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Exploring extrinsic and biological factors shaping *Aedes albopictus* vector competence: Assessing the role of *Wolbachia* in intestinal homeostasis maintenance

Tatiana Barbar

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La Réunion University
Thesis for the degree of
DOCTOR OF UNIVERSITY

Graduate School (542): Sciences, Technologies and Health (EDSTS)

Specialization in Vector-pathogen interaction, Virology, and Molecular Biology

**Exploring extrinsic and biological factors shaping
Aedes albopictus vector competence:
Assessing the role of *Wolbachia* in intestinal homeostasis maintenance**

Presented and publicly defended on **Monday the 13th of May 2024** by

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***“In life, nothing is to be feared, everything is to be understood.
Now is the time to understand more,
so that we may fear less.”***

- Marie Curie



***“Just Like Science,
Emerging Infectious Diseases Know No Borders.”***

Foreword:

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Summary

Arboviruses (Arthropod-Borne Viruses), specifically those belonging to the *Flaviviruses* genus, including Dengue virus, Zika virus, Japanese Encephalitis Virus, and others, pose significant threats to public health. These emerging infectious diseases put millions at risk of infection, causing thousands of deaths worldwide, each year. Tropical and subtropical regions have been highly susceptible to these viruses due to a combination of several factors, including conducive humid climate, rich natural biodiversity, abundant breeding sites, rapid urbanization, increased globalization, extensive travel, as well as limited resources for vector control. Dengue is considered one of the fastest and most dangerous tropical fever diseases transmitted to humans by mosquitoes. Due to its widespread occurrence and high morbidity and mortality rates, it is identified as the leading cause of fever after Malaria. La Réunion Island, a French department located in the Southwest of the Indian Ocean, has been significantly impacted by the emergence and re-emergence of dengue epidemics since 1977. Since then, this has been considered a major health problem as it has caused thousands of infections and hundreds of fatalities. These epidemics were triggered by the primary mosquito vector for Dengue virus on the island, *Aedes albopictus*, one of the most invasive mosquito species. Based on specific environmental and biological factors, mosquito populations in La Réunion had developed a susceptibility to the infection by the four-dengue virus (DENV) serotypes, contributing to high transmission efficiency and creating a complex interplay between environmental and biological factors. Thus, understanding the biology of *Aedes albopictus* competence towards the DENV serotypes circulating locally is crucial for devising effective strategies to mitigate Dengue transmission in La Réunion. As of today, no medically confirmed treatment has been established for Dengue virus. Thus, vector control, limiting the density of vector mosquitoes is the most used method to break the transmission chain. An emphasized biological control strategy is the use of *Wolbachia* bacterium. Naturally present in many insects, notably *Aedes albopictus*, it is one of the biological intrinsic factors that might affect vector competence toward DENV. Different studies have shown that *Wolbachia* can limit the DENV infection in *Aedes* mosquitoes by manipulating several physiological traits.

My thesis aims to characterize the genetic and phylogeny of the DENV-1 circulating in La Réunion Island during the last epidemic and to explore both extrinsic and intrinsic factors influencing the competence of *Aedes albopictus* towards the DENV-1 and DENV-2 serotypes that have emerged. A significant portion of the research is dedicated to exploring the symbiotic relationship between *Aedes albopictus* and the *Wolbachia* bacterium, emphasizing its influence on intestinal balance both after ingestion of a blood meal and the Dengue virus.

Keywords: *Aedes albopictus*, Dengue Virus, *Wolbachia*, Vector competence, La Réunion Islands, Dengue epidemics, midgut, Intestinal Stem Cells, Apoptosis, Intestinal homeostasis.

Résumé :

Les *arbovirus* (virus transmis par les arthropodes), en particulier ceux appartenant au genre des *Flavivirus*, tels que le virus de la Dengue, le virus Zika, le virus de l'Encéphalite Japonaise, et d'autres, représentent une menace importante pour la santé publique. Ils sont à l'origine de maladies infectieuses émergentes qui mettent en danger la santé des populations infectées par ces virus et causent de milliers de décès dans le monde chaque année. Les régions tropicales et subtropicales sont particulièrement touchées par la circulation de ces virus en raison d'une combinaison de plusieurs facteurs, notamment un climat humide propice, une riche biodiversité naturelle, des sites de reproduction abondants pour les vecteurs, une urbanisation rapide, une mondialisation accrue, ainsi que des ressources limitées pour le contrôle des vecteurs. La Dengue est considérée comme l'une des maladies fébriles tropicales les plus rapides et dangereuses transmises à l'homme par les moustiques. En raison de sa prévalence généralisée et de ses taux élevés de morbidité et de mortalité, elle est identifiée comme la principale cause de fièvre après le paludisme. L'île de La Réunion, un département français situé au sud-ouest de l'océan Indien, a été fortement impactée par l'émergence et la réémergence d'épidémies de Dengue depuis 1977. Depuis lors, il s'agit d'un problème de santé majeur, ayant causé des milliers d'infections et des centaines de décès. Ces épidémies ont été déclenchées par le moustique vecteur principal sur l'île, *Aedes albopictus*, l'une des espèces de moustiques les plus invasives. Sur la base de facteurs environnementaux et biologiques spécifiques, les populations de moustiques à La Réunion ont développé une susceptibilité à l'infection par les quatre sérotypes du virus de la Dengue (DENV), contribuant à une efficacité élevée de transmission et créant une interaction complexe entre les facteurs environnementaux et biologique. Ainsi, comprendre la compétence vectorielle d'*Aedes albopictus* vis-à-vis des sérotypes de DENV circulant localement est crucial pour élaborer des stratégies efficaces de lutte contre la transmission de la Dengue à La Réunion. À ce jour, aucun traitement médicalement confirmé n'a été établi pour le virus de la Dengue. Ainsi, le contrôle des vecteurs, en limitant la densité des moustiques vecteurs, est la méthode la plus utilisée pour rompre la chaîne de transmission. Une stratégie de contrôle biologique mise en avant est l'utilisation de la bactérie *Wolbachia*. Naturellement présente chez de nombreux insectes, notamment chez *Aedes albopictus*, elle est l'un des facteurs intrinsèques biologiques qui pourraient affecter la compétence vectorielle vis-à-vis de DENV. Différentes études ont montré que cette dernière peut limiter l'infection par DENV chez les moustiques *Aedes* en manipulant plusieurs traits physiologiques.

Ma thèse a visé à caractériser la génétique et la phylogénie du DENV-1 circulant à La Réunion lors de la dernière épidémie et à explorer les facteurs extrinsèques et intrinsèques influençant la compétence d'*Aedes albopictus* vis-à-vis des sérotypes DENV-1 et DENV-2 qui ont émergé à cette époque. Une part importante de la recherche a été consacrée à l'exploration de la relation symbiotique entre *Aedes albopictus* et la bactérie *Wolbachia*, en mettant l'accent sur son influence sur l'équilibre intestinal tant après l'ingestion d'un repas sanguin que du virus de la Dengue.

Mots-clés : *Aedes albopictus*, Virus de la Dengue, *Wolbachia*, Compétence vectorielle, Îles de La Réunion, Épidémies de Dengue, Intestin moyen, Cellules souches intestinales, Apoptose, Homéostasie intestinale.

Scientific publications

Article I: Hafsia S., **Barbar T.**, Wilkinson A.D., Atyame C., Biscornet L. , Bibi J. , Louange M., Gedeon J. , Santis O. , Flahault A. , Cabie A. , Bertolotti A. and Mavingui P. 2023. “Genetic characterization of dengue virus serotype 1 circulating in Reunion Island, 2019–2021, and the Seychelles, 2015–2016”. BMC Infectious Diseases. 23:294 <https://doi.org/10.1186/s12879-023-08125-y>.

Article II: Hafsia S., Baudino F., Lebon C., Alout H., Gomard Y., Wilkinson D., **Barbar T.**, Fourié T., Mavingui P., and Atyame C. 2024. Submitted. “Vector competence of *Aedes albopictus* field populations from Reunion Island exposed to local epidemic dengue viruses”.

Article under submission

Article III: **Barbar T.**, Lebon C, Baudino F.,, Kambris Z., Osman D. & Mavingui P. 2024. Submitted. “*Wolbachia* plays a major role in the maintenance of midgut homeostasis in *Aedes albopictus*”

Article under submission

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Abbreviation list

A

Ae.: *Aedes*

ADE: Antibody-Dependent Enhancement

ARS : Agence Régionale de Santé

B

BL : Basal Lamina

C

C.: Capsid Protein

CDC: United States Centers for Disease Control and Prevention

CHIKV: Chikungunya Virus

D

DENV: Dengue Virus

DF: Mild Asymptomatic Dengue

DHF: Severe Dengue Hemorrhagic Fever

DPE: Days Post-exposure

DSS: Dengue Shock Syndrome

E

E.: Envelope protein

EB: Enteroblast

EC: Enterocytes

EE: Enteroendocrine

EID: Emerging Infectious Diseases

EIP: Extrinsic Incubation Period

ER: Endoplasmic Reticulum

I

IAP: Inhibitor of Apoptosis

ISC: Intestinal Stem Cell

J

JEV: Japanese Encephalitis

M

M.: Membrane Protein

MEB: Midgut Escape Barrier

MIB: Midgut Infection Barrier

MOH: Ministry Of Health

N

NS: Non-structural protein

O

ORF: Open Reading Frame

P

prM: Pre-Membrane Protein

R

ROS: Reactive oxygen Species

RVF: Rift Valley Fever Virus

S

SPF: Santé Publique France

Spp.: Species

T

TGN: Trans-Golgi Network

U

USUV: Usutu Virus

UTR: Untranslated Region

V

VC: Vector Competence

W

WNV: West Nile Virus

Y

YFV: Yellow Fever Virus

Z

ZIKV: Zika Virus

Thesis structure

This Ph.D. thesis manuscript is organized into five distinct parts, enabling a thorough exploration of the complex interactions between the Dengue virus, its vectors, and the environmental and biological factors influencing its transmission in La Réunion Island, contributing significantly to the field of virology and vector biology:

Part I. Introduction to *Flaviviruses*: This initial section provides a comprehensive introduction to the history of Flaviviruses, with a particular focus on the global emergence of the Dengue virus and the widespread distribution of its primary vectors, the *Aedes* spp. mosquitoes. It lays the foundation for understanding the context and significance of the research conducted.

Part II. Thesis research program: In this section, we will address the general background of the thesis as well as the axes and the objectives of the study.

Part III. Research results: This part is divided into 3 chapters. In each one, we will present the results of the thesis in the form of an article.

Chapter 1: Dengue virus emergence in La Réunion island: This part investigates the history of the Dengue virus emergence on La Réunion Island. It specifically examines the recent appearance of the DENV-1 serotype during the epidemic from 2018 to 2021.

- **First key research question:** Were the DENV-1 strains, which appeared in the neighboring islands—the Seychelles during 2015-2016 and La Réunion during 2019-2021, introduced from a common source?
- **Research objective:** To perform molecular and phylogenetic characterization of DENV-1 in La Réunion Island and the Seychelles through partial and complete genome sequencing of DENV-1 isolated from patients in both locations.

Chapter 2: Vector competence of *Aedes albopictus*: This part assesses the vector competence of various *Aedes albopictus* populations from La Reunion Island for the circulating DENV-1, exploring infection rates, dissemination efficiency, transmission efficiency, and both extrinsic and intrinsic factors influencing this competence.

- **Second key research question:** Do biological factors, particularly the presence of the *Wolbachia* endosymbiont, impact the vector competence of the Reunionese *Aedes albopictus* populations for Dengue virus strains?
- **Research objective:** To better understand the vector competence of *Aedes albopictus* populations from Reunion Island for local DENV epidemic strains, considering their *Wolbachia* infection status.

Chapter 3: *Wolbachia* effect on midgut structure and homeostasis in *Aedes albopictus*: This section focuses on the structure and homeostasis of the *Aedes albopictus* midgut, emphasizing its role as a primary barrier against foreign invaders. It specifically highlights the role of the *Wolbachia* bacterium in maintaining midgut homeostasis.

- **Third key research question:** Does *Wolbachia* maintain intestinal homeostasis in *Aedes albopictus* after a normal blood meal and DENV-1 infection?
- **Research objective:** To investigate *Wolbachia*'s role in preserving the integrity of the intestinal epithelium of *Aedes albopictus*, including its impact on cellular division,

apoptosis, and visceral muscle structure, under normal conditions and following Dengue virus ingestion.

Part IV. General discussion and conclusions: The final part offers a comprehensive discussion on the thesis work, addressing its limitations and outlining perspectives that provide insights into potential strategies for understanding vector competence and reducing Dengue virus transmission by the *Aedes albopictus* mosquito.

Part V. References

General introduction

Part I: General introduction

“Emerging Infectious Diseases, a global threat”

In this general introduction, first, we will present vector-borne diseases, specifically those induced by the *Flavivirus*, which are recognized as a global health problem affecting many countries worldwide.

Second, we will present the most dangerous tropical fever disease caused by the Dengue virus (DENV), with a focus on its incurred incidence over centuries.

Third, we will introduce the primary and secondary vectors of DENV, *Aedes aegypti* and *Aedes albopictus*, respectively. We will focus on the rapid expansion of *Aedes albopictus* population, recognized as an important threat, putting millions of people at risk of infection every year.

