low transmission rates for the local population of *Cx. quinquefasciatus*, with maximum values of 20% at 14 dpi. Goddard *et al.* [41] recorded maximum transmission efficiencies of 36–52% in California at 14 dpi. In China, Jiang *et al.* [42] reported a transmission efficiency of 37% at 14 dpi. In Europe, vector competence studies for WNV with the siblings species *Cx. pipiens sensu stricto* (*Cx. pipiens s.s.*) showed a maximum transmission efficiency of 21–33% at 32 dpi in Italy [9] and 40–53% at 21 dpi in Germany [43]. Overall, these values align with those obtained in the present study for Cape Verdean *Cx. quinquefasciatus*. Nonetheless, comparisons of WNV vector competence must be made with caution, as variations in transmission can be influenced by several factors, including the genetic variability of virus strains, incubation temperature, viral titer in the infected blood and mosquito populations studied [44–46]. These disparities underscore the need to standardize these parameters across studies to ensure comparability and consistency.

A recent study showed that *Cx. quinquefasciatus* from the island of Santiago prefers to feed on human blood, followed by chicken blood [47]. This mammophilic and ornithophilic tendency translates into a high potential for this mosquito to act as a bridge vector between avian and human hosts. In addition to this species, *Cx. pipiens s.s.* and hybrids between *Cx. pipiens s.s.* and *Cx. quinquefasciatus* are also present in Cape Verde [20, 48]. This may pose an increased risk for WNV transmission in the country since experimental infections have demonstrated a higher vector competence of hybrids compared to their parental species [49].

The lack of recent reports of WNV circulation in Cape Verde may reflect the absence of active virus surveillance. Cape Verde lies along the migration route of numerous African and European bird species [50–52], including the Eurasian collared dove (*Streptopelia decaocto*) and various species of herons and storks, all of which are well-known WNV reservoirs and could potentially initiate local transmission in the presence of competent vectors. Genetic surveillance studies suggest substantial WNV circulation between Europe and West Africa, consistent with the migratory flyways of Afro-Palearctic birds, including movements to and from neighboring countries of Cape Verde [3, 53]. In this context, the introduction of WNV surveillance in migratory and wild birds in Cape Verde, as well as in mosquitoes and humans living near the nesting areas, with the aim to confirm the current status of WNV circulation in the country, would be an important initiative.

Our results showed that the treated *Cx. quinquefasciatus* (wPip^{-}; *Wolbachia* free) exhibited a higher WNV dissemination rate than their wild-type counterparts, resulting in an earlier virus presence in the saliva of the former. Glaser and Meola [17] documented similar results, showing that *Cx. quinquefasciatus* lacking *Wolbachia* had a significantly higher viral dissemination rate than those harboring native *Wolbachia*. Alomar *et al.* [18] showed that the absence of *Wolbachia* in *Cx. quinquefasciatus* infected with WNV produces a higher viral load in conditions of low competition stress. In *Aedes albopictus*, native *Wolbachia* also appears to contribute to differences in arbovirus dissemination in mosquito tissues. Mousson *et al*. [54] showed that *Ae. albopictus* naturally super-infected with the *Wolbachia* strains *w*AlbA and *w*AlbB exhibited reduced Dengue virus (DENV) dissemination to the wings compared to mosquitoes without *Wolbachia*. In another study with *Aedes albopictus*, Tsai *et al.* [31] found that the presence of native *Wolbachia* was linked to reduced DENV titters in mosquito tissues.

Although our findings indicate that native *Wolbachia* confers an antiviral phenotype during WNV infection, other studies have reported conflicting results. Leggewie *et al*. [55] found no association between *Wolbachia* and WNV infection in experimentally exposed *Cx. pipiens s.s.* from Germany. Micieli and Glaser [30] observed a protective effect of *Wolbachia* against WNV in a long-established laboratory colony of *Cx. quinquefasciatus* but reported that a similar effect was absent in new colonies of the same mosquito species. More recently, however, Novakova *et al.* [56] demonstrated that an increased prevalence of WNV in field-caught *Culex pipiens sensu lato* from Canada was correlated with a reduced level of native *Wolbachia*, suggesting that the endosymbiont can induce protection in natural settings.

Wolbachia can be found in different tissues of the *Cx. pipiens*, including the midgut [57]. The lower dissemination rate we observed in *Cx. quinquefasciatus* harboring *Wolbachia* suggests that the endosymbiont may potentially integrate either the midgut infection barrier or the midgut escape barrier, delaying the virus dissemination to the legs, wings, and saliva. Our data revealed that WNV load in mosquito bodies lacking *Wolbachia* was significantly higher than that in those harboring *Wolbachia* and this disparity may have contributed to the observed increase in viral dissemination in *Wolbachia*-free mosquitoes. Several studies have demonstrated that viral dissemination within mosquitoes requires the virus load to reach a threshold in the insect's midgut [58, 59], implying that mosquitoes with midgut-to-hemocoel dissemination must have a higher viral load in their body than those without dissemination. Furthermore, it has been shown that manipulation of the RNA interference (RNAi) pathway in the midgut to either enhance or reduce virus replication resulted in a corresponding decrease or increase in viral disseminated infection [60]. These findings suggest that virus replication in the midgut significantly impacts dissemination, and our data indicate that native *Wolbachia* may play a role in impairing this process in *Cx. quinquefasciatus* infected with WNV.

Salivary gland barriers are another critical aspect for vector competence [61, 62]. In our study, although the transmission rate was higher for *Wolbachia*-free mosquitoes, the difference was not significant. These results suggest that the native *Wolbachia* protective effect did not occur at the salivary gland level and that differences in transmission rate likely resulted from varying levels of WNV dissemination from the mosquito midgut.

Previous studies showed that antiviral protection conferred by native *Wolbachia* is correlated with its density in host tissues [63–65]. We found no significant changes in *Wolbachia* density between the initial F1 generation and the F7 generation used in artificial infection. These results suggest that the protection provided by native *Wolbachia* in the mosquitoes in our study was not attributable to variations in the endosymbiont density within host tissues.

How *Wolbachia* impairs viral replication in mosquitoes is unclear. Two mechanisms have been suggested. In the first, *Wolbachia* may compete with the virus for cellular resources, such as cholesterol, which is necessary for virus replication [66–69]. The second proposed mechanism is that the symbiont triggers the activation of the host's immune system, including the Toll, IMD, and Jak/STAT signaling pathways and antimicrobial peptides [16, 70–72]. *Wolbachia* appears to be inefficient in activating the immune system in mosquitoes where it naturally resides, such as *Cx. quinquefasciatus*, a phenomenon attributed to the extended period of co-evolution between the endosymbiont and the host [73–75]. Therefore, it is reasonable to hypothesize that inhibition of WNV by *Cx. quinquefasciatus* native *Wolbachia* may be primarily due to nutrient competition rather than be immune-related. Supporting this hypothesis are studies that found no differences in the regulation of immune genes in *Culex* and *Aedes* mosquitoes due to their native *Wolbachia* [10, 74]. Further investigations on this mosquito species are warranted to validate this hypothesis.