Part I. General introduction

I. A. Origin and history of infectious vector borne-disease and transmission

I.A.1. Vector-Borne diseases and *Flaviviruses*

Throughout history, Emerging Infectious Diseases (EIDs) have been among the most feared scourges of the past. Infections have either recently appeared or resurfaced with higher occurrence and wider spread in the population, driven by advantages related to evolving conditions (1,2). Each year, over 700,000 deaths caused by EIDs are reported, such as Dengue, Zika, Chikungunya, Yellow Fever, Japanese Encephalitis, Rift Valley fever, West Nile virus; Malaria, Schistosomiasis, Human African Trypanosomiasis, Leishmaniasis, Chagas, and Onchocerciasis (3). Data from 1952 to 2017, showed that at least one arbovirus (arthropod-borne virus) was reported in one country/territory by 146 nations, with 123 reporting more than one. Specifically, 85 countries/territories reported autochthonous occurrences of Zika virus (ZIKV), 111 of dengue virus (DENV), 106 of Chikungunya (CHIKV), 43 of Yellow Fever Virus (YFV), and 39 of Rift Valley fever (RVF) (4) (Figure 1).

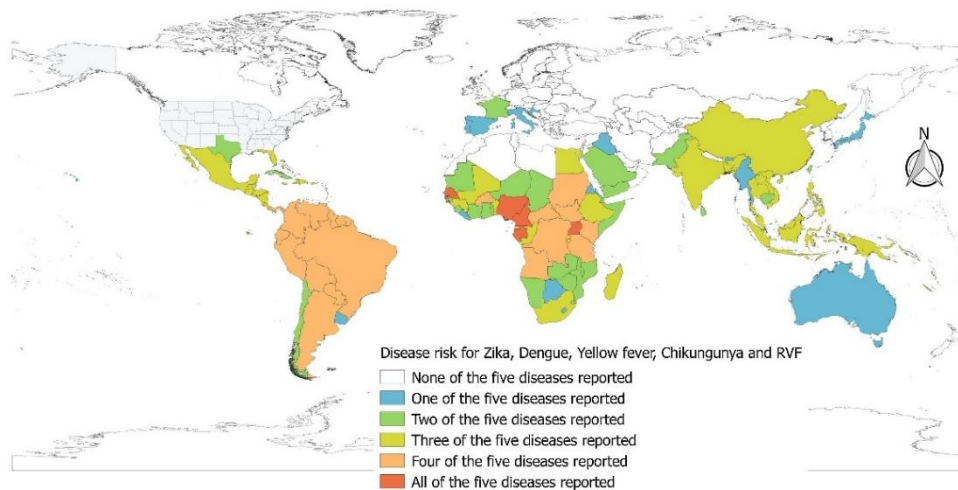


Figure 1 : Global occurrences of the DENV, ZIKV, CHIKV, YFV, and RVF arboviral diseases at the country level. The color gradient ranges from white, indicating no occurrences, to red, representing the presence of all selected arboviral diseases (Leta S. et al., 2017).

For decades, even centuries, the global emergence of arboviral diseases has been caused by viruses belonging to the *Flaviviridae* family, tormenting humanity and causing millions of deaths worldwide. They appear unexpectedly in human populations, often causing spectra of potentially serious diseases and are a major cause of death. These are considered major pathogens for both humans and animals. They are transmitted mainly by arthropods and ticks. So far, more than 70 *Flavivirus* genera have been identified as arthropod-borne viruses. Unlike other genera in the *Flaviviridae* family, *Flaviviruses* can infect both vertebrate and invertebrate species (5,6).

The temporal origin of *Flaviviruses* diversification has been subject to debate due to inherent challenges in dating the deep evolution of RNA viruses. Phylogenetic analysis suggests that *Flaviviruses* originated approximately ~85,000 (64,000–110,000) years ago from a common ancestor. The split of the first group of *Flaviviruses* into three subgroups — those transmitted by mosquitoes, ticks, and those with unknown vectors — dates back to around 40,000 years (7,8).

- **Tick-transmitted *Flaviviridae***, including tick-borne encephalitis virus, Louping Ill virus, Omsk Hemorrhagic Fever virus, and Kyasanur Forest Disease virus, form a closely related and monophyletic group known as of a "serocomplex," despite variations in the diseases they caused (8).
- **The mosquito-transmitted *Flaviviridae*** subgroup is more diverse and it includes the serological complex of Japanese encephalitis virus (JEV), Yellow Fever Virus (YFV), West Nile Virus (WNV), and the four serotypes of Dengue Virus (DENV) (DENV-1, DENV-2, DENV-3, and DENV-4) (8).
- **The "No-Known-Vector" group** constitutes the third group within the *Flavivirus* genus, where vertebrates such as rodents (transmitting Modoc virus,) and bats (transmitting Rio Bravo virus) are the sole hosts, with no arthropod vectors involved in their maintenance or transmission so far (8).

Our study focuses on the **mosquito-transmitted *Flaviviridae* group**, which can be broadly divided into two sub-groups:

- **Viruses transmitted by *Culex* spp. Mosquitoes**, primarily associated with encephalitic *Flaviviruses* like JEV and WNV, which cause invasive neurological diseases.
- **Viruses transmitted by *Aedes* spp. Mosquitoes**, primarily associated with viscerotropic *Flaviviruses* such as DENV, ZIKV, and YFV, which cause severe hemorrhagic fever (7) (Figure 2) (9).

While the main mosquito-transmitted viruses have existed for thousands of years, the separation of DENV, YFV, and JEV groups dates back over 3000 years. The cladogenesis period for DENV and JEV occurred in the last two centuries, with most other lineages diverging more recently (10). Initially discovered in tropical regions like Africa, South America, and certain parts of Asia, the majority of *Flaviviruses* have now become widely dispersed and are causing diseases globally (11).

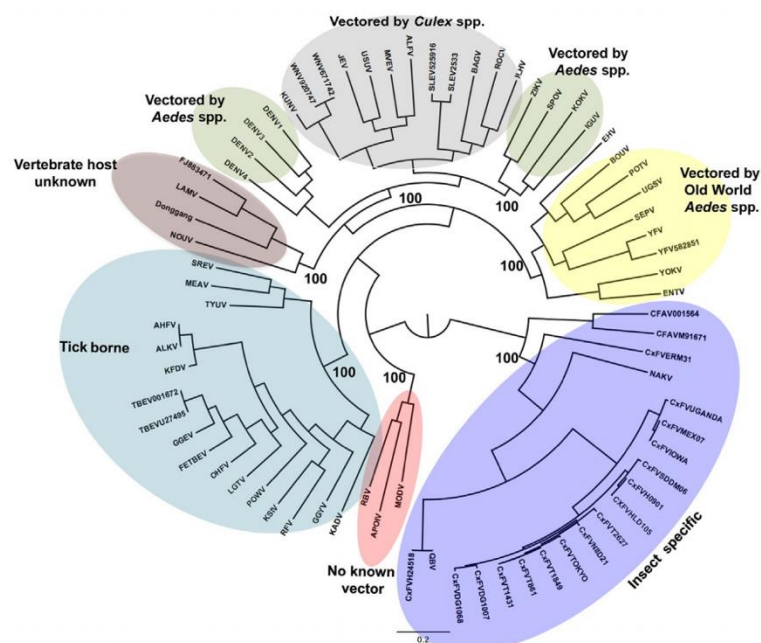


Figure 2 : Phylogeny of *Flaviviruses*: Maximum likelihood analysis of selected members of the *Flavivirus* genus. Scale bars indicate amino acid substitutions per site. Branch labels denote virus abbreviations. (Roudy CM et al., 2023).

I.A.2. Flavivirus infection symptoms

Flavivirus infection can result in a spectrum of diseases, ranging from subclinical infection to neurological or hemorrhagic illnesses leading to organ impairment and death (Figure 3). For instance, in the case of YFV disease, the severe form typically follows a biphasic pattern. The “infection phase” presents symptoms resembling flu, including fever, fatigue, headaches, and muscle pains, which may progress to complications such as hyperemia, conjunctival injection, and liver tenderness. Subsequently, some patients enter the “toxicity phase” characterized by hemorrhagic disease and organ dysfunction, as evidenced by jaundice, nausea, vomiting, and neurological and hemorrhagic manifestations (8,12).

Similarly, infection with JEV and WNV manifests in symptoms such as headaches, muscle pains, diarrhea, and vomiting. As neurotropic viruses, JEV and WNV can lead to complications, including neurological signs such as opisthotonos, acute flaccid paralysis, seizures, mental confusion, fixed facies, and cogwheel rigidity. Severe forms of the disease may progress to severe encephalitis, meningitis, loss of consciousness, coma, and death in 20-30% of patients. Additionally, approximately 30-50% of individuals who survive a severe form of the disease may experience neurological sequelae occur, including seizures, physical disabilities, and cognitive deficits (13).

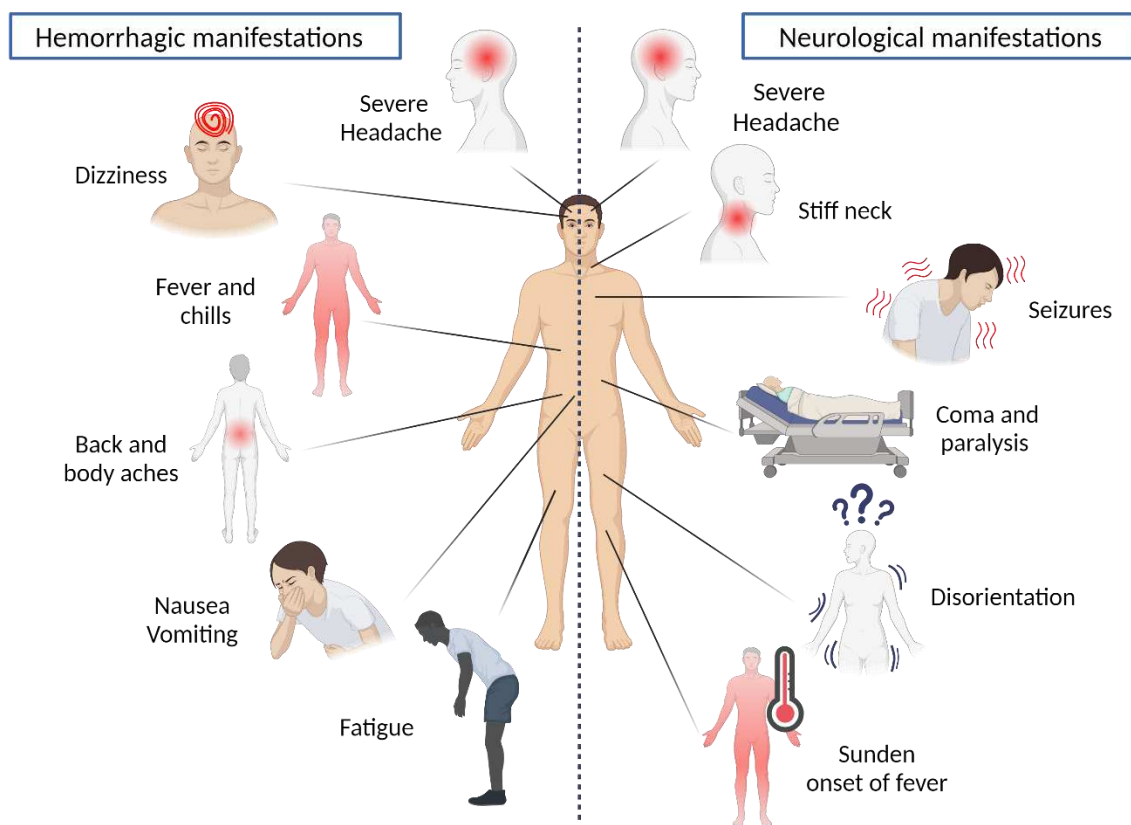


Figure 3: Flaviviruses general neurological and hemorrhagic symptoms

I.A.3. Historical epidemics caused by *Flavivirus*

I. A. On the global scale and in the tropical and sub-tropical regions

Over the past 50 years, the emergence of several arboviruses, has led to countless global epidemics. The burden of these diseases is highest in tropical and subtropical regions. Ecological factors have been proposed to explain the increase in human infections, linked to heightened transmission by *Aedes* mosquito vectors. Potential factors that have contributed to the rise in vector populations and their transmission include changes in land use and demographic changes that expose individuals to increased contact with a previous/unknown microbe or its natural host, thereby facilitating its propagation, climate changes, population growth, population migration to urban areas, and the lack of vaccination leading to low immunity among populations (14–16). Since 2014, significant outbreaks of DENV, CHIKV, YFV, and ZIKV have afflicted populations in tropical territories, causing deaths, and overwhelming healthcare systems (17). Here we will present the global history of emergences of major arboviral diseases, related to the Yellow Fever Virus, Japanese Encephalitis Virus, Zika virus, and Usutu Virus (arthropod-borne viruses). Dengue virus history, origin of emergence, and circulation will be presented in the next sections since we will focus on its emergence on La Réunion Island as well as its interaction with the mosquito vector.

Yellow Fever Virus (YFV), transmitted by *Aedes aegypti* and *Aedes albopictus* mosquito species, was the first filterable pathogen demonstrated to cause human disease and the first virus proven to be transmissible by an arthropod vector (18). The first documented probable outbreak of YFV occurred in 1495 mainly in America, in Hispaniola, today known as the Dominican Republic (19). However, the first reported epidemic took place either in Barbados or St. Kitts in 1647, and in 1648, an epidemic in the Caribbean spread to Yucatan, Mexico, and caused afterward regular outbreaks in Cuba (20). Subsequently, major epidemics began to affect America. In 1793, the YFV caused the death of about 10% of the population in Philadelphia, followed by the largest outbreak in 1878 along the southern Mississippi River between Memphis and New Orleans. This epidemic resulted in over 20,000 death cases, with estimates of around 120,000 reported cases (21). YFV epidemics persisted in the United States until 1905 when the last documented outbreak occurred in New Orleans before the appearance of the vaccine (22). However YFV it was still endemic in tropical and subtropical regions of Africa and South America, especially among children and the elderly with weakened immune systems (23,24). According to the World Health Organization, in 2016-2017, an outbreak of YFV in Angola caused 2,267 infections and the death of 293 individuals (25). Sporadic cases have been reported primarily in the Amazonian states, with incursions into the Southeast in 2002 and 2008, and in the south from 2008 to 2018. A recent epidemic peak was recorded in Brazil from 2016 to 2018, with 2,050 registered cases and 681 deaths (19) (Figure 4.a).