3.5 Conclusion

Our findings showed that *Cx. quinquefasciatus* from Cape Verde transmitted the tested L1 strain of WNV, highlighting the significance of continued surveillance efforts to prevent virus transmission within the country. Regarding the effect of the mosquito species native *Wolbachia* on WNV transmission, our results showed that when the endosymbiont was removed from *Cx. quinquefasciatus*, the mosquitoes exhibited higher WNV body titers compared to that in their counterparts carrying *Wolbachia*, which resulted in earlier dissemination but not transmission of WNV. Additional research is needed to elucidate the mechanisms underlying *Wolbachia*-mediated viral protection in natural hosts and the extent to which this protection may affect the epidemiology of WNV.

Abbreviations

BSL3: Biosafety Level 3

CTAB: Cetyltrimethylammonium bromide

dpi: days post-infection

PFU: Plaque-forming Units

RT-qPCR: Quantitative real-time PCR

WNV: West Nile virus

VIASEF: *In vivo* Arthropod Security Facility

wsp: *Wolbachia* surface protein gene.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-024-06609-7.

Additional file 1: Table S1. Primer sequences employed in the molecular identification of the *Culex pipiens* complex species collected in Santiago Island, Cape Verde. **Table S2.** Primer sequences used to detect and quantify *Wolbachia* in *Culex quinquefasciatus* from Santiago Island, Cape Verde. **Table S3.** Probe and primer sequences used for West Nile virus detection by RT-qPCR.

Acknowledgments

We would like to express our appreciation to the National Institute of Public Health for offering field and laboratory support in Cape Verde.

Authors' contributions

AJFM, CAS, and JP designed the study. AJFM conducted fieldwork, insectary-related tasks, viral stock production, and mosquito infections. FT and GS performed viral stock production and mosquito infections. TM performed insectary-related tasks. CAS, AJFM, and JP drafted the manuscript. All authors read and approved the final manuscript.

Funding

Fundação para a Ciência e Tecnologia (FCT) for funds to GHTM - UID/04413/2020 and LA-REAL – LA/P/0117/2020. Aires da Moura received funding through the Ph.D. fellowship program of Camões I.P.

Availability of data and materials

No datasets were generated or analyzed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

3.6 References

1. Karim SU, Bai F. Introduction to West Nile virus. Methods Mol Biol. 2023;2585:1–7.

2. Clark MB, Schaefer TJ. West Nile virus. Treasure Island: StatPearls Publishing; 2024.

3. Mencattelli G, Ndione MHD, Rosà R, Marini G, Diagne CT, Diagne MM, *et al*. Epidemiology of West Nile virus in Africa: an underestimated threat. PLoS Negl Trop Dis. 2022;16:e0010075.

4. Ulbert S. West Nile virus vaccines– current situation and future directions. Hum Vaccin Immunother. 2019;15:2337–2342.

5. Fiacre L, Pagès N, Albina E, Richardson J, Lecollinet S, Gonzalez G. Molecular determinants of West Nile virus virulence and pathogenesis in vertebrate and invertebrate hosts. Int J Mol Sci. 2020; 21:9117.

6. Garrigós M, Garrido M, Panisse G, Veiga J, Martínez-de la Puente J. Interactions between West Nile virus and the microbiota of *Culex pipiens* vectors: a literature review. Pathogens. 2023;12:1287.

7. Koenraadt CJM, Möhlmann TWR, Verhulst NO, Spitzen J, Vogels CBF. Effect of overwintering on survival and vector competence of the West Nile virus vector *Culex pipiens*. Parasit Vectors. 2019;12:147.

8. Romo H, Papa A, Kading R, Clark R, Delorey M, Brault AC. Comparative vector competence of north American *Culex pipiens* and *Culex quinquefasciatus* for African and European lineage 2 West Nile viruses. Am J Trop Med Hyg. 2018; 98:1863-1869.

9. Fortuna C, Remoli ME, Di Luca M, Severini F, Toma L, Benedetti E, *et al*. Experimental studies on comparison of the vector competence of four Italian *Culex pipiens* populations for West Nile virus. Parasit Vectors. 2015; 8:463.

10. Tokash-Peters AG, Jabon JD, Fung ME, Peters JA, Lopez SG, Woodhams DC. Trans-generational symbiont transmission reduced at high temperatures in a West Nile virus vector mosquito *Culex quinquefasciatus*. Front Trop Dis. 2022; 3:1–12.

11. Fall G, Diallo M, Loucoubar C, Faye O, Sall AA. Vector competence of *Culex neavei* and *Culex quinquefasciatus* (Diptera: Culicidae) from Senegal for lineages 1, 2, Koutango and a putative new lineage of West Nile virus. Am J Trop Med Hyg. 2014; 90:747-754.

12. Rochlin I, Faraji A, Healy K, Andreadis TG. West Nile virus mosquito vectors in North America. J Med Entomol. 2019;56:1475–1490.

13. Landmann F. The *Wolbachia* endosymbionts. Microbiol Spectr; 2019;7:10.1128.

14. Flores HA, de Bruyne JT, O'Donnell TB, Nhu VT, Giang NT, Trang HTX, *et al*. Multiple *Wolbachia* strains provide comparative levels of protection against Dengue virus infection in *Aedes aegypti*. PLoS Pathog. 2020;16:e1008433.

15. Hugo LE, Rašić G, Maynard AJ, Ambrose L, Liddington C, Thomas CJE, *et al*. *Wolbachia w*AlbB inhibit dengue and Zika infection in the mosquito *Aedes aegypti* with an Australian background. PLoS Negl Trop Dis. 2022;16:e0010786.

16. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, *et al*. A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, chikungunya and *Plasmodium*. Cell. 2009;139:1268–1278.

17. Glaser RL, Meola MA. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One. 2010;5:e11977

18. Alomar AA, Pérez-Ramos DW, Kim D, Kendziorski NL, Eastmond BH, Alto BW, *et al*. Native *Wolbachia* infection and larval competition stress shape fitness and West Nile virus infection in *Culex quinquefasciatus* mosquitoes. Front Microbiol. 2023; 14:1138476.

19. Leal SDV, Varela IBF, Monteiro DDS, Ramos de Sousa CM, da Luz Lima Mendonça M, De Pina AJ, *et al.* Update on the composition and distribution of the mosquito fauna (Diptera: Culicidae) in Cabo Verde, a country at risk for mosquito-borne diseases. J Med Entomol. 2024; 61:919-924.

20. da Moura AJF, Valadas V, Da Veiga Leal S, Montalvo Sabino E, Sousa CA, Pinto J. Screening of natural *Wolbachia* infection in mosquitoes (Diptera: Culicidae) from the Cape Verde islands. Parasit Vectors. 2023; 16:142.

21. Instituto de Higiene e Medicina Tropical. Relatório do grupo de trabalho de luta contra o paludismo e erradicação dos culicídeos na república de Cabo Verde em colaboração com a brigada de luta contra o paludismo em Cabo Verde. Lisbon: IHMT; 1986.

22. Silver JB. Mosquito ecology: field sampling methods. 3rd ed. Dordrecht: Springer Netherlands; 2007.

23. Ribeiro H, Ramos HC. Guia ilustrado para a identificação dos mosquitos de Angola. 4th ed. Lisbon: Boletim da Sociedade Portuguesa de Entomologia; 1995.