Japanese Encephalitis Virus (JEV) is an arbovirus primarily transmitted during the warm season, in most temperate regions in Asia, which can lead to significant epidemics. Its first detection dates to the year 1871 in Japan. Later, in 1924, a major epidemic affected 6,000 people and resulted in a 60% fatality rate (26). Subsequent outbreaks occurred in 1927, 1935, and 1964. JEV was first isolated from *Culex tritaeniorhynchus* mosquitoes in 1938 (26). In the

following years, initial cases were recorded in several regions worldwide, including mainland China in 1940, the Philippines in 1950, Pakistan in 1983, Papua New Guinea in 1995, and the northern extension to Australia (27). Three billion people living in 24 areas were affected by JEV (28). In 2006, the annual incidence of JEV was estimated between 30,000 and 50,000 cases (29) but in 2011 it increased to approximately 67,900 cases (with 50% of these cases reported in China), with an estimated fatality rate between 14,000 and 20,500 per year. In endemic regions, the incidence rate depends on vaccination, as 55,000 cases (81%) occur in areas with developed JEV vaccination programs, while nearly 12,900 cases (19%) occur in areas without vaccination programs (30) (Figure 4.b). In tropical and subtropical areas, transmission may persist throughout the year but typically intensifies during the rainy season, characterized by an increase in vector populations. Major outbreaks occur at intervals of 2-15 years (31).

Zika virus (ZIKV), was first isolated in 1947 from the serum of a sentinel rhesus macaque monkey in the Zika Forest of the Entebbe Peninsula in Uganda and secondly from *Aedes africanus* mosquito species (32). After its discovery, the virus remained confined for the first 60 years within the equatorial zone across Africa and Asia. The virus began its first emergence outside this zone in 2007, when it has primarily emerged in the Pacific and the Americas, spreading to Yap Island with 49 confirmed cases (33). In 2013-2014, it was spread eastward to New Caledonia, Pacific islands, and French Polynesia, with an estimated 28,000 cases (11% of the population) (34,35). It finally reached Latin America and Brazil in 2015, causing significant incidents (36), and further disseminated into North America in 2016 (34,37) (Figure 4.c). Until today, ZIKV continues its emergence throughout the tropical regions of the Americas. As of now, a total of 89 countries and territories in Africa and Asia have reported evidence of ZIKV infection transmitted by mosquitoes. These epidemics have underscored the potential of the ZIKV to traverse its territory and spread to new geographic locations, capable of causing large-scale outbreaks. Through species distribution modeling techniques, a recent study predicted that a significant portion of the tropical and subtropical regions of the globe present favorable environmental conditions but have not yet reported symptomatic cases of Zika virus infection. In these regions, over two billion people living in these areas are at risk of being affected by ZIKV as populations of *Flavivirus* vector mosquitoes are expanding (38,39).

The Usutu Virus (USUV), is a “newly discovered *Flavivirus*”, an African Mosquito-Borne *Flavivirus* of the Japanese Encephalitis Virus Group, it was first identified in Austria in 2001 when it was found outside of the African continent (40). In the following years, USUV causes unusual bird mortality in neighboring countries including Italy, Germany, Spain, Hungary, Switzerland, Poland, England, Czech Republic, Greece, and Belgium. The first two USUV human infections were reported in Italy in 2009 and in Croatia in 2013. Its natural life cycle relies on ornithophilic mosquitoes, primarily *Culex* spp., as a vector, and wild birds, predominantly, as an amplifying host. The primary clinical symptoms of USUV infection include nausea, vomiting, headache, fever, nuchal rigidity, hand tremor, and hyperreflexia. In more severe cases, it can lead to encephalitis or meningoencephalitis, especially affecting immunocompromised patients (41–43). Despite its harmful effects on bird populations and the potential threat to human health, there is currently a lack of molecular tools available for studying USUV (Figure 4.d).

Many other major *Flaviviruses* continue to emerge and re-emerge in populations worldwide, by different vector species, causing significant rates of morbidity and mortality. The emergences of these viruses are presented in Table 1.

I.A.3.2. In La Réunion Island

La Réunion is a French Island located in the southwestern Indian Ocean and has been at risk of the emergence of several *Flavivirus* species for hundreds of years.

JEV, YFV, and ZIKV emergences had never been reported on the island. However, there is an increased risk of introduction of these viruses. Indeed, there are numerous ongoing human and commercial exchanges between Southeast Asia, where JE is endemic, and other regions including Africa, the Americas, and the Pacific where ZIKV and YF are endemic. Additionally, factors related to vectors such as the expansion of vector populations like *Culex* and *Aedes* mosquitoes, and amplifying hosts such as pigs and chickens in the island increase the risk of the viruses being introduced (39,44).

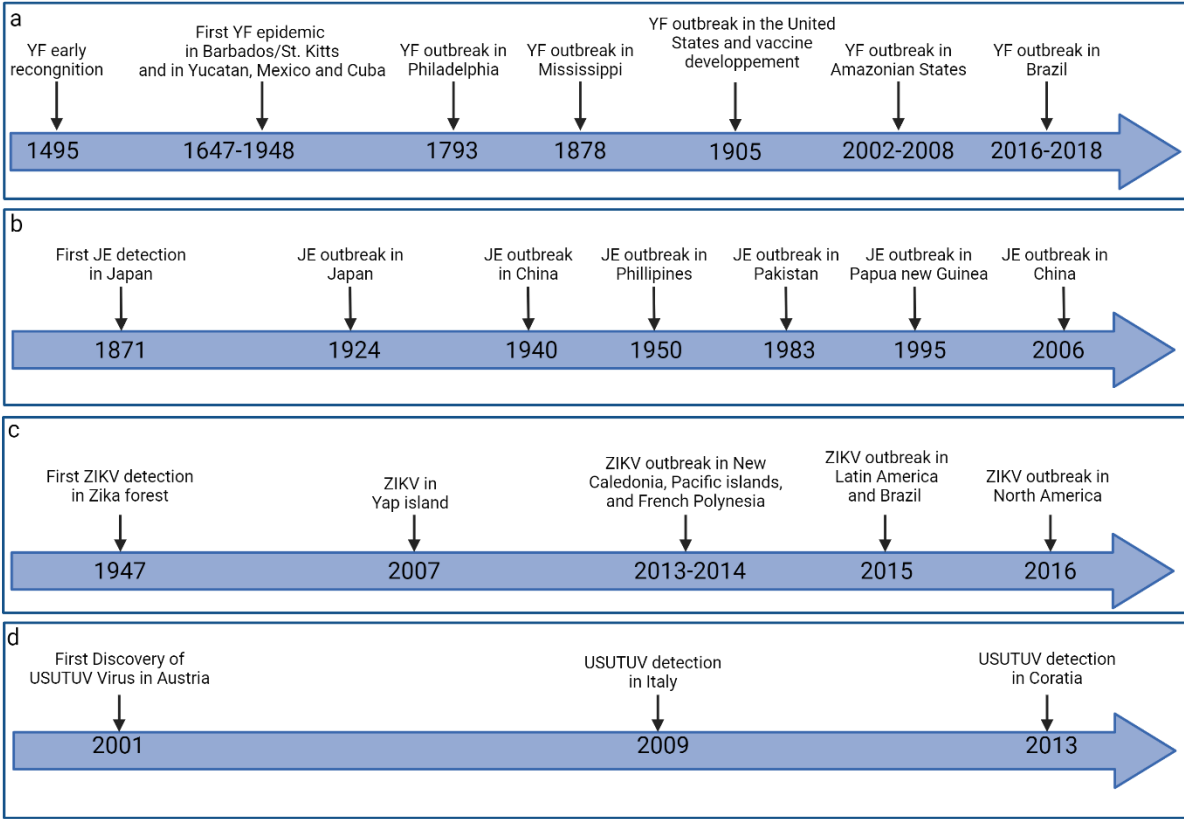


Figure 4: Worldwide Historical highlights of Mosquito-borne Flavivirus epidemics (YF: Yellow Fever Virus; JE: Japanese Encephalitis Virus; USUTU: USUTUV Virus and ZIKV: Zika Virus)

Table 1: The table below describes the transmission route and diseases caused by Flaviviruses, outlining the main geographical distribution, zoonotic reservoir, clinical syndrome, and estimated number of infections for a given Flavivirus (Pierson T. et al., 2020)

Virus	Primary geographical distribution	Vectorial transmission	Human disease	Cases per year
Dengue	Central America, North America, Asia, Australia, Africa	<i>Aedes aegypti</i> <i>Aedes albopictus</i>	Dengue fever, Hemorrhagic Severe Dengue	390 millions cases per year
Zika	Central America, South America, Africa, Asia, North America	<i>Aedes aegypti</i> <i>Aedes albopictus</i> Sexual transmission Vertical (mother to fetus)	Febrile syndrome, Guillain-Barré syndrome, Congenital anomaly, Microcephaly	Thousands to millions, depending on the year (since 2013)
West Nile	North America, Middle East, Africa, Europe, Australia	<i>Culex pipiens</i> <i>Culex tarsalis</i>	Febrile syndrome, Meningitis, Encephalitis, Acute flaccid paralysis	<10 000 cases per year
Japanese encephalitis	Asia, Australia	<i>Culex tritaeniorhynchus</i> <i>Culex annulirostris</i>	Febrile syndrome, Meningitis, Encephalitis	70 000 cases per year
Yellow fever	Africa, South America	<i>Aedes aegypti</i>	Febrile syndrome, Hepatic failure, Hemorrhagic syndrome	130,000 severe cases per year (case fatality rate > 50%)
Powassan	North America, Eastern Europe	<i>Ixode cookei</i> <i>Ixode scapularis</i>	Febrile syndrome, Meningitis, Encephalitis	Hundreds
Usutu	Africa - Europe	<i>Culex pipiens</i>	Febrile syndrome, Meningitis Encephalitis, Acute flaccid paralysis	Hundreds to thousands
Ilheus	South America, Central America	<i>Culex pipiens</i> <i>Ochlerotatus serratus</i> <i>Sabethes</i> <i>Haemagogus</i>	Febrile syndrome, Encephalitis	Unknown
Rocio	South America (Brazil only)	<i>Culex pipiens</i> <i>Culex tarsalis</i> <i>Psorophora ferox</i>	Febrile syndrome Encephalitis	Unknown
Wesselsbron	Africa	<i>Aedes</i> spp. (<i>Aedes caballus</i> and <i>Aedes circumluteolus</i>)	Febrile syndrome	Unknown
Spondweni	Africa, North America (?)	<i>Aedes</i> , <i>Culex</i> , <i>Eretma podites</i> & <i>Mansonia</i>	Febrile syndrome Vascular leakage (shock), Neurological impairment	Unknown

I. B. Dengue virus

The Dengue virus is a vector-borne disease, currently considered one of the fastest, most dangerous, and neglected tropical diseases transmitted by mosquitoes worldwide. Due to its high incidence and mortality rate, it has been classified as the most dangerous and diagnosed cause of fever after Malaria (45). The incidence of DENV is increasing over time due to climate changes, urbanization, the distribution of vector mosquitoes, and the geographic expansion of its two main mosquito vectors, *Aedes aegypti* and *Aedes albopictus* considered as its primary and secondary vector, respectively (16,46) (Table 2).

I.B.1. History of the Dengue virus expansion

The earliest suspected cases resembling dengue-like epidemics were documented in 1635 in Martinique and Guadeloupe, and in 1699 in Panama. However, the precise origins of the first reported outbreaks remain uncertain. Benjamin Rush's account of the first recognized outbreak in Philadelphia, United States, in 1780, offers a clear depiction of the first dengue fever syndrome caused by DENV (47,48). Since then the virus has already become a cosmopolitan agent, causing almost simultaneous epidemics in the three continents, Asia, Africa and North America, suggesting that these viruses and their mosquito vectors have been widely distributed across the tropics worldwide for over 200 years (10,48). One hundred years later, in 1889, the virus was detected in the eastern end of the Mediterranean. In July 1901, the city of Beirut was touched by an epidemic of dengue, and two years later, Graham discovered its transmission by the vector *Aedes* mosquito (49,50). From this epidemic until 1940, diseases associated with symptoms similar to those of DENV were characterized by relatively infrequent but often significant outbreaks (15).

The ecological disruption that occurred in the regions of Southeast Asia and the Pacific during and after World War II created ideal conditions for an increase in the transmission of mosquito-borne diseases, especially DENV (15,51). In 1943, the first DENV epidemic detected in the Southwest Indian Ocean was in Comoros (52). In the United States and its territories, the transmission of DENV primarily occurs in tropical and subtropical areas such as Puerto Rico, the U.S. Virgin Islands, American Samoa, and the Pacific Islands associated with the United States. Hawaii has experienced DENV outbreaks since 1944 (53). During this epidemic, two DENV strains circulated in Hawaii originating from Tahiti. This illustrates the ease with which DENV can be transported over distances of several thousand miles. Later, several epidemics occurred sporadically in non-endemic regions, such as Florida, where mosquito vectors exist, and their circulation increases due to favorable ecological and climatic conditions (54–56). DENV continued its initial introductions in Southeast Asia in 1950, and in 1953 it emerged for the first time in the Philippines. Emergences spread 20 years later throughout Southeast Asia (15,52).