24. Siria DJ, Batista EPA, Opiyo MA, Melo EF, Sumaye RD, Ngowo HS, *et al.* Evaluation of a simple polytetrafluoroethylene (PTFE)-based membrane for blood-feeding of malaria and dengue fever vectors in the laboratory. Parasit Vectors. 2018;11:236.

25. Weeks AR, van Opijnen T, Breeuwer JA. AFLP fingerprinting for assessing intraspecific variation and genome mapping in mites. Exp Appl Acarol. 2000; 24:775-793.

26. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids and other sibling species (Diptera: Culicidae). Am J Trop Med Hyg. 2004;70:339-45.

27. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294-9.

28. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc B Biol Sci. 1998; 265:509-15.

29. Shemshadian A, Vatandoost H, Oshaghi MA, Abai MR, Djadid ND, karimian F. Relationship between *Wolbachia* infection in *Culex quinquefasciatus* and its resistance to insecticide. Heliyon. 2021;7:e06749.

30. Micieli MV, Glaser RL. Somatic *Wolbachia* (Rickettsiales: Rickettsiaceae) levels in *Culex quinquefasciatus* and *Culex pipiens* (diptera: Culicidae) and resistance to West Nile virus infection. J Med Entomol. 2014;51:189-99.

31. Tsai CH, Chen TH, Lin C, Shu PY, Su CL, Teng HJ. The impact of temperature and *Wolbachia* infection on vector competence of potential dengue vectors *Aedes aegypti* and *Aedes albopictus* in the transmission of dengue virus serotype 1 in southern Taiwan. Parasit Vectors. 2017;10:551.

32. Parreira R, Severino P, Freitas F, Piedade J, Almeida APG, Esteves A. Two distinct introductions of the West Nile virus in Portugal disclosed by phylogenetic analysis of genomic sequences. Vector Borne Zoonotic Dis. 2007;7:344-352.

33. Brien JD, Lazear HM, Diamond MS. Propagation, quantification, detection and storage of West Nile virus. Curr Protoc Microbiol. 2013;31:15D.3.1-15D.3.18.

34. McAuley AJ, Beasley DWC. Propagation and titration of West Nile virus on Vero cells. Methods Mol Biol. 2016;1435:19–27.

35. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, *et al.* Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol. 2000;38:4066-4071.

36. Ndione MHD, Ndiaye EH, Faye M, Diagne MM, Diallo D, Diallo A, *et al*. Re-Introduction of West Nile virus lineage 1 in Senegal from Europe and subsequent circulation in human and mosquito populations between 2012 and 2021. Viruses. 2022; 14:2720.

37. Lutomiah JL, Koka H, Mutisya J, Yalwala S, Muthoni M, Makio A, *et al*. Ability of selected Kenyan mosquito (Diptera: Culicidae) species to transmit West Nile virus under laboratory conditions. J Med Entomol. 2011;48:1197-1201.

38. LaBeaud AD, Sutherland LJ, Muiruri S, Muchiri EM, Gray LR, Zimmerman PA, *et al*. Arbovirus prevalence in mosquitoes, Kenya. Emerg Infect Dis. 2011;17:233-241.

39. Nyamwaya D, Wang'ondu V, Amimo J, Michuki G, Ogugo M, Ontiri E, *et al*. Detection of West Nile virus in wild birds in Tana river and Garissa counties, Kenya. BMC Infect Dis. 2016;16:696.

40. Richards SL, Anderson SL, Lord CC. Vector competence of *Culex pipiens quinquefasciatus* (Diptera: Culicidae) for West Nile virus isolates from Florida. Trop Med Int Health. 2014;19:610-617.

41. Goddard LB, Roth AE, Reisen WK, Scott TW. Vector competence of California mosquitoes for West Nile virus. Emerg Infect Dis. 2002;8:1385-1391.

42. Jiang SF, Zhang YM, Guo XX, Dong Y De, Xing D, Xue R De, *et al.* Experimental studies on comparison of the potential vector competence of four species of *Culex mosquitoes* in China to transmit West Nile virus. J Med Entomol. 2010;47:788-790.

43. Holicki CM, Ziegler U, Răileanu C, Kampen H, Werner D, Schulz J, *et al.* West Nile virus lineage 2 vector competence of indigenous *Culex* and *Aedes* mosquitoes from Germany at temperate climate conditions. Viruses. 2020;12:561.

44. Vogels CBF, Fros J J, Göertz G P, Pijlman G P, Koenraadt CJM. Vector competence of northern European *Culex pipiens* biotypes and hybrids for West Nile virus is differentially affected by temperature. Parasit Vectors. 2016;9:393.

45. Anderson SL, Richards SL, Tabachnick WJ, Smartt CT. Effects of West Nile virus dose and extrinsic incubation temperature on temporal progression of vector competence in *Culex pipiens quinquefasciatus*. J Am Mosq Control Assoc. 2010;26:103–7.

46. Kilpatrick AM, Fonseca DM, Ebel GD, Reddy MR, Kramer LD. Spatial and temporal variation in vector competence of *Culex pipiens* and *Cx. restuans* mosquitoes for West Nile virus. Am J Trop Med Hyg. 2010;83:607-13.

47. Gonçalves AALM, Dias AHC, Monteiro DDS, Varela IBF, da Veiga Leal S. Blood meal survey reveals insights into mosquito-borne diseases on the island of Santiago, Cape Verde. Front Trop Dis. 2023; 4:1070172.

48. Gomes B, Alves J, Sousa CA, Santa-Ana M, Vieira I, Silva TL, *et al*. Hybridization and population structure of the *Culex pipiens* complex in the islands of Macaronesia. Ecol Evol. 2012;2:1889-1902.

49. Ciota AT, Chin PA, Kramer LD. The effect of hybridization of *Culex pipiens* complex mosquitoes on transmission of West Nile virus. Parasit Vectors. 2013;6:305.

50. Hazevoet C. Seventh report on birds from the Cape Verde Islands, including records of nine taxa new to the archipelago. Zool Caboverdiana. 2012;3:1-28.

51. Hazevoet C. Eighth report on birds from the Cape Verde Islands, including records of nine taxa new to the archipelago. Zool Caboverdiana. 2014;5:29–56.

52. BirdLife International. Data Zone, Cape Verde. Cambridge: BirdLife International; 2024. https://datazone.birdlife.org/country/cape-verde. Accessed 6 May 2024.

53. García-Carrasco JM, Muñoz AR, Olivero J, Segura M, Real R. Mapping the risk for West Nile virus transmission, Africa. Emerg Infect Dis. 2022;28:777–85.

54. Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB. The native *Wolbachia* symbionts limit transmission of Dengue virus in *Aedes albopictus*. PLoS Negl Trop Dis. 2012;6:e1989.

55. Leggewie M, Krumkamp R, Badusche M, Heitmann A, Jansen S, Schmidt-Chanasit J, *et al*. *Culex torrentium* mosquitoes from Germany are negative for *Wolbachia*. Med Vet Entomol. 2018 Mar; 32:115-120.

56. Novakova E, Woodhams DC, Rodríguez-Ruano SM, Brucker RM, Leff JW, Maharaj A, *et al.* Mosquito microbiome dynamics, a background for prevalence and seasonality of West Nile virus. Front Microbiol. 2017;4:526.

57. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, *et al. Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Biol. 1999;29:153–60.