The surge in disease incidence began in 1970 and continues to the present day. Lately, large and dangerous epidemics are being recorded worldwide. This is attributed to the increased prevalence of DENV hemorrhagic fever due to the extensive expansion of mosquito vectors in many new countries with suitable socio-environmental conditions (15,51,57–59).

I.B.2. Dengue recent emergences and epidemiology on the global & tropical scales

The geographical and genetic evolution of DENV and its vectors triggered increase epidemics, leading to a global pandemic characterized by the simultaneous circulation of several serotypes of the virus (DENV-1, DENV-2, DENV-3, and DENV-4). Infection with one DENV serotype provides lifelong immunity to the particular serotype but subsequent infection with another DENV serotype increases the risk of developing severe DENV (15,51).

In tropical and subtropical regions, Dengue is considered one of the most dangerous diseases, as its incidence has increased more than 30 times in the last two decades (16). In these regions, approximately 2.5 billion people are at risk of infection, resulting in 50 to 100 million infections each year (4,60). Worldwide, since 1950, sociological and climatic changes have been reshaping and expanding geographically EIDs, notably DENV, contributing to urgent consequences for public health (61,62). These conditions are causing the emergence of the virus in more remote and new regions, not only in the tropical and subtropical regions but in the entire world (63,64). According to the World Health Organization, before 1970, only nine countries had experienced severe dengue epidemics. Today, the disease is endemic in more than 100 countries. It has emerged and re-emerged in many tropical and subtropical regions, as well as in the Americas and Asia, causing numerous epidemics over the years (65).

- **From 1970 until 2000**

After 1970, Dengue fever epidemics grew larger and spread to new areas, becoming a major cause of childhood hospitalization and death (15). The disease re-emerged in the Pacific Islands, Americas, and tropical islands. Large outbreaks occurred, like the one in Seychelles (1976-77) infecting 80% of the population (66). The Caribbean also saw an epidemic in 1977, followed by outbreaks on other islands. The US reported its first case since 1945 in 1980, and Cuba had the first dengue hemorrhagic fever epidemic in the Americas in 1981 (57). The US reported its first case since 1945 in 1980, and Cuba had the first dengue hemorrhagic fever epidemic in the Americas in 1981 (67). The 1980s and 1990s saw intensified transmission, with outbreaks in Comoros (1993) and imported cases among travelers returning from Southeast Asia and the Americas (68–70).

- **From 2000 until 2010**

Over the past 2 decades, explosive epidemics have occurred worldwide, and the number of DENV cases has multiplied by more than 8, increasing from 505,430 cases in 2000 to over 2.4 million in 2010, and then 5.2 million in 2019. The reported number of deaths has increased from 960 in 2000 to 4,032 in 2015. Asia accounts for approximately 70% of the global disease burden (71–74). Several micro-epidemics occurred in the 2000s during an inter-epidemic period (46). From 2001 to 2006, a major epidemic was reported in the Pacific and Asia (53). From 2003 to 2004, more than 400 patients were diagnosed in Seychelles (75). Two years later, DENV caused an epidemic in the east of Madagascar (76). In 2009, in Mauritius, 234 cases were reported, and in the following year, 54 autochthonous cases were reported in Mayotte (77).

- **From 2011 until 2018**

Dengue outbreaks have been observed worldwide in recent years. In non-endemic regions such as the United States and Europe, indigenous cases have been reported annually.

In Croatia, France, and Portugal, local transmissions were recorded between 2010 and 2015. (73). Tropical regions also experienced DENV circulation, notably in the Comoros in 2011 and in Mayotte and La Réunion in subsequent years (77). With Malaysia Tropical zones battled ongoing outbreaks, experiencing a significant surge from 2014 to 2015 (78–83). Similarly, the Americas saw a major outbreak in Brazil in 2016 (73,84). Even Sri Lanka wasn't immune, with a record-breaking number of cases in 2017 (85,86). The spread even reached Europe, with over 3,000 cases reported across 27 countries in 2017-2018.(87,88).

- **From 2019 until today**

Dengue fever cases have continued to rise globally, with the highest number reported in 2019. All regions were affected, including previously unaffected areas like Afghanistan. The Americas alone reported 3.1 million cases, including over 25,000 severe cases. Significant outbreaks were also reported in Bangladesh, Malaysia, the Philippines, and Vietnam in Asia (45). Between 2020 and 2021 numerous countries in the Americas, Asia, and several islands experienced significant outbreaks(Figure 5-6) (73,89,90). As of December 2023, over 6 million cases and over 6000 dengue-related deaths were reported from 92 countries/territories (Figure 7) (91). In January 2024, over half a million dengue cases and over 100 dengue-related deaths were reported globally (92). Far this year, 392,724 probable cases of dengue have been documented with 54 deaths confirmed as attributable to the disease (93) (Table 2) (94).

Today, the virus threatens more than 120 countries globally, including regions in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, the Western Pacific, and the Indian Ocean. Its annual global incidence is estimated at 3,97 billion people at risk of infection each year, with 50 million infections, of which 96 million present clinical symptoms (71–74). The sporadic outbreaks in non-endemic regions emphasize the importance of vector surveillance and control measures, even in areas where DENV is not commonly present. This highlights the need for early detection, a rapid response, and public health interventions to prevent the establishment and spread of DENV in new regions and countries.

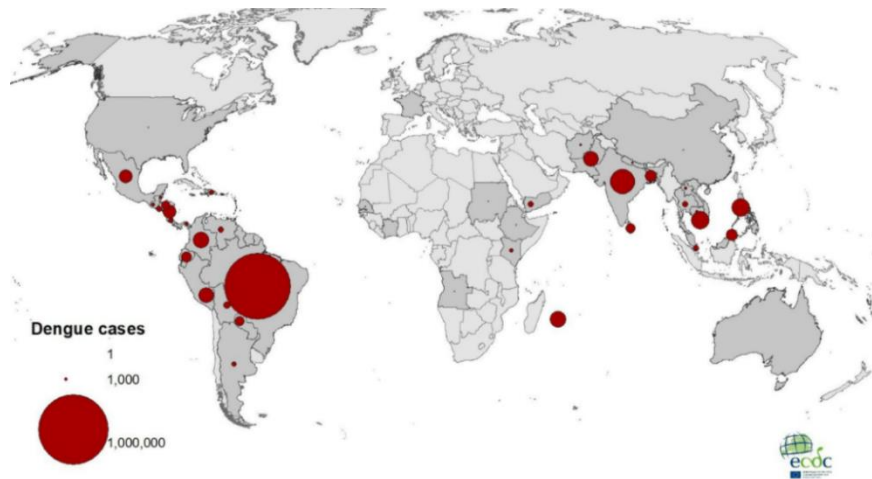


Figure 5: Geographical Distribution of Dengue Cases Worldwide in 2021 (European Centre for Disease Prevention and Control – 2021)

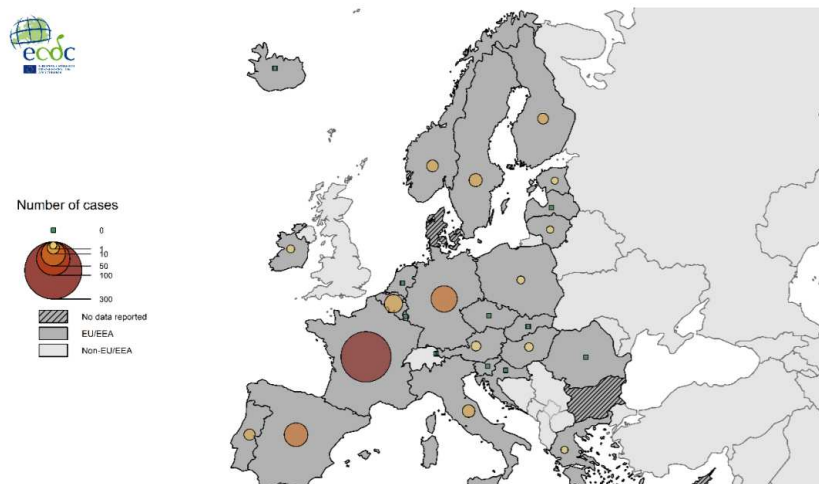


Figure 6 : Geographical distribution of Dengue cases by country of EU/EEA, in 2021 (European Center for Diseases Prevention and Control - 2021).

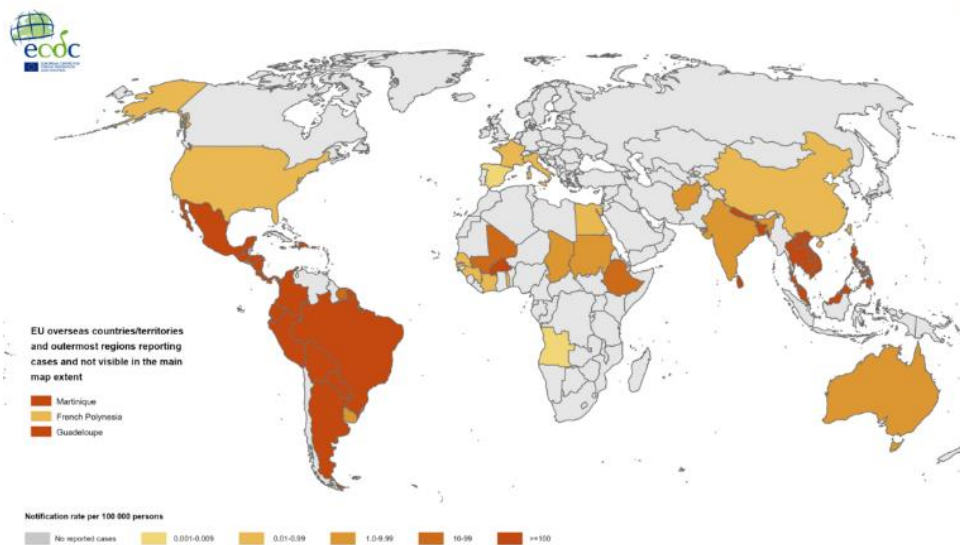


Figure 7: Geographical Distribution of Dengue Cases Worldwide between September 2023 and february 2024 (European Centre for Disease Prevention and Control – 2023).

Regions	Outbreak Years
First introduction in the Americas	1600
Philadelphia, Java, Jakarta, Bavaria, Cairo and Alexandria	1779-1780
eastern end of the Mediterranean	1889
Beyrouth	1901
Multiple countries (World war era)	1916-1918
Hawaii	1944
Comoro Islands	1948
Southeast Asia	1950
Philipines	1953
Pacific Islands	1970
La Réunion	1975
Seychelles	1976-1977
Caribbean	1977
Puerto , Southeastern Mexico	1978
Seychelles	1978-1979
America	1980
Cuba	1981
Worldwide spread	1980-1990
Comoros	1993
The Pacific and Asia	2001-2006
Seychelles and La Réunion	2003-2004
Madagascar	2006
La Réunion	2007-2008
Mauritius	2009
Croatia	2010
Comoros	2011
France	2010-2015
Portugal, Mayotte, La Réunion	2012
Western Pacific Region, Comoros, Mayotte, La Réunion	2014
Malaysia	2014-2015
The Pacific, Asia, the seychelles, Madagascar, Martitus and La Réunion	2015-2016
Americas - Brazil	2016
Highest Number of Dengue cases	2019
Afghnista, Bangladesh, Malaysia, the Philipines, and Vietnam	2019
Bangladesh, Brazil, the Cook Islands, Ecuador, India, Indonesia, Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka, Sudan, Thailand, and Timor-Leste	2020

Table 2: Worldwide highlighted and most important reported Dengue outbreaks since 1600 until 2020

Regions	Outbreak Years
First introduction in the Americas	1600
Philadelphia, Java, Jakarta, Bavaria, Cairo and Alexandria	1779-1780
eastern end of the Mediterranean	1889
Beyrouth	1901
Multiple countries (World war era)	1916-1918
Hawaii	1944
Comoros Islands	1948
South East Asia	1950
Philippines	1953
Worldwide spread	1970-2023
Pacific Islands	1970
La Réunion	1975
Seychelles	1976-1977
Caribbean	1977
Puerto Rico, Southeastern Mexico	1978
Seychelles	1978-1979
America	1980
Cuba	1981
Americas	1980-1990
Comoros	1993
Tamaulipas	1995-1996
South-east Asia, western Pacific, and Latin America	1998
Thailand, Philippines, India	1998-1999
The Pacific and Asia	2001-2006
Seychelles and La Réunion	2003-2004
Madagascar	2006
La Réunion	2007-2008
Mauritius	2009
Croatia	2010
Comoros	2011
France	2010-2015
Portugal, Mayotte, La Réunion	2012
Western Pacific Region, Comoros, Mayotte, La Réunion	2014
Malaysia	2014-2015
The Pacific, Asia, the Seychelles, Madagascar, Mauritius and La Réunion	2015-2016
Americas – Brazil	2016
Sri-Lanka	2017
Europe – 27 countries	2017-2018
Highest Number of Dengue cases	2019
Afghanistan, Bangladesh, Malaysia, the Philippines, and Vietnam	2019
Bangladesh, Brazil, the Cook Islands, Ecuador, India, Indonesia, Maldives, Mauritania, Mayotte (Fr), Nepal, Singapore, Sri Lanka, Sudan, Thailand, and Timor-Leste	2020
Brazil, India, Vietnam, Philippines, Colombia and 27 countries in Europe	2021
Italy, France, and Spain, also in Africa, Americas, South-East Asia, Western Pacific and Eastern Mediterranean	2022-2024

I.B.3. Dengue virus genome and structure

The Dengue virus, roughly 50 nm in diameter, features a lipid bilayer enveloping the particle, housing two transmembrane viral proteins. This lipid bilayer forms a glycoprotein envelope with of 90 pairs of envelope proteins (E), facilitating virus' attachment to host cells (95). The second layer comprises the membrane protein (prM/M), which, in immature DENV particles, forms spikes on the virion's surface with trimeric arrangement (96). These proteins govern viral penetration into human cells. The capsid protein houses the viral genome, consisting of a single-stranded positive-sense RNA around 11 Kb in size, encapsidated in a lipid membrane. The RNA consists of a single open reading frame flanked by untranslated regions (UTR) at the 5' and 3' ends. The 5'UTR (~100 nucleotides) has a methylated cap, while the 3'UTR (~450 nucleotides) lacks a polyadenylated tail (97,98). The ORF flanking by UTRs encodes for three structural proteins (capsid, membrane, and envelope) and for seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (18). Dengue comprises four antigenically and genetically similar serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) with 60% to 70% amino acid sequence identity (99). Despite shared complications and clinical manifestations, biological differences may arise due to a 30% divergence in polyproteins among the four serotypes and factors influencing interactions, such as serotype-vector and serotype-host relationships (46,99) (Figure 8).