58. Girard YA, Klingler KA, Higgs S. West Nile virus dissemination and tissue tropisms in orally infected *Culex pipiens quinquefasciatus*. Vector Borne Zoonotic Dis. 2004;4:109–22.

59. Vazeille M, Madec Y, Mousson L, Bellone R, Barré-Cardi H, Sousa CA, *et al.* Zika virus threshold determines transmission by European *Aedes albopictus* mosquitoes. Emerg Microbes Infect. 2019;8:1668–78.

60. Carpenter A, Clem RJ. Factors affecting arbovirus midgut escape in mosquitoes. Pathogens. 2023;12:220.

61. Lewis J, Gallichotte EN, Randall J, Glass A, Foy BD, Ebel GD, *et al*. Intrinsic factors driving mosquito vector competence and viral evolution: a review. Front Cell Infect Microbiol. 2023;13:1330600.

62. Kramer LD, Ebel GD. Dynamics of flavivirus infection in mosquitoes. Adv Virus Res. 2003;60:187–232.

63. Wei Y, Wang J, Wei Y-H, Song Z, Hu K, Chen Y, *et al*. Vector competence for DENV-2 among *Aedes albopictus* (Diptera: Culicidae) populations in China. Front Cell Infect Microbiol. 2021;11:649975.

64. Martinez J, Tolosana I, Ok S, Smith S, Snoeck K, Day JP, *et al.* Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. Mol Ecol. 2017;26:4072-84.

65. Martinez J, Longdon B, Bauer S, Chan YS, Miller WJ, Bourtzis K, *et al.* Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* Strains. PLOS Pathog. 2014;10:e1004369.

66. Edwards B, Ghedin E, Voronin D. *Wolbachia* interferes with Zika Virus replication by hijacking cholesterol metabolism in mosquito cells. Microbiol Spectr. 2023; 11:e0218023.

67. Geoghegan V, Stainton K, Rainey SM, Ant TH, Dowle AA, Larson T, *et al*. Perturbed cholesterol and vesicular trafficking associated with dengue blocking in *Wolbachia*-infected *Aedes aegypti* cells. Nat Commun. 2017;8:526.

68. Cloherty APM, Olmstead AD, Ribeiro CMS, Jean F. Hijacking of lipid droplets by Hepatitis C, Dengue and Zika viruses- from viral protein moonlighting to extracellular release. Int J Mol Sci. 2020;21:7901.

69. Osuna-Ramos JF, Reyes-Ruiz JM, del Ángel RM. The role of host cholesterol during flavivirus infection. Front Cell Infect Microbiol. 2018;8:388.

70. Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS, *et al. Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control Dengue virus in the mosquito *Aedes aegypti*. Proc Natl Acad Sci U S A. 2012;109:E23-E31.

71. Asad S, Parry R, Asgari S. Upregulation of *Aedes aegypti* vago1 by *Wolbachia* and its effect on Dengue virus replication. Insect Biochem Mol Biol. 2018;92:45–52.

72. Vandana V, Dong S, Sheth T, Sun Q, Wen H, Maldonado A, *et al. Wolbachia* infection-responsive immune genes suppress *Plasmodium falciparum* infection in *Anopheles stephensi*. PLOS Pathog. 2024;20:e1012145.

73. Zhang D, Wang Y, He K, Yang Q, Gong M, Ji M, *et al*. *Wolbachia* limits pathogen infections through induction of host innate immune responses. PLoS One. 2020;15: e0226736.

74. Molloy JC, Sinkins SP. *Wolbachia* do not induce reactive oxygen species-dependent immune pathway activation in *Aedes albopictus*. Viruses. 2015;7:4624–4639.

75. Zug R, Hammerstein P. *Wolbachia* and the insect immune system: what reactive oxygen species can tell us about the mechanisms of *Wolbachia*-host interactions. Front Microbiol. 2015;6:1201.

3.7 Supplementary information

Table S1- Primer sequences employed in the molecular identification of *Culex pipiens* complex species collected in Santiago Island, Cape Verde.

Species	Primers	Sequences (5'- 3')	References
Culex pipiens complex	ACEquin	CCTTCTTGAATGGCTGTGGCA	[1]
	ACEpip	GGAAACAACGACGTATGTACT	
	B1246s	TGGAGCCTCCTCTTCACGG	
Other species (COI)	LCOI1490_F1	GGTCAACAAATCATAAAGATATTG	[2]
	HCOI2198_R1	TAAACTTCAGGGTGACCAAAAAATCA	

Table S2- Primer sequences used to detect and quantify *Wolbachia* in *Culex quinquefasciatus* from Santiago Island, Cape Verde.

Target	Primers sequences (5'-3')	Size (bp)	References
wsp (conventional PCR)	81F: TGGTCCAATAAGTGATGAAGAAA 691R: AAAAATTAAACGCTACTCCA	610	[3]
wsp (qPCR)	Forward: GCAAACAGTGTGGCAGCATT Reverse: CACCAACACCAACACCAACG	100	[This study]
18S ribossomal	Cx18S-F: TCAGATGTTGATACCGTCGGC Cx18S-R: ATCAGGTCACACTACACCGC	94	[This study]

Table S3- Primers and probes used for West Nile Virus detection by RT-qPCR.

Target	Primers sequences (5'-3')	Size (bp)	References
WNV Env	WNENV-F: TCAGCGATCTCTCCACCAAAG WNENV-R: GGGTCAGCACGTTTGTCATTG	70bp	[4]
Probe	FAM 5´-TGCCCGACCATGGGAGAAGCTC-3´ TAMRA	-	

References:

1. Smith JL, Fonseca DM. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids and other sibling species (Diptera: Culicidae). Am J Trop Med Hyg. 2004;

2. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 1994;3:294–9.

3. Zhou W, Rousset F, O'Neill S. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc R Soc B Biol Sci. 1998;265:509–15.

4. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, *et al*. Rapid Detection of West Nile virus from Human Clinical Specimens, Field-Collected Mosquitoes and Avian Samples by a TaqMan Reverse Transcriptase-PCR Assay. J Clin Microbiol. 2000;38:4066–71.

CHAPTER 4: General Discussion

In this study, we explored the prevalence and genetic diversity of *Wolbachia* in mosquitoes from Cape Verde while also investigating how this naturally occurring endosymbiont influences the vector competence of its mosquito hosts.

Our findings revealed a high prevalence of *Wolbachia* in mosquitoes belonging to the *Cx. pipiens* complex (*Cx. pipiens s.s*., *Cx. quinquefasciatus* and their hybrids), as well as in *Cx. tigripes*, while absent in key vectors such as *Ae. aegypti* and *An. arabiensis*. Additionally, experiments involving artificial infection suggested that the presence of native *Wolbachia* in *Cx. quinquefasciatus* may impact the susceptibility of this mosquito to WNV.

Our study provides the first genetic characterization of *Wolbachia* in *Cx. pipiens s.l.* from Cape Verde. Given that *Wolbachia* is maternally transmitted across generations, analyzing its genetic diversity in *Cx. pipiens s.l.* populations allows to trace host lineage and migration patterns, thereby offering insights into the origins and dispersal history of these mosquitoes [1]. The diversity of *w*Pip groups (*w*Pip-II, *w*Pip-III, and *w*Pip-IV) detected in *Cx. pipiens s.l.* from Cape Verde sheds light on the origins of the species in the archipelago, indicating that multiple introduction events have contributed to their establishment in the archipelago.

Since its discovery by the Portuguese in the 1460s, Cape Verde has held a key role within the transatlantic trade network, marked by significant maritime activity between the archipelago, Europe, West Africa, and the Americas [2], which likely facilitated the introduction of *Culex* species from these regions. Notably, all *w*Pip groups identified in Cape Verde are also present in Europe, suggesting a probable European origin [3,4]. However, the possibility of a North African contribution to *w*Pip-IV remains compelling, especially considering recent findings in Tunisia, Algeria, and Morocco [5].

The presence of *w*Pip-III on Fogo Island may also suggest an origin from the Americas, given the predominance of *w*Pip-III in that continent [4]. This perspective is further supported by Fonseca *et al.* [6], whose study presents evidence of a recent introduction of *Cx. quinquefasciatus* into West Africa from the New World [6]. The absence of *w*Pip-I in Cape Verde is particularly intriguing, given its widespread prevalence in both sub-

Saharan West African countries and in Portugal [4,7], regions that have maintained strong commercial and historical ties with Cape Verde for centuries.

Given that both *Wolbachia* and mitochondria are predominantly inherited through the maternal line, with limited influence from horizontal transmission, a combined analysis of mitochondrial DNA and *Wolbachia* in *Culex* mosquitoes from Cape Verde could provide valuable insights into the origins of mosquito populations across the different islands. Numerous studies have already shown associations between specific COI haplotypes and *w*Pip groups [3,7,8], emphasizing the significance of this approach for unraveling the evolutionary history of mosquito populations. This joint analysis could thereby offer a more precise understanding of their genetic lineage and migration patterns in Cape Verde.

Beyond providing insights into the origins of the mosquito population, these findings provide perspectives on how *Wolbachia*-based control programs could be effectively implemented to control *Cx. pipiens s.l.* populations in Cape Verde. Several studies have shown that *w*Pip strains from the same group generally promote compatibility between their hosts, whereas crosses between different groups are more likely to result in CI [9,10]. Therefore, understanding the diversity and distribution of *w*Pip groups in *Cx. pipiens s.l.* populations from Cape Verde is essential for designing effective IIT approaches. This knowledge will enable targeted laboratory crossing experiments to identify the most suitable *w*Pip strain for IIT deployment, optimizing population suppression efforts, without relying on costly and labor-intensive *Wolbachia* microinjection techniques. This approach has previously proven successful in controlling *Culex* species in Burma and has also been effectively tested under semi-field conditions in La Réunion [11,12].

Experimental infection studies revealed high susceptibility of *Cx. quinquefasciatus* from Santiago Island to WNV, highlighting the considerable risk of virus transmission in Cape Verde. This emphasizes the importance of employing effective vector control strategies to mitigate the potential for an outbreak. Moreover, *Cx. pipiens/Cx. quinquefasciatus* hybrids were found on the islands of Santo Antão, Fogo, and Maio, which further amplifies the country's vulnerability to WNV transmission. This concern is supported by Ciota *et al.* [13], who demonstrated that hybridization significantly enhances vector competence for WNV. Thus, the widespread presence of these hybrids throughout the

archipelago may considerably elevate the risk of WNV transmission, underscoring the critical need for proactive surveillance and effective vector control measures.

Artificial infection experiments demonstrated that, in the absence of *Wolbachia*, *Cx. quinquefasciatus* exhibited a more rapid dissemination of WNV, potentially driven by higher viral titers in the thorax and abdomen during the initial stages of infection. Similar protective effects of native *Wolbachia* against viruses have been observed in studies involving several hosts, including *Cx. pipiens s.l.*, *Ae. albopictus* and *D. melanogaster* [14–16]. However, once the virus reached the mosquito's saliva, the protective effect of native *Wolbachia* seemed to fade, demonstrating no impact on limiting viral transmission. This suggests that the protection provided by native *Wolbachia* is only effective during the early stages of infection and is insufficient to completely block transmission. These results contrast with experiments involving transinfected *Wolbachia*, in which the endosymbiont shows a strong ability to inhibit virus infection and transmission throughout the entire extrinsic incubation period [17,18].

The reduced impact of *Wolbachia* on vector competence in its native hosts, compared to transinfected hosts, may be attributed to the lower densities of the endosymbiont in natural populations. Several studies have shown that novel *Wolbachia* transinfections enable a more extensive dissemination of the endosymbiont across the mosquito's somatic tissues, resulting in an overall higher density compared to native hosts [19–21]. Additionally, *Wolbachia* density-dependent protection has been observed consistently in both laboratory and field conditions [21–23]. Together, these findings imply that the lower *Wolbachia* density typically found in the somatic tissues of native hosts may be insufficient to provide a high viral resistance.

The impact of native *Wolbachia* on WNV dissemination sparks intriguing questions about its role in natural ecosystems. *Wolbachia* prevalence in *Cx. quinquefasciatus* populations can vary substantially, from 30% to nearly 100% [24–26] and elevated temperatures have been linked to reduced *Wolbachia* densities and transgenerational transmission [27,28]. This implies that environmental factors affecting *Wolbachia* prevalence might shape WNV dynamics in natural mosquito populations. Evidence from Novakova *et al*. [23] supports this, as higher temperatures were correlated with reduced *Wolbachia* density and increased WNV infection in field-collected *Cx. pipiens*. Despite these observations, the

epidemiological impact of native *Wolbachia* on WNV infection is not fully understood and more extensive field studies are required.

The pathogen-blocking effect mediated by native *Wolbachia*, as demonstrated here, presents an opportunity to uncover its underlying protective mechanisms. A key focus of this investigation could be related to understanding how *Wolbachia* density is regulated in natural hosts. Recent findings suggest that this regulation is driven by genetic factors inherent to the endosymbiont itself [29] and exploring or manipulating these genetic factors could potentially increase *Wolbachia* density within its hosts. Advances in this field are encouraging, and a recently developed forward genetics screening method enabled the isolation of *Wolbachia* mutants in *Drosophila* that exhibited over-proliferation, resulting in enhanced antiviral effects in their natural hosts [30]. Future research could adopt similar strategies for mosquitoes with native *Wolbachia* to improve their antiviral protection further.

In the course of the field-based investigations, a novel strain of *Wolbachia* (without an assigned MLST profile) was discovered in *Cx. tigripes*. This discovery marks the first detection of *Wolbachia* in this mosquito species. Its genetic proximity to the *Wolbachia* strains *w*AlbB from *Ae. albopictus* and *w*Pip from *Cx. pipiens s.l.* opens perspectives about its potential utility for vector control. The *w*AlbB and *w*Pip strains have been used to control major vectors such as *Ae. aegypti, Ae. albopictus* and *Cx. pipiens s.l.*, effectively inducing reproductive incompatibility or blocking the transmission of arboviruses [11,31,32]. Future research is needed to assess the potential of this newly identified and closely related *Wolbachia* strain detected in *Cx. tigripes* to control major vector species. This will include evaluating its stability in transinfected vectors, ensuring its reliable maternal transmission, and assessing its impact on host fitness, as well as its ability to induce CI and inhibit pathogen transmission. Validation of these phenotypic traits would provide a foundation for the development of an effective *Wolbachia*-based vector control strategy using this new strain.