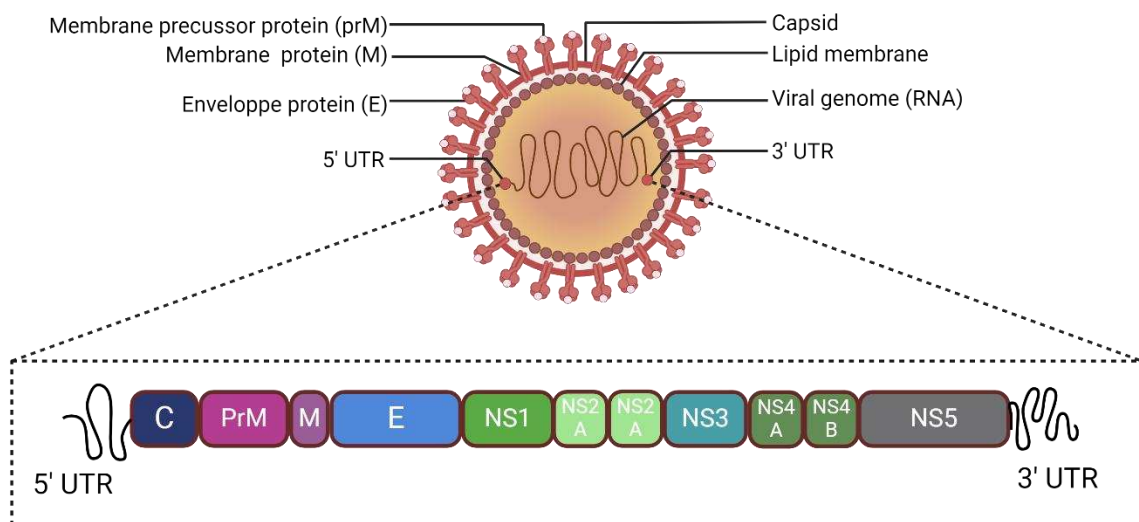


Figure 8 : Structural Organization of Dengue Virus Genome. The structure of the Dengue virus is encased within a lipid layer, comprising the capsid protein (C) as its core component. This core is further enveloped with an envelope protein (E) and the precursor membrane protein (prM) during the immature phase of virions. As the virus matures, the prM transforms into the M protein, solidifying its anchorage within the viral structure. The viral genome consists of the 5' UTR, an open reading frame (ORF), and the 3' UTR. The ORF encoding the polyprotein serves as a template for the translation of three structural proteins (C, PrM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

I.B.4. Cycle of dengue virus

The virus attaches to host cells, primarily monocytes, through interactions with surface glycoproteins and several receptors that are not yet well-defined. Monocytes are important target cells for DENV, as they express a crucial receptor, Fc λ , through which the Virus-Antibody complex internalizes the cell through endocytosis in a clathrin-coated pit. The early endosome is marked by a pH of around 6.5, which matures to a lower acidic pH, approximately 5.5, once separated from the surface. This acidic pH leads to various conformational changes allowing the fusion of the host membrane with the viral envelope by the E protein and the release of the encapsulated viral genome which is presented to the rough Endoplasmic Reticulum (ER) (100). In the ER, viral RNA undergoes translation into a singular polyprotein. This process is catalyzed by a multiprotein complex composed of viral and host proteins. The viral RNA is replicated through an intermediate negative strand, which serves as a template to produce excessive amounts of positive-strand progeny. It is then translated into a single polypeptide cleaved into ten proteins, and afterward, the viral genome will be replicated. The newly synthesized structure proteins and the newly formed RNA are assembled to form new immature virions. Immature viral particles cluster by budding on the surface of the endoplasmic reticulum and are transported through the host's secretory pathway, the trans-Golgi network. During release, virions transform into their infectious form by undergoing a maturation step defined by the cleavage of the prM protein by the furin protease. Mature viruses are then released from the cell and can infect other cells (Figure 9) (101,102).

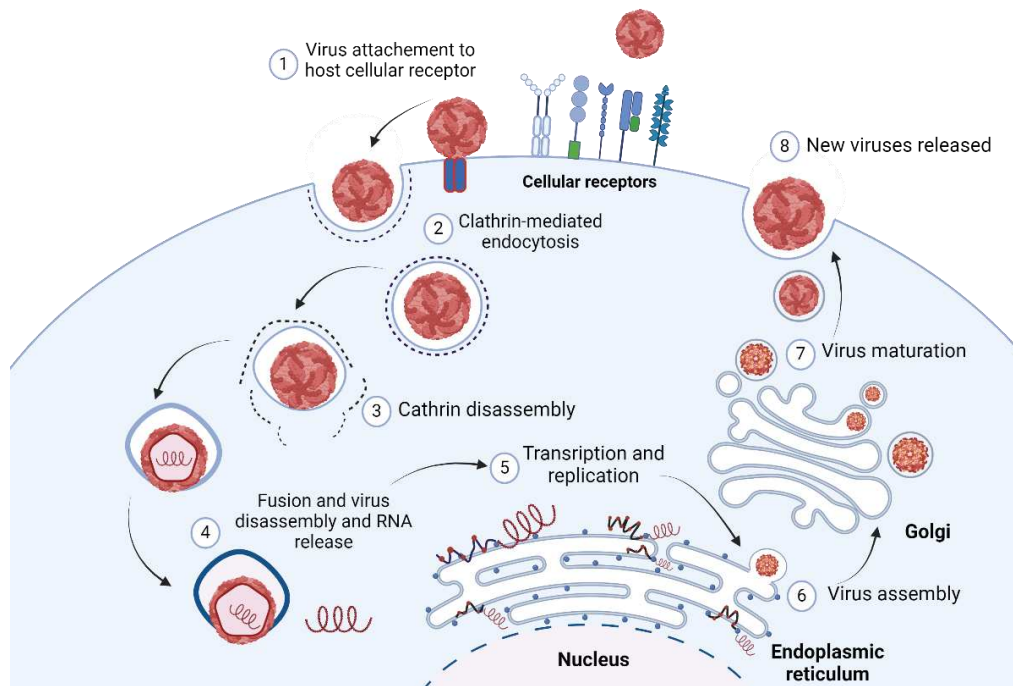


Figure 9: Schema illustrating intracellular viral cycle. (1) DENV binds to various host cell receptors for entry through receptor-mediated endocytosis. (2) A clathrin-coated pit serves as the site of internalization, and the virus is endocytosed into a clathrin-coated vesicle. (3) Clathrin is released, initiating endocytosis. (4) Conformational changes lead to the fusion of the viral envelope on the host membrane and the release of the encapsulated viral genome (capsid-bound RNA) into the cytoplasm. (5) In the Endoplasmic Reticulum, the viral RNA serves as a template for translation and replication. (6) The replicated genome and translated viral proteins are assembled, forming an immature viral particle in the ER. (7) This immature virus undergoes furin-mediated maturation in the trans-Golgi network (TGN). (8) The mature virus is then excreted from the infected cell, completing the infection cycle

I.B.5. Dengue disease's symptoms

Each of the four serotypes can contribute to DENV infection, ranging from Asymptomatic, Mild or Dengue fever (DF), to Severe Dengue Hemorrhagic Fever (DHF), or Dengue Shock Syndrome (DSS), potentially fatal (73,99,103). DF can be debilitating, causing spontaneously severe fever (5 to 7 days), severe headaches, retro-orbital pain, myalgia, arthralgia, nausea, vomiting, petechiae, leukopenia, thrombocytopenia, severe general body pains, and rashes. These symptoms may sometimes be accompanied by hemorrhagic complications (99). Along with the DF complications, DHF is characterized by hemorrhagic manifestations and plasma leakage that determine the severity of the disease. DHF can reach a critical phase marked by a rapid increase in temperature and circulatory disturbances, including plasma leakage, hemoconcentration, and thrombocytopenia. If not properly treated, DHF can progress to DSS infection, characterized by a rapid drop in blood pressure, caused by excessive bleeding and blood loss, leading to circulatory shock and organ failure. It can be fatal in most cases and kill the patient within 12 to 24 hours (104).

The assessment of DHF/DSS infection can be correlated with host immunity, age, virus genomics, and other factors, but the greatest risk is a heterotypic secondary infection by DENV—multiple successive infections or co-infections with different serotypes in a single patient. Usually, this triggers Antibody-Dependent Enhancement (ADE). ADE postulates that non-neutralizing antibodies secreted during the first infection at a specific concentration can interact with the virus from the second infection and instead of neutralizing the virions, they facilitate viral entry into cell lines (105,106). The progression of dengue to its severe form can be initiated by an increase in viral uptake, leading to increased viral replication and viral load (105). Large and dangerous hyperendemic typically occur during the simultaneous co-circulation of multiple serotypes and in the absence of collective immunity against one of the

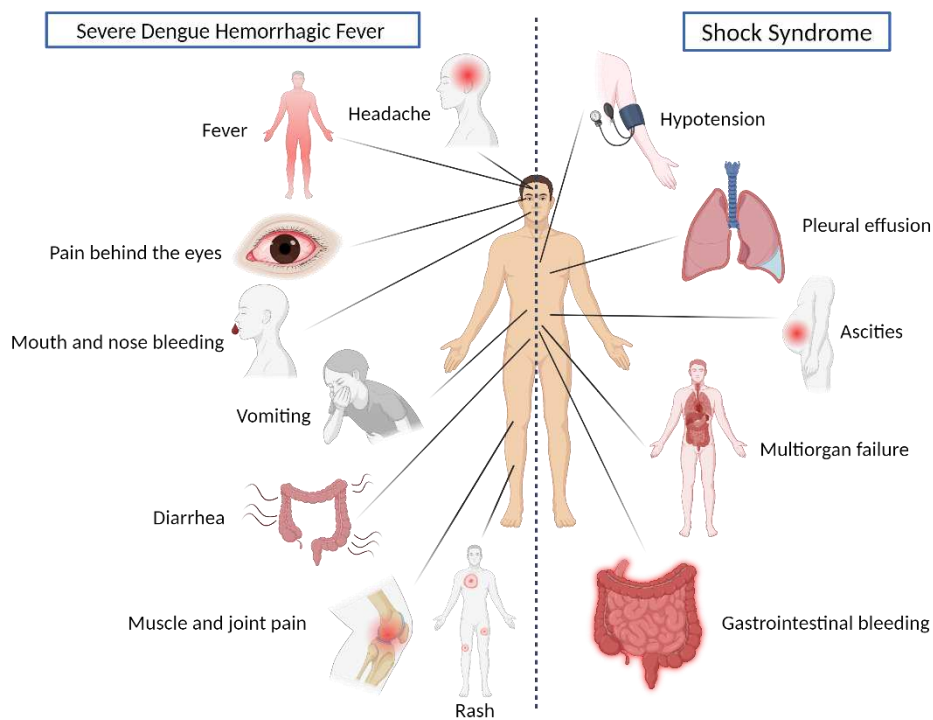


Figure 10: Severe Dengue Hemorrhagic fever and Shock Syndrome Symptoms

four serotypes, or when a new epidemic strain of the virus emerges in an area where another serotype has already been introduced (46).

I. C. Dengue virus main vectors – *Aedes aegypti* and *Aedes albopictus*

Except Antarctica, all other parts of the world are home to mosquitoes, both within the Arctic Circle and in oases amidst deserts (107). Numerous significant mosquito vectors play a crucial role in transmitting dangerous pathogens.

Firstly, the *Culex* mosquito serves as a vector for a range of mosquito-borne viruses, including the WNV, USTUV, RFV, Sindbis virus, Tahyna virus, and others. Secondly, the *Anopheles* mosquito is equally significant as it is linked to the transmission of diseases such as Malaria, Filariasis, and various arbovirus infections. Additionally, mosquitoes belonging to the *Aedes* species, particularly *Aedes aegypti* and *Aedes albopictus*, transmit dangerous mosquito-borne like DENV, CHIKV, ZIKV, YFV, and RFV. These species have caused important emergences and epidemics throughout history and pose significant public health risks (9,17,108–110).

According to Mitchell C et al. 1995, confirming the vector-host transmission cycle, specifically implicating *Aedes* spp. as a vector of DENV, involves various field evidence and criteria: i) Isolating DENV from mosquito populations collected in nature; ii) demonstrating DENV infection in a mosquito after feeding on an infected host; iii) demonstrating virus transmission through a bite to the host; iv) detection the virus in the vector's saliva; v) aligning the timing of epidemics with the emergence of mosquito populations in the same region (111,112).