The absence of *Wolbachia* in *Anopheles* species from Cape Verde adds further complexity to the understanding of *Wolbachia* dynamics in vector populations. While no evidence of infection was observed in this study, recent research has identified the presence of native *Wolbachia* strains in several malaria vector populations in West Africa [33,34]. This

divergence highlights potential regional or species-specific factors influencing *Wolbachia* prevalence in *Anopheles* species and suggests that Cape Verde may represent a unique ecological context in which *Wolbachia* is absent or undetectable in *Anopheles* populations. However, the lack of detection in *Anopheles* could also be attributed to the limited sampling of this genus in the present study, which may have reduced the likelihood of detecting the endosymbiont. This highlights the need for larger-scale sampling efforts to draw more definitive conclusions regarding *Wolbachia* prevalence in *Anopheles* populations from Cape Verde.

Laboratory findings suggest that predominance of the bacterium *Asaia* in the *Anopheles* microbiome presents a biological obstacle to the establishment and maternal transmission of *Wolbachia*, which may explain the low prevalence of *Wolbachia* in *Anopheles* species [35,36]. This unique predominance of *Asaia* in *Anopheles* species is now being explored as a foundation for developing innovative malaria control strategies centered around this bacterium.

While transinfected *Anopheles* lines harboring *Wolbachia* have been successfully developed in laboratory settings, their practical application in malaria control remains a distant goal [37]. Additionally, the lack of robust tools for the genetic transformation of *Wolbachia* hampers its development as a paratransgenic agent despite recent advances in attempts to culture the endosymbiont under cell-free conditions [38,39]. In this context, the easily cultivable and genetically modified bacterium *Asaia* has emerged as a viable alternative to *Wolbachia*, offering distinct advantages for malaria control interventions [40]. Genetic engineering of *Asaia* has enabled its use in producing and delivering RNAi molecules targeting mosquito fitness or parasite development [41]. The genetically modified *Asaia* is also efficient in expressing anti-plasmodial molecules such as scorpine and immunotoxins (anti-Pbs21 scFv-Shiva1) that reduce the number of *P. berghei* oocysts in the mosquito midgut [42]. Additionally, *Asaia* colonization has been shown to activate mosquito immune genes, such as defensin and cecropin, which reduced *P. berghei* development in *An. stephensi* [43]. Semi-field trials using *Asaia*-infected *An. stephensi* and *An. gambiae* showed that the symbiont could be effectively transmitted within mosquito populations, confirming its feasibility for large-scale interventions [44]. Further studies and trials are needed to ensure the effectiveness of this approach against

Plasmodium species that infect humans, such as *P. falciparum* or *P. vivax*, before it can be used on a larger scale in the field.

Another bacterium that has garnered significant interest for its potential use in paratransgenesis to control malaria transmission is *Serratia spp*. Foundational studies highlight the potential of a genetically modified strain of the bacteria (*Serratia* AS1) to colonize *Anopheles* tissues such as midguts and reproductive organs, coupled with its ability to express anti-*Plasmodium* effector proteins that disrupt the *P. falciparum* life cycle [45]. The bacterium's versatility is underscored by its ability to be transmitted via multiple acquisition routes across major vectors such as *An. stephensi*, *Cx. pipiens s.s.* and *Cx. quinquefasciatus*, enhancing its potential for dissemination in natural populations [46]. Despite these encouraging advances, *Serratia*-based approaches have yet to transition into field trials, a critical step for assessing their real-world applicability.

Wolbachia was not detected in *Ae. aegypti* populations from Cape Verde. This finding aligns with the majority of studies, reinforcing the rarity of *Wolbachia* in natural populations of this species and supporting the prevailing hypothesis that reports of *Wolbachia* detection in *Ae. aegypti* are likely attributable to laboratory or field contamination rather than the presence of a stable symbiotic relationship [47,48]. The absence of a natural association between *Wolbachia* and *Ae. aegypti* contrasts with its widespread occurrence in other major vectors, such as *Ae. albopictus* and *Cx. pipiens s.l.,* suggesting that intrinsic genetic or ecological factors may inhibit the establishment of *Wolbachia* in *Ae. aegypti*.

Given the natural absence of *Wolbachia* infection in *Ae. aegypti*, recent research has concentrated on investigating the potential of other native bacterial symbionts within this mosquito species, recognizing their potential for playing an important role in advancing vector control strategies. For instance, *Bacillus*, *Enterobacter,* and *Stenotrophomonas* strains isolated from *Ae. aegypti* larvae have demonstrated significant larvicidal activity when reintroduced into the mosquito, highlighting their potential as novel bioinsecticides targeting the vector [49]. Furthermore, Ramirez *et al.* [50] demonstrated that *Chromobacterium sp.* (*Csp_P* isolate), derived from the midgut of field-captured *Ae. aegypti,* is capable of degrading viral envelope proteins of DENV and ZIKV. Additionally,

Csp_P exhibited efficacy against *P. falciparum* in studies involving *An. gambiae*, underscoring its broad-spectrum antipathogenic properties [51].

Despite the promising potential of paratransgenesis-based strategies leveraging microbial symbionts such as *Chromobacterium sp*., their application in controlling arbovirus transmission by *Ae. aegypti* remains predominantly experimental and less developed compared to *Wolbachia*-based transinfection approaches, which demonstrated practical effectiveness in field applications [52,53].

Currently, vector control in Cape Verde relies primarily on diesel application in breeding sites (posing significant environmental harm), the use of natural predators (*Gambusia sp*.), and chemical insecticides such as temephos and deltamethrin [54]. However, the emerging insecticide resistance in *Ae. aegypti, Cx. pipiens s.l.* and *An. arabiensis* raises significant concerns about the efficacy of these methods [55–57]. The recent outbreaks of Zika and dengue [58,59], combined with the high levels of trade and air travel between the islands and endemic areas, emphasize the urgent need for innovative alternatives. The introduction of *Wolbachia* into vector control in Cape Verde could offer a promising and sustainable strategy to address the pressing challenges faced by the archipelago without the negative environmental consequences of existing methods while also addressing the growing problem of insecticide resistance.

Conclusions

The present study revealed a high prevalence and genetic diversity of *Wolbachia* in *Culex* mosquitoes from Cape Verde. The distinct genetic groups of *Wolbachia* observed in *Cx. pipiens s.l.* across the islands reflect multiple introduction and colonization events shaping the evolutionary history of these mosquito species in the archipelago. Additionally, the study identified a novel *Wolbachia* strain in *Cx. tigripes,* which holds potential for further exploration as a means to develop innovative vector control strategies through CI.

In *Cx. quinquefasciatus*, the natural presence of *Wolbachia* was shown to significantly reduce the initial dissemination of WNV in infected mosquitoes, suggesting the potential for a protective role of this bacterium against arboviruses, even in its native hosts. However, this protective effect diminished once the virus reached the mosquito's saliva, having no significant impact on limiting viral transmission. These findings highlight that the antiviral effects of native *Wolbachia* are primarily effective during the early stages of infection but are insufficient to block transmission. To this extent, future investigations should focus on the characterization and selection of *Wolbachia* genetic variants that exhibit enhanced antiviral effects when infecting their natural hosts.