In the next section, we will introduce both vectors responsible for transmitting DENV: *Aedes aegypti* and *Aedes albopictus*. Our focus will be on *Aedes albopictus*, given its role as the primary vector of the DENV virus in La Réunion.

I.C.1. *Aedes aegypti* mosquito Vector – Origin, history & emergences



Figure 11: *Aedes aegypti* mosquito

The *Aedes aegypti* species (Figure 11) is an invasive mosquito thriving primarily in tropical and subtropical regions but also found to some extent in temperate climates worldwide (109,113) . It serves as the primary urban vector for DENV. Studies have shown varying degrees of susceptibility to different DENV serotypes among mosquito populations during epidemics (114,115). Climate and temperature play a crucial role in determining the

geographic range of this mosquito species. However, several other factors also impact the suitability of its habitat. Notably, the close association of *Aedes aegypti* with humans and domestic environments allows it to thrive in areas that might otherwise be deemed unsuitable based solely on climatic conditions (113,116).

The emergence of *Aedes aegypti* was first thought to be from the New World, but the presence of similar species in Africa suggests its likely origin there (117–119). From the 15th to 19th centuries, the slave trade and globalization led to its spread, causing DENV epidemics in Africa, Asia, and North America (120). *Aedes aegypti* is believed to have arrived in tropical Asia in the late 19th century, coinciding with the onset of urban dengue (121). In the 1990s, its distribution decreased in North America due to the rise of *Aedes albopictus* (122). *Aedes aegypti* thrived in warm regions of the Americas but couldn't survive in the cold northeast, where new outbreaks could occur through ship arrivals (123). At present *Aedes aegypti* is found in 143 countries/islands worldwide across the tropics, and sub-tropical regions, including the southeastern United States, the Middle East, Southeast Asia, the Indian Ocean territories, the Pacific and Indian Islands and Northern Australia (109,124–126).

The initial emergence of significant epidemics occurred in the Americas from 1600 to 1946. Later, in the 1960s, intensive eradication efforts led to a decline in the abundance and spread of *Aedes aegypti* mosquitoes (123). In Europe, notable outbreaks occurred, including a significant one in Athens and neighboring regions of Greece from 1927 to 1928 (109). Subsequent re-infestations resulted in two dengue epidemic waves between 1971 and 1999, and again between 2000 and 2010 (47). In the Indian Ocean, *Aedes aegypti* was responsible for the emergence of DENV-1 in the Comoros in 1993 (77). This latter is still causing a re-emergence of dengue and the occurrence of hemorrhagic dengue fever is still reported worldwide (Figure 12) (48,127,128).

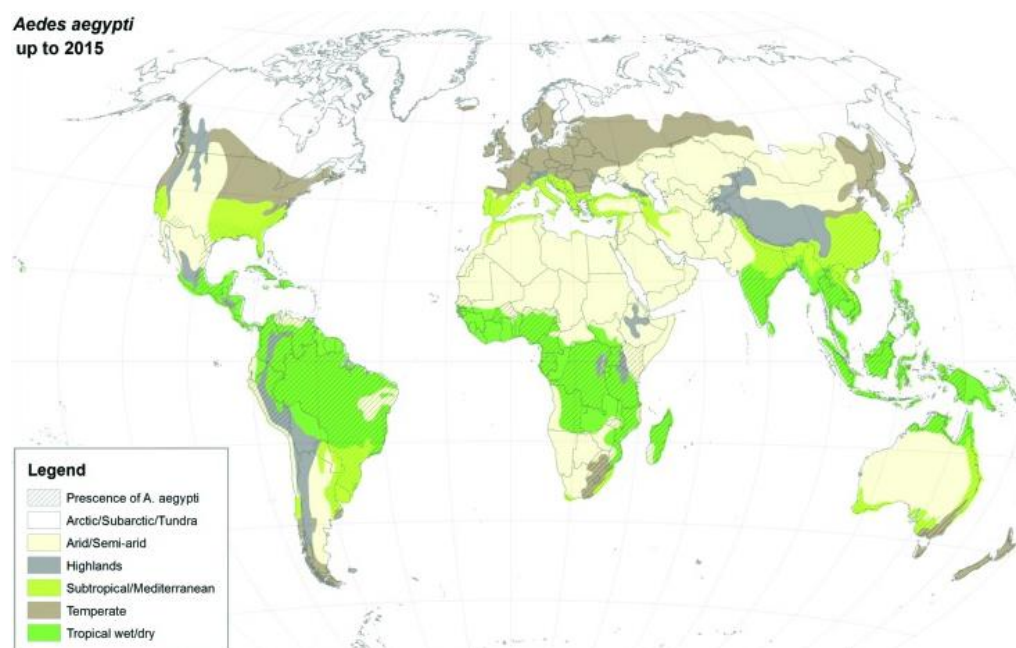


Figure 12: Global distribution of *Aedes aegypti* using climatic & surveillance data collected up to 2015 (Lwande et al., 2019).

I.C.2. *Aedes albopictus* vector

I.C.2.1- Origin and history

The tiger mosquito (Figure 13), or the striped mosquito of Bengal *Aedes albopictus* is at the forefront of public health concerns as it transmits numerous pathogens to both humans and animals. It is considered one of the 100 most important invasive species with a rapidly expanding worldwide population. In laboratory conditions, *Aedes albopictus* has been shown to transmit more than 22 viruses (111,129). Despite the frequent isolation of DENV from mosquitoes captured in the wild, there is no evidence indicating that *Aedes albopictus* is a significant urban vector for dengue, except in a limited number of countries where *Aedes aegypti* is absent, such as parts of China, the Seychelles, historically in Japan, and most recently in Hawaii (111). However, lately, the occurrence of DENV epidemic outbreaks in the absence of *Aedes aegypti*, but in the presence of *Aedes albopictus*, led to the conclusion that the latter species is an important vector (111).



Figure 13: *Aedes albopictus* Tiger mosquito

It is native to Southeast Asia (119,121), and was introduced in the Indian Ocean in the last centuries when it was first reported in Madagascar and, the Seychelles (130). Over the past decades, it has spread to new countries in Africa, the Middle East, Europe, Islands in the Indian Ocean, and the Americas (North and South) after extending its distribution eastward through Pacific islands in the early 20th century (131). *Aedes albopictus* can currently be found in temperate and tropical Asia (its original zone), Europe, America, and Africa, in addition to various locations in the Pacific and Indian Oceans (119). Between 1940 and 2020, *Aedes albopictus* was reported for the first time in 86 countries. The initial DENV outbreak in Japan from 1942 to 1944 implicated *Aedes albopictus*. In 1944, due to human movements and goods transportation during World War II, this species expanded to Australia and Pacific countries for the first time (111).

I.C.2.2- Recent emergences

Over the past 30 years, *Aedes albopictus* has invaded several countries worldwide causing important emergences of DENV all over the regions, including the Mediterranean basin. From 1970 onwards, it has been detected in several Pacific countries (Papua New Guinea, Solomon Islands, and Fiji). It was confirmed in Albania in 1979, followed by Italy in 1990. In 2005, the tiger mosquito was detected in the Torres Strait Islands, but vector surveillance and control prevented *Aedes albopictus* from establishing itself on the Australian continent (132,133). From the year 2000, its emergence has been reported in new several countries such as Hawaii in 2001 (134), the Greek islands in 2003, Madagascar in 2006 (76), Gabon in 2007 (135), China in 2004 and 2010 (136,137), Malta in 2009, Ibiza in 2014, and the Tyrrhenian Islands in 2016 (138). The first epidemic likely attributed to *Aedes albopictus* in Europe occurred in 2007 in Ravenna, Italy, followed by a second epidemic in 2010 in southern France. Recent findings indicate that in the insular regions of Madagascar, *Aedes albopictus* has demonstrated a competitive advantage over its counterpart, *Aedes aegypti*. Specifically, it was identified in 13 out of 15 breeding sites in Madagascar, whereas *Aedes aegypti* was only found in six sites within native forests and natural reserves (141) (Figure 14) (142). Future climate changes and wetter/warmer conditions anticipate the risk of *Aedes albopictus* establishment in northern Europe, including regions of Germany and the southernmost parts of the UK. The introduction of *Aedes albopictus* into countries where it has not been previously identified is commonly regarded as a serious threat and thus it is seen as a potential or actual vector of arboviruses and new epidemics (143,144).

Important emergences of *Aedes albopictus* in La Réunion island have been recorded for decades. A detailed description of its history in the island will be presented in the next chapter.

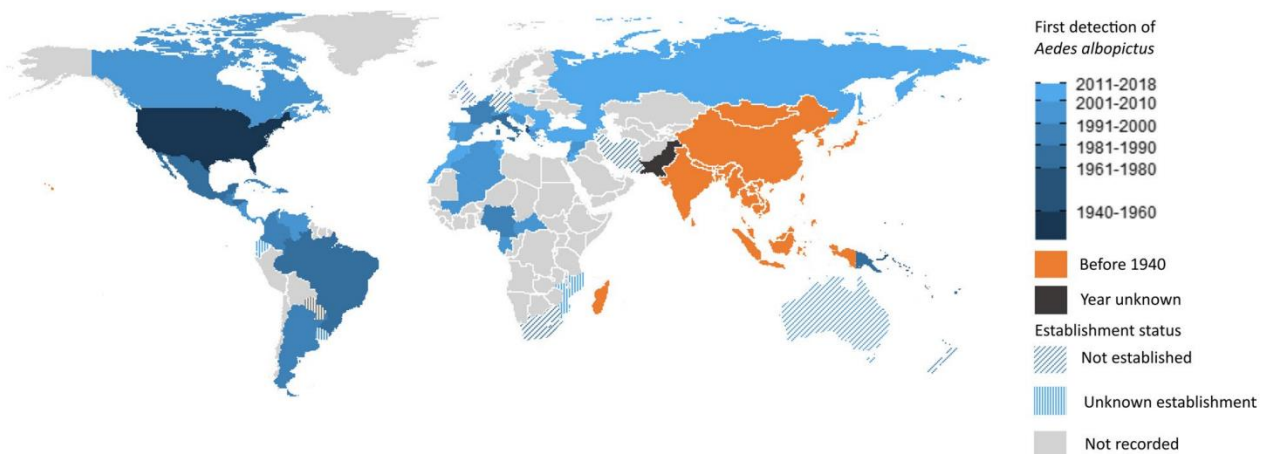


Figure 14 : Distribution range of *Aedes albopictus*. The map indicates the year of first detections (interceptions and vector surveillance of *Aedes albopictus*) by country and whether established populations have formed (solid color). (Swan T. et al., 2022)

I.C.2.3- Rapid Expansion of *Aedes albopictus* population

Aedes aegypti, taking to emerge predominantly in tropical world, contrasts with the more rapid emergence of *Aedes albopictus*. The latter has swiftly spread across all continents within a few decades, now present on five of the six continents, posing a significant threat to humanity. For *Aedes aegypti* and *Aedes albopictus* emergences regions, there is substantial variation in the suitability range among countries and territories, with many tropical regions

globally exhibiting high suitability over extensive areas. *Aedes albopictus* has suitability foci in 197 countries/territories, whereas *Aedes aegypti* is suitable in 188 countries (Figure 15) (4). The rapid expansion compared to the primary vector, is likely facilitated by factors such as human population growth, increased mobility, urbanization, modern transportation, and the global trade of goods, including potential mosquito breeding sites like used tires and bamboo (figure 16).

Additionally, the rapid emergence and expansion of *Aedes albopictus* are partly linked to its biology and particularly to its ability to withstand various environmental stresses under highly contrasting climates. This adaptability is exemplified by the eggs of this species, which can enter diapause and withstand desiccation to persist in the environment throughout the dry season. During the following rainy season, the larvae and adults are capable of hatching and traveling over long distances on inert surfaces containing water (145,146).

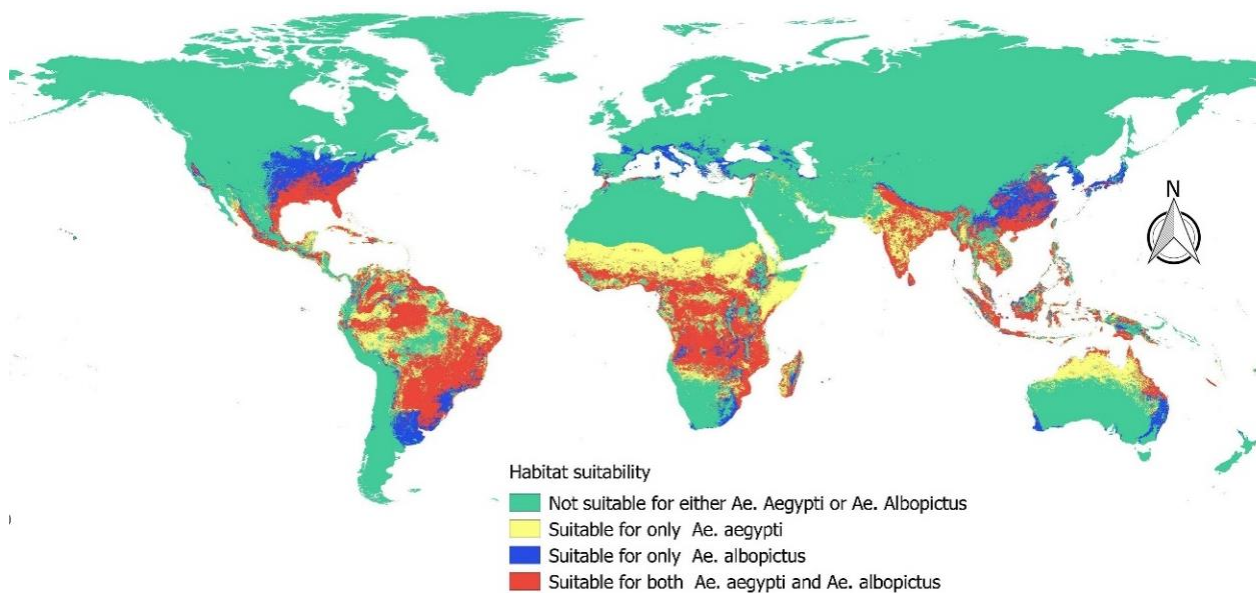


Figure 15: Global predicted habitat suitability of *Aedes aegypti* and *Aedes albopictus* (Leta S. et al., 2017)

I.C.2.4- Rapid expansion in La Réunion Island

For decades until today, La Réunion Island has been severely affected by DENV epidemics, primarily caused by the circulation of *Aedes albopictus* which is considered as its main vector on the island (131,147–151). This is primarily due to its abundance on the island, including regions at altitudes higher than 1,200 m, and to the relative absence of its sister vector species, *Aedes aegypti* (127,129,152). Moreover, this involvement of *Aedes albopictus* in DENV transmission in La Réunion is evidenced by the observation of DENV transmission peaks aligning with periods of elevated population densities of this vector (153). Additionally, a DENV-1 strain was identified in pools of *Aedes albopictus* collected during the 2004 epidemic in Réunion (Santé Publique France 2007), beside other studies that have demonstrated that *Aedes albopictus* from Réunion can be infected with both DENV-1 (154) and DENV-2 (152).

Part II. Thesis research program

II.A. Rationale

The frequency and severity of Dengue virus epidemics, transmitted by *Aedes* mosquito vectors worldwide, are alarmingly increasing annually. La Réunion Island has been grappling with the resurgence of DENV for decades. Despite the vector control measures implemented by the ARS (Agence Régionale de Santé) from 2016 to 2021, the transmission intensity has continued to rise. Hospital services reported a surge in cases and deaths annually, with symptoms becoming more severe. This escalation is attributed to the successive and/or simultaneous circulation of three DENV serotypes (DENV-1, DENV-2, and DENV-3) during the latest outbreak. Therefore, the molecular and genetic characterization of DENV, along with exploring their emergence origins, are crucial for tracing their introduction route and understanding the link between epidemic territories.

Moreover, distinct *Aedes albopictus* populations in Réunion may be involved in the DENV transmission on the island. These populations have probably undergone significant adaptation and competence for transmitting the virus under specific regional conditions. Hence, investigating the influence of socio-environmental conditions and biological factors on vector competence is crucial for understanding how the vector adapts to regional and biological conditions.

Furthermore, delving into the intricate biological factors influencing vector competence for DENV is crucial for comprehensively addressing and mitigating its transmission. Both historical and contemporary literature emphasize the ability of the *Wolbachia* bacterium to modulate various biological mechanisms within its host, particularly *Aedes albopictus*. In this context, our research aims to elucidate *Wolbachia's* potential role in regulating intestinal homeostasis under normal physiological conditions and its response following DENV ingestion, which could potentially exacerbate stress and epithelial damage.

II.B. Axes and objectives

To address these inquiries, my Ph.D. thesis was structured around three complementary chapters presented in the next part (Part III).

- **The first chapter focused on the genetic characterization of DENV-1 and investigating its emergence origin in La Réunion and neighboring Seychelles.**

Objective of the first study: Perform molecular and phylogenetic analysis on circulating DENV-1 strains in La Réunion Island and Seychelles by sequencing partial and full genomes of DENV-1 isolated from patients in both regions.

- **The second chapter involved exploring the impact of extrinsic geographical and intrinsic factors on the development of vector competence toward DENV-1 and DENV-2 strains among various wild populations of *Aedes albopictus* during the recent outbreak.**

Objective of the second study: Gain insights into the vector competence of *Aedes albopictus* populations from Reunion Island for endemic DENV strains, while accounting for their *Wolbachia* infection status.

- **The third chapter examined the effect of *Wolbachia* on intestinal homeostasis in a laboratory population of *Aedes albopictus* under normal conditions and following DENV-1 infection**

Objective of the third study: Investigate the role of *Wolbachia* in maintaining the integrity of the intestinal epithelium in *Aedes albopictus*. This entails examining the effects of *Wolbachia* on cellular division, apoptosis, and visceral muscle, as well as the expression of candidate genes associated with cellular division and apoptosis.

In the forthcoming part, each identified axis will be thoroughly examined within its respective chapter. Every chapter will be initiated with a detailed literature review to furnish the necessary context and background, setting the stage for the ensuing study. Subsequently, the findings will be articulated in the form of a scholarly article. After each chapter, a detailed summary will encapsulate the research implications, seamlessly bridging to the next chapter and enhancing the overall coherence of the study.

Part III. Chapter 1:
Transmission of Dengue
virus by *Aedes* mosquito in
La Réunion Island

Part III. Chapter 1:

Transmission of Dengue virus by *Aedes* mosquito in La Réunion island

“La Réunion, a target for the Dengue virus?”

In this chapter, we will focus on Dengue virus emergence and the epidemics caused by *Aedes albopictus* vector, in La Réunion island.

First, we will track the history of the primary vector on the Island, *Aedes albopictus*.

Second, we will present the DENV epidemiology since 1977 until today.

Third we will trace the origin of the emergence of DENV during the last epidemic.

In this axis, we will present our findings related to the genetic characterization of the DENV-1 serotype and explore the origin territory of its emergence to the island.

Part III. Chapter 1: Dengue virus transmission by *Aedes* mosquito in La Réunion Island

III.1.A. *Aedes* mosquito's emergence in La Réunion Island

Reunion Island is an overseas French department located in the South-Western Indian Ocean, about 700 km east of Madagascar. Between 1901 and 1902, the *Aedes aegypti* mosquito was the first recorded mosquito species on the island. The population distribution was limited to a few artificial or natural larval habitats such as rock holes and certain ravines on the west coast, at elevations between 300 and 650 meters (155). The insecticide treatment campaigns against Malaria for vector elimination contributed to its disappearance in 1950, but it was rediscovered in 1980. From 1985, its population began to diminish again and was replaced by the population of *Aedes albopictus*. The Asian tiger mosquito, *Aedes albopictus*, was first reported in La Réunion in 1913, but it may have been introduced several years earlier through trade with the Indian/Indonesian subcontinent or from the coast of Madagascar (149,155). Later, in the period between 1985 and 1986, during the mosquito-control campaigns, only 73 larval habitats containing *Aedes aegypti* larvae were found, compared to 177 habitats containing *Aedes albopictus* larvae. *Aedes albopictus* started dominating the *Aedes aegypti* population and this latter was no longer found in their habitats, and its populations were pushed to less anthropized areas (127,155,156).

In La Réunion, among the 12 species of *Culicidae*, since the 1950s, *Aedes albopictus* has been the dominant species in inhabited areas below 1,200 meters and was responsible for the last two epidemics of Dengue virus, which took place in 1977 and 2017 (131,150,157). During the last decades, *Aedes albopictus* contributed to significant outbreaks of arboviruses, including DENV and CHIKV, not only in La Réunion but in all the neighboring Islands in the south west of the Indian Ocean (77,151,158)

Aedes albopictus is considered as an urban vector, the main dispersion pathways of the tiger mosquito on the island results from the transportation of used tires and used vehicles. Additionally, the trade of bamboo containing eggs and the passive transport of *Aedes albopictus* by riverboat also pose potential risks of dispersion. Furthermore, fluctuations in temperature, rainy seasons, urbanization, and increased global transit all heighten the risk of vector transportation. Adapted breeding sites near residential areas, such as stagnant water, create favorable conditions for vector adaptation and sustained circulation (142). Additionally, human populations with inadequate sanitation and low immunity are particularly vulnerable to the expansion of vector populations. These factors elevate the risk of DENV infection over time (159–161) (Figure 16).

All these factors have contributed to the increased circulation of DENV on the island, facilitated by the expansion of the vector population, *Aedes albopictus*, in favorable environmental conditions.

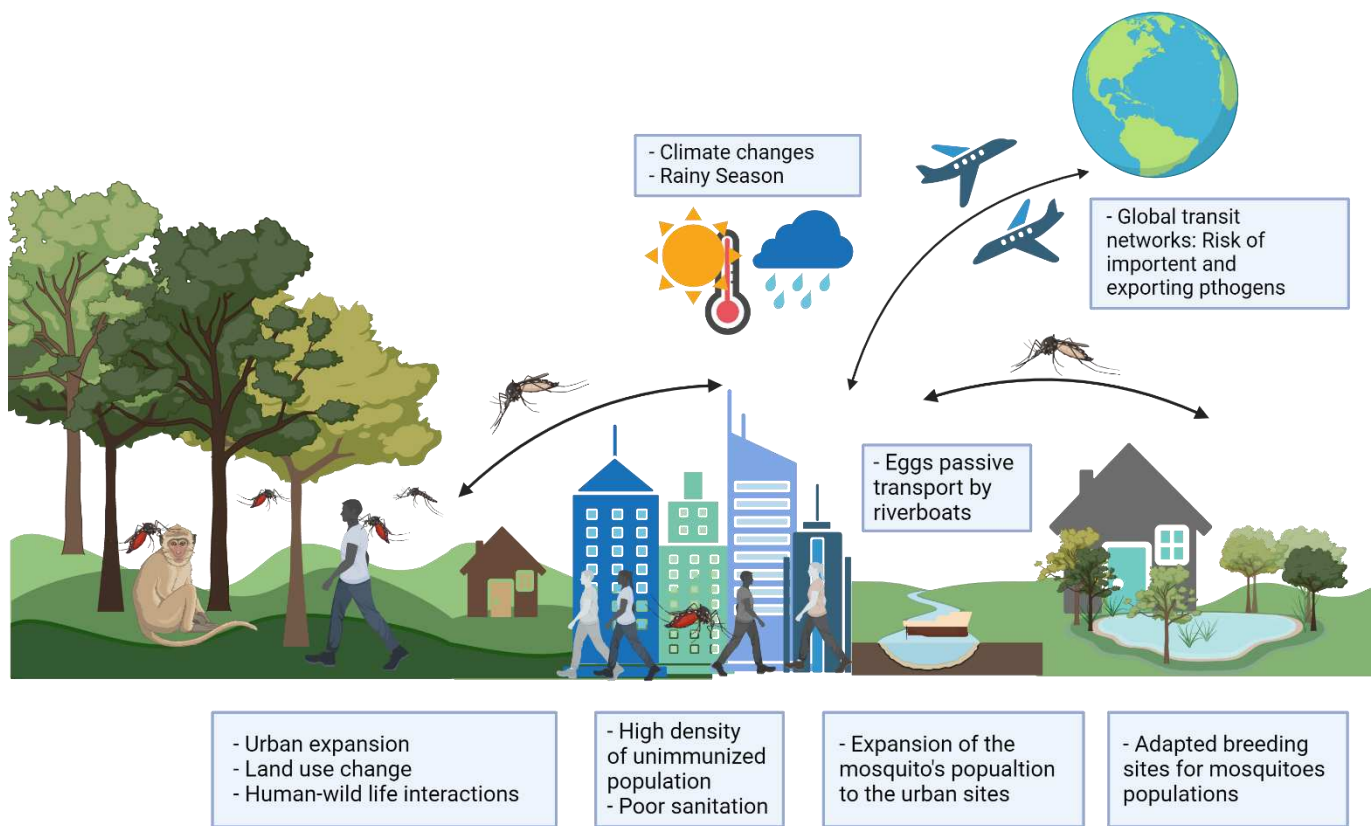


Figure 16: Favorable factors contributing to the expansion of mosquito's vector populations and pathogens

III.1.B. Epidemiology of Dengue virus in La Réunion Island

La Réunion Island has been a victim of the emergence and re-emergence of the 4 DENV serotypes since 1977, DENV-1, DENV-2, and DENV-3 in autochthonous cases, and all four serotypes in imported cases (77). In 1977 and 1978, the DENV-2 strain emerged, contributing to a significant epidemic over two years, affecting 30% of the population (162). The second epidemic occurred in 2004, involving the circulation of DENV-1, causing 228 suspected cases. From 2004 to 2015, there was an inter-epidemic period on the island with sporadic recorded cases. Between January 1, 2007, and October 5, 2009, five autochthonous cases were confirmed, along with five imported cases (from Southeast Asia) and 71 probable cases. All five confirmed autochthonous cases occurred in Saint-Louis during two consecutive clusters (75). Subsequently, moderate dengue episodes occurred in 2016 and 2017. In 2016, 230 cases were recorded, with an incidence rate of 26 autochthonous cases per 100,000 people, and no death related to dengue was confirmed. In 2017, a slight decrease in the annual number of cases was observed with 103 dengue cases and an incidence rate of 11 autochthonous cases per 100,000 people (77) (Table 3).

Since late 2017, a significant epidemic began and continued until 2022. This recent DENV outbreak on the island is considered one of the largest and most dangerous epidemics and was reposted across all the regions of the island (Figure 17) (163). Epidemic circulation has been on the rise, with a very high number of confirmed cases. By the end of 2018, 6,701 autochthonous cases were confirmed with 149 hospitalizations and 6 deaths (164). Until August 2021, 29,000 cases of DENV, over 1,000 hospitalizations, and 33 deaths were recorded (147) (Figure 18). Up until 2022, 70,978 confirmed cases, 2,672 hospitalizations, and 75 deaths have been reported since the beginning of the epidemic (77,147). This significant burden of morbidity and mortality were caused as all three dengue serotypes (DENV-1, DENV-2, and DENV-3) were circulating simultaneously on the island. DENV-2 was the first to circulate widely in the population in 2018. DENV-1 was introduced and co-circulated with DENV-2 in 2019 and it showed dominance starting at the end of 2019. A very low circulation of DENV-3 in 2020 was isolated in the east of the island (77,147,165) (Table 3).