4.1 References

1. Atyame CM, Duron O, Tortosa P, Pasteur N, Fort P, Weill M. Multiple *Wolbachia* determinants control the evolution of cytoplasmic incompatibilities in *Culex pipiens* mosquito populations. Mol Ecol. 2011;20:286–98.

2. Lobban RA. Cape Verde: Crioulo Colony To Independent Nation. Routledge; 1995.

3. Lilja T, Lindström A, Hernández-Triana LM, Luca M Di, Lwande OW. European *Culex pipiens* populations carry different strains of *Wolbachia pipientis.* Insects. 2024;15:639.

4. Dumas E, Atyame CM, Milesi P, Fonseca DM, Shaikevich E V., Unal S, *et al*. Population structure of *Wolbachia* and cytoplasmic introgression in a complex of mosquito species. BMC Evol Biol. 2013;13:181

5. Tmimi FZ, Bkhache M, Mounaji K, Failloux AB, Sarih M. First report of the endobacteria *Wolbachia* in natural populations of *Culex pipiens* in Morocco. J Vector Ecol. 2017;42:349–51.

6. Fonseca DM, Smith JL, Wilkerson RC, Fleischer RC. Pathways of expansion and multiple introductions illustrated by large genetic differentiation among worldwide populations of the southern house mosquito. Am J Trop Med Hyg. 2006; 74:284–9.

7. Shaikevich E V., Vinogradova EB, Bouattour A, Gouveia De Almeida AP. Genetic diversity of *Culex pipiens* mosquitoes in distinct populations from Europe: Contribution of Cx. quinquefasciatus in Mediterranean populations. Parasit and Vectors. 2016;9:1–16.

8. Atyame C, Delsuc F, Pasteur N, Weill M, Duron O. Diversification of *Wolbachia* endosymbiont in the *Culex pipiens* mosquito. Mol Biol Evol. 2011;28:2761–72.

9. Atyame C, Labbé P, Dumas E, Milesi P, Charlat S, Fort P, *et al*. *Wolbachia* divergence and the evolution of cytoplasmic incompatibility in *Culex pipiens*. PLoS One. 2014;9:21–6.

10. Bonneau M, Atyame C, Beji M, Justy F, Cohen-Gonsaud M, Sicard M, *et al*. *Culex pipiens* crossing type diversity is governed by an amplified and polymorphic operon of *Wolbachia*. Nat Commun. 2018;9:1–10.

11. Atyame CM, Cattel J, Lebon C, Flores O, Dehecq JS, Weill M, *et al*. *Wolbachia*-based

population control strategy targeting *Culex quinquefasciatus* mosquitoes proves efficient under semi-field conditions. PLoS One. 2015;10:e0119288.

12. Laven H. Eradication of *Culex pipiens fatigans* through Cytoplasmic Incompatibility. Nat.1967;216:383–4.

13. Ciota AT, Chin PA, Kramer LD. The effect of hybridization of *Culex pipiens* complex mosquitoes on transmission of West Nile virus. 2013;6:305.

14. Glaser RL, Meola MA. The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. PLoS One. 2010;5:e11977.

15. Mousson L, Zouache K, Arias-Goeta C, Raquin V, Mavingui P, Failloux AB. The Native *Wolbachia* Symbionts Limit Transmission of Dengue virus in *Aedes albopictus*. PLoS Negl Trop Dis. 2012;6:e1989.

16. Chrostek E, Martins N, Marialva MS, Teixeira L. *Wolbachia*-Conferred Antiviral Protection Is Determined by Developmental Temperature. MBio. 2021;12:e0292320.

17. Flores HA, de Bruyne JT, O'Donnell TB, Nhu VT, Giang NT, Trang HTX, *et al*. Multiple *Wolbachia* strains provide comparative levels of protection against Dengue virus infection in *Aedes aegypti*. PLoS Pathog. 2020;16:e1008433.

18. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Hedges LM, *et al*. A *Wolbachia* Symbiont in *Aedes aegypti* Limits Infection with dengue, Chikungunya and *Plasmodium*. Cell. 2009;139:1268–78.

19. Ant TH, Sinkins SP. A *Wolbachia* triple-strain infection generates self-incompatibility in *Aedes albopictus* and transmission instability in *Aedes aegypti*. Parasit Vectors. 2018;11:1–7.

20. Blagrove MSC, Arias-Goeta C, Failloux AB, Sinkins SP. *Wolbachia* strain *w*Mel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. Proc Natl Acad Sci U S A. 2012;109:255–60.

21. Lu P, Bian G, Pan X, Xi Z. *Wolbachia* induces density-dependent inhibition to Dengue virus in mosquito cells. PLoS Negl Trop Dis. 2012;6:e1754.

22. Micieli MV, Glaser RL. Somatic *Wolbachia* (Rickettsiales: Rickettsiaceae) levels in *Culex quinquefasciatus* and *Culex pipiens* (Diptera: Culicidae) and resistance to West Nile virus infection. J Med Entomol. 2014;51:189–99.

23. Novakova E, Woodhams DC, Rodríguez-Ruano SM, Brucker RM, Leff JW, Maharaj A, *et al*. Mosquito Microbiome Dynamics, a Background for Prevalence and Seasonality of West Nile virus. Front Microbiol. 2017;8:526.

24. Carvajal TM, Hashimoto K, Harnandika RK, Amalin D, Watanabe K. Detection of *Wolbachia* in Field-Collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. Parasit Vectors. 2019;12:361.

25. Goindin D, Cannet A, Delannay C, Ramdini C, Gustave J, Atyame C, *et al*. Screening of natural *Wolbachia* infection in *Aedes aegypti*, *Aedes taeniorhynchus* and *Culex quinquefasciatus* from Guadeloupe (French West Indies). Acta Trop. 2018; 185:314–7.

26. Karami M, Moosa-Kazemi SH, Oshaghi MA, Vatandoost H, Sedaghat MM, Rajabnia R, *et al*. *Wolbachia* Endobacteria in Natural Populations of *Culex pipiens* of Iran and Its Phylogenetic Congruence. J Arthropod Borne Dis. 2016;10:347.

27. Tokash-Peters AG, Jabon JD, Fung ME, Peters JA, Lopez SG, Woodhams DC. Trans-generational symbiont transmission reduced at high temperatures in a West Nile virus vector mosquito *Culex quinquefasciatus*. Front Trop Dis. 2022;3:1–12.

28. Caragata EP. Susceptibility of *Wolbachia* mosquito control to temperature shifts. Nat Clim Chang. 2023;13:767–8.

29. Bénard A, Henri H, Noûs C, Vavre F, Kremer N. *Wolbachia* load variation in *Drosophila* is more likely caused by drift than by host genetic factors. Peer Community Evol Biol. 2021;1.

30. Duarte EH, Carvalho A, López-Madrigal S, Costa J, Teixeira L. Forward genetics in *Wolbachia*: Regulation of *Wolbachia* proliferation by the amplification and deletion of an addictive genomic island. PLOS Genet. 2021;17: e1009612.

31. Caputo B, Moretti R, Manica M, Serini P, Lampazzi E, Bonanni M, *et al*. A bacterium against the tiger: preliminary evidence of fertility reduction after release of *Aedes albopictus* males with manipulated *Wolbachia* infection in an Italian urban area. Pest

Manag Sci. 2020; 76:1324–32.

32. Maciel-de-Freitas R, Sauer FG, Kliemke K, Garcia GA, Pavan MG, David MR, *et al*. *Wolbachia* strains *w*Mel and *w*AlbB differentially affect *Aedes aegypti* traits related to fecundity. Microbiol Spectr. 2024;12:e0012824.

33. Niang EHA, Bassene H, Makoundou P, Fenollar F, Weill M, Mediannikov O. First report of natural *Wolbachia* infection in wild *Anopheles funestus* population in Senegal. Malar J. 2018;17:408.

34. Jeffries CL, Lawrence GG, Golovko G, Kristan M, Orsborne J, Spence K, *et al*. Novel *Wolbachia* strains in *Anopheles* malaria vectors from Sub-Saharan Africa. Wellcome Open Res. 2018;3:113.

35. Hughes GL, Dodson BL, Johnson RM, Murdock CC, Tsujimoto H, Suzuki Y, *et al*. Native microbiome impedes vertical transmission of *Wolbachia* in *Anopheles* mosquitoes. Proc Natl Acad Sci U S A. 2014;111:12498–503.

36. Rossi P, Ricci I, Cappelli A, Damiani C, Ulissi U, Mancini MV, *et al*. Mutual exclusion of Asaia and *Wolbachia* in the reproductive organs of mosquito vectors. Parasit Vectors. 2015;8:1–10.

37. Joshi D, Pan X, McFadden MJ, Bevins D, Liang X, Lu P, *et al*. The maternally inheritable *Wolbachia w*AlbB induces refractoriness to Plasmodium berghei in *Anopheles stephensi*. Front Microbiol. 2017;8:366

38. Krafsur AM, Ghosh A, Brelsfoard CL. Phenotypic Response of *Wolbachia pipientis* in a Cell-Free Medium. Microorganisms. 2020; 8:1–18.

39. Minwuyelet A, Petronio GP, Yewhalaw D, Sciarretta A, Magnifico I, Nicolosi D, *et al*. Symbiotic *Wolbachia* in mosquitoes and its role in reducing the transmission of mosquito-borne diseases: updates and prospects. Front Microbiol. 2023;14:1267832.

40. Rami A, Raz A, Zakeri S, Dinparast Djadid N. Isolation and identification of *Asaia sp*. in *Anopheles spp*. mosquitoes collected from Iranian malaria settings: Steps toward applying paratransgenic tools against malaria. Parasit Vectors. 2018;11:1–8.

41. Asgari M, Ilbeigikhamsehnejad M, Rismani E, Dinparast Djadid N, Raz A. Molecular

characterization of RNase III protein of *Asaia sp*. for developing a robust RNAi-based paratransgenesis tool to affect the sexual life cycle of *Plasmodium* or *Anopheles* fitness. Parasit Vectors. 2020; 13:1–19.

42. Ratcliffe NA, Furtado Pacheco JP, Dyson P, Castro HC, Gonzalez MS, Azambuja P, *et al*. Overview of paratransgenesis as a strategy to control pathogen transmission by insect vectors. Parasit Vectors. 2022; 15:112.

43. Cappelli A, Damiani C, Mancini MV, Valzano M, Rossi P, Serrao A, *et al*. *Asaia* Activates Immune Genes in Mosquito Eliciting an Anti-*Plasmodium* Response: Implications in Malaria Control. Front Genet. 2019; 10:836.

44. Mancini MV, Spaccapelo R, Damiani C, Accoti A, Tallarita M, Petraglia E, *et al*. Paratransgenesis to control malaria vectors: a semi-field pilot study. Parasit Vectors. 2016;9:140.

45. Wang S, Dos-Santos ALA, Huang W, Liu KC, Oshaghi MA, Wei G, *et al*. Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. Science. 2017;357:1399–402.

46. Koosha M, Vatandoost H, Karimian F, Choubdar N, Oshaghi MA. Delivery of a Genetically Marked *Serratia AS1* to Medically Important Arthropods for Use in RNAi and Paratransgenic Control Strategies. Microb Ecol. 2019;78:185–94.

47. Gloria-Soria A, Chiodo TG, Powell JR. Lack of Evidence for Natural *Wolbachia* Infections in *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. 2018;55:1354–6.

48. Ross PA, Hoffmann AA. Revisiting *Wolbachia* detections: Old and new issues in *Aedes aegypti* mosquitoes and other insects. Ecol Evol. 2024;14: e11670.

49. de Oliveira JC, de Melo Katak R, Muniz VA, de Oliveira MR, Rocha EM, da Silva WR, *et al*. Bacteria isolated from *Aedes aegypti* with potential vector control applications. J Invertebr Pathol. 2024;204:108094.

50. Ramirez JL, Short SM, Bahia AC, Saraiva RG, Dong Y. *Chromobacterium Csp_P* reduces malaria and dengue infection in vector mosquitoes and has entomopathogenic and *in vitro* anti-pathogen activities. PLoS Pathog. 2014;10:1004398.

51. Saraiva RG, Huitt-Roehl CR, Tripathi A, Cheng YQ, Bosch J, Townsend CA, *et al*. *Chromobacterium spp.* mediate their anti-*Plasmodium* activity through secretion of the histone deacetylase inhibitor romidepsin. Sci Rep. 2018;8:6176.

52. Scolari F, Casiraghi M, Bonizzoni M. *Aedes spp.* and their microbiota: a review. Front Microbiol. 2019;10:469067.

53. Ross PA, Robinson KL, Yang Q, Callahan AG, Schmidt TL, Axford JK, *et al*. A decade of stability for *w*Mel *Wolbachia* in natural *Aedes aegypti* populations. PLOS Pathog. 2022;18: e1010256.

54. Salgueiro P, Serrano C, Gomes B, Alves J, Sousa CA, Abecasis A, *et al*. Phylogeography and invasion history of *Aedes aegypti*, the dengue and Zika mosquito vector in Cape Verde islands (West Africa). Evol Appl. 2019; 12:1797–811.

55. da Cruz DL, Paiva MHS, Guedes DRD, de Souza Gomes EC, Pires SG, Gomez LF, *et al*. First report of the L1014F *kdr* mutation in wild populations of *Anopheles arabiensis* in Cabo Verde, West Africa. Parasit Vectors. 2021; 14:582.

56. Pires S, Alves J, Dia I, Gómez LF. Susceptibility of mosquito vectors of the city of Praia, Cabo Verde, to Temephos and *Bacillus thuringiensis* var *israelensis*. PLoS One. 2020;15: e0234242.

57. Rocha HDR, Paiva MHS, Silva NM, de Araújo AP, de Azevedo Camacho D dos R da R, da Moura AJF, *et al*. Susceptibility profile of *Aedes aegypti* from Santiago Island, Cabo Verde, to insecticides. Acta Trop. 2015; 152:66–73.

58. INSP. Boletim Epidemiológico da dengue-Cabo Verde. Semana Epidemiológica 45 de 2024. Praia; 2024. Available from: https://insp.gov.cv/wp-content/uploads/2024/11/BO-dengue-43.pdf. Accessed 28 November 2024.

59. Faye O, de Lourdes Monteiro M, Vrancken B, Prot M, Lequime S, Diarra M, *et al*. Genomic Epidemiology of 2015-2016 Zika Virus Outbreak in Cape Verde. Emerg Infect Dis. 2020;26:1084-90.