Over the past two years (2022-2023), La Réunion Island has not experienced any significant epidemics. In 2022, there was no notable epidemic peak, with only the DENV-1 serotype circulating. The number of confirmed cases decreased by over a thousand compared to previous years, resulting in 3 deaths and 61 hospitalizations (148) (Figure 18). In 2023, the DENV-2 serotype began to circulate. There have been 218 reported cases of dengue, leading to 12 hospitalizations, including 2 severe cases primarily in the southern region, similar to the situation observed in 2022 (166) (Table 3).

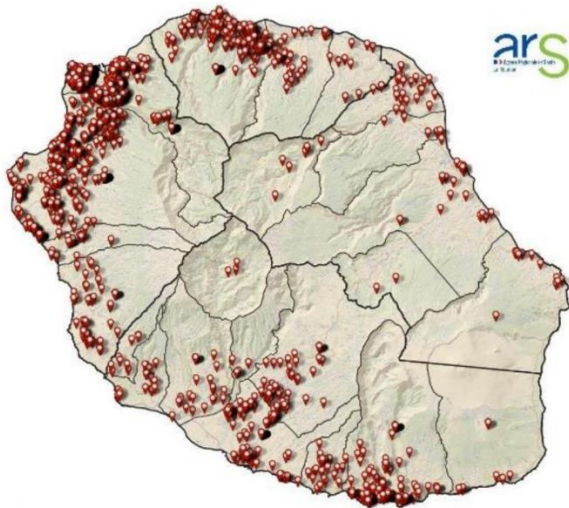


Figure 18 : Localization of confirmed Dengue cases across La Réunion in 2021, by Health Data Department (S14 & 15), (source ARS-LAV)

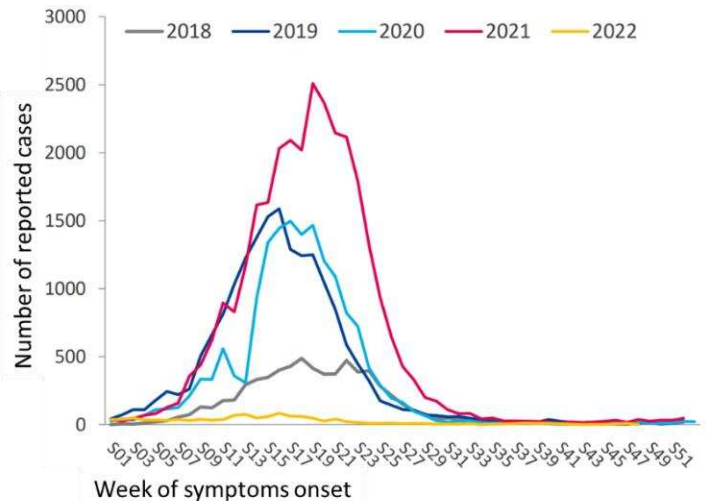


Figure 17 : Distribution of the number of Dengue cases per week of symptom onset, Réunion Island (S01/2018 to S48/2022).

Public Health France, regional epidemiology report-2022

Table 3: Summary of the number of confirmed cases of dengue and severe dengue disease in Reunion from 1977 to 2023. (Modified from Hafsia et al.,2022).

Year	Total number of cases	Autochthonous cases	Imported cases	Dengue Severity			DENV serotypes
				Hospitalizations	Severe cases	Deaths	
1977- 1978	ND	ND	ND	ND	ND	ND	2 ^a
2004	228	228	ND	ND	ND	ND	1 ^a
2005	ND	ND	ND	ND	ND	ND	ND
2006	ND	ND	ND	ND	ND	ND	ND
2007	28	28	ND	7	0	0	1 ^a
2008	48	44	4	ND	ND	ND	1 ^a ,3 ⁱ
2009	12	8	4	ND	ND	ND	2 ⁱ
2010	32	18	14	1	0	0	1 ⁱ +3 ^{a+i} ,4 ⁱ
2011	4	2	2	6	0	0	ND
2012	38	31	7	0	0	0	1 ^a ,3 ^a
2013	33	21	12	4	0	0	1 ^{a+i} , 3 ^{a+i}
2014	44	29	15	0	0	0	2 ^{a+i}
2015	10	6	4	0	0	0	ND
2016	231	221	10	18	3	0	1 ^a ,2 ^a ,3 ^a
2017	103	94	9	14	0	0	1 ⁱ ,2 ^{a+i} ,4 ⁱ
2018	6,771	6,759	11	154	27	6	2 ^a
2019	18,217	18,217	ND	620	75	14	1 ^a ,2 ^a ,3 ^a
2020	16,414	16,414	ND	787	108	22	1 ^{a+i} ,2 ^{a+i} ,3 ^a
2021*	29,577	29,577	ND	1,111	245	33	1 ^a
2022	1,183	1,183	ND	61	15	3	1 ^a
2023	218	121	ND	12	2	0	2 ^a

^aSerotype identified in autochthonous cases.

ⁱSerotype identified in imported cases.

*In 2021, data available only from January to the beginning of October.

ND, unavailable data

III.1.C. Thesis first publication: The origin of different dengue strains circulating in La Réunion from 2018 to 2021

III.1.C.1. Articles' background and objectives

Islands located in the SWIO exhibit common conditions conducive to the spread of DENV. These conditions include the presence of DENV-competent vectors, similar environmental and climatic factors, limited mosquito control measures, rapid population growth, uncontrolled urbanization, in addition to inadequate water supply and waste management systems (167,168). These common factors shared between the islands in the same region explain the simultaneous outbreaks of DENV experienced over time suggesting a probable ongoing but low-level transmission of the disease across all the neighboring islands of the SWIO.

As mentioned above, several documented epidemics support this association. In 1976 and 1977 a large outbreak of DENV-2 was recorded in the Seychelles and subsequently in La Réunion islands in 1977 and 1978 and the dispersion of the virus to the Mayotte island was also suggested (80,169). Sporadic imported cases have been reported in La Réunion Island since 2005. It was demonstrated that these cases were originated from neighboring islands including Madagascar and Mayotte (81). In 2010, the same DENV circulation was reported in the Comoros and Mayotte islands (170). Similarly, in 2012, DENV micro epidemics took place in Mayotte and La Réunion. Likewise, in 2014, the Comoros, Mayotte, and La Réunion Islands were also affected by the same small epidemics (77).

In 2016, a notable epidemic was documented in the Seychelles, spreading to nearby islands such as Madagascar and Mauritius, and subsequently affecting La Réunion locally (78–82). The onset of this epidemic can be traced back to La Réunion towards the end of 2017, with the circulation of DENV-2, followed by the emergence of DENV-1 by the conclusion of 2018. This succession of viral strains contributed to an epidemic crisis that endured until 2022 (77,171). Between March and May 2018, 2 autochthonous cases of DENV-2 were reported in La Réunion, whose sequences showed similarity to those of Israeli travelers infected in the Seychelles in 2015. Alongside the strain circulating in the Seychelles, La Réunion strain was grouped within the Cosmopolitan genotype of DENV-2. This genotype contains sequences from China and India, from which both La Réunion and Seychelles strains may originate (172). Sequencing and alignment of both DENV-2 strains, isolated from La Réunion in 2018 and Seychelles in 2015, revealed that the two genomes shared a similarity of 99.8% and exhibited 93% identity with the genomic sequence of a DENV-2 strain from Thailand (79).

During the recent epidemic in La Réunion Island, DENV-1 was introduced and co-circulated with DENV-2 in 2019, and it became the dominant strain circulating in 2021 (157). The same introduction of DENV-1 was recorded also in Seychelles in 2016 (MOH Seychelles). Simultaneously with the circulation of the 2 serotypes DENV-1 and DENV-2, circulation of the DENV-3 serotype was detected in 2020 (SPF, August 11, 2020), increasing the risk of the Antibody-Dependent Enhancement phenomenon (46,105).

Given the circulation of the DENV-2 strain in Réunion in 2018 having the same origins as the DENV-2 circulating strain in Seychelles in 2015 (172), **we hypothesized a possible link**

between the DENV-1 strain that emerged in Réunion Island in 2019 and DENV-1 that circulated few years ago in Seychelles. Thus, the objectives of our first study were to characterize genetically the DENV-1 strains from the two Islands and deduce, when possible, its origin.

III.1.C.2. Thesis first publication

Genetic characterization of dengue virus serotype 1 circulating in Reunion Island, 2019–2021, and the Seychelles, 2015–2016

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RESEARCH

Open Access



Genetic characterization of dengue virus serotype 1 circulating in Reunion Island, 2019–2021, and the Seychelles, 2015–2016

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Abstract

Background An unprecedented increase in the number of cases and deaths reported from dengue virus (DENV) infection has occurred in the southwestern Indian ocean in recent years. From 2017 to mid-2021 more than 70,000 confirmed dengue cases were reported in Reunion Island, and 1967 cases were recorded in the Seychelles from 2015 to 2016. Both these outbreaks displayed similar trends, with the initial circulation of DENV-2 which was replaced by DENV-1. Here, we aim to determine the origin of the DENV-1 epidemic strains and to explore their genetic characteristics along the uninterrupted circulation, particularly in Reunion.

Methods Nucleic acids were extracted from blood samples collected from dengue positive patients; DENV-1 was identified by RT-qPCR. Positive samples were used to infect VERO cells. Genome sequences were obtained from either blood samples or infected-cell supernatants through a combination of both Illumina or MinION technologies.

Results Phylogenetic analyses of partial or whole genome sequences revealed that all DENV-1 sequences from Reunion formed a monophyletic cluster that belonged to genotype I and were closely related to one isolate from Sri Lanka (OL752439.1, 2020). Sequences from the Seychelles belonged to the same major phylogenetic branch of genotype V, but fell into two paraphyletic clusters, with greatest similarity for one cluster to 2016–2017 isolate from Bangladesh, Singapore and China, and for the other cluster to ancestral isolates from Singapore, dating back to 2012. Compared to publicly available DENV-1 genotype I sequences, fifteen non-synonymous mutations were identified in the Reunion strains, including one in the capsid and the others in nonstructural proteins (NS) (three in NS1, two in NS2B, one in NS3, one in NS4B, and seven in NS5).

Conclusion In contrast to what was seen in previous outbreaks, recent DENV-1 outbreaks in Reunion and the Seychelles were caused by distinct genotypes, all likely originating from Asia where dengue is (hyper)endemic in many countries. Epidemic DENV-1 strains from Reunion harbored specific non-synonymous mutations whose biological significance needs to be further investigated.

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Keywords Dengue, Outbreaks, Genetics, Genomes, Reunion Island, Seychelles

Background

Dengue virus (DENV) is the most widespread mosquito-borne flavivirus worldwide. More than 390 million people in over 129 countries are exposed to dengue virus infections with an estimated 20,000 deaths every year [1, 2]. DENV is transmitted through the bite of infected female mosquitoes belonging to the *Aedes* genus; the species *Aedes aegypti* being a primary vector while *Aedes albopictus* plays a secondary role [3]. The DENV genome consists of a positive-sense single-stranded linear RNA of approximately 11 kilobases. Four DENV serotypes (DENV-1 to DENV-4) are currently known. Molecular analyses have highlighted the diversity of DENV genotypes belonging to each of the four serotypes [4, 5].

The southwestern Indian ocean islands have a history of periodic outbreaks of DENV, but Reunion Island has been an exception in recent years. Since an outbreak of the virus in 2017, there has been continuous circulation of DENV on the island with over 70,000 confirmed cases between 2017 and 2022 (Table 1). This continuous presence of the virus on the island may suggest that dengue has become endemic [6].

At the beginning of the epidemic wave, DENV-2 was the first serotype detected on the island [9, 10]. At the end 2019, among the 25,000 confirmed cases and 20 deaths, DENV-2 still represents the major serotype, but a few cases of DENV-1 and DENV-3 were also reported [11]. During the course of the epidemic, DENV-1 was also observed to co-circulate with DENV-2, and rapidly became the dominant serotype. DENV-1 was the only identified serotype among the 29,577 confirmed cases by week 35 in 2021 [12]. A similar trend was also observed in the Seychelles where an outbreak of DENV-2 started in 2015, being replaced by DENV-1 in 2016 (MOH Seychelles). Interestingly, the DENV-2 strains that circulated at the beginning of these outbreaks are 99.8% similar to each other and 93% similar to a 2013 strain from Singapore, indicating a single introduction of DENV-2, presumably from Asia [10]. As DENV-1 subsequently succeeded DENV-2 in these two islands, it would be

interesting to see whether the DENV-1 outbreaks were also initiated by a single introduction event.

In 2021, DENV-1 circulation in Reunion coincided with a significant increase of severe cases and hospitalizations when compared to 2018 and 2019 [12]. For instance, several severe ophthalmological cases related to DENV-1 infection were reported, notably in 2020 and 2021 [12]. Moreover, while severe forms and deaths were most frequently observed in older patients with comorbidities such as diabetes and hypertension, increasing numbers of DENV1-induced mortalities were observed in younger patients (including children) without comorbidities [12]. Although such an increase in severity was observed for the first time in Reunion, these symptoms were already described in Asia where dengue is endemic [13, 14]. To investigate whether the current epidemic DENV-1 strains from Reunion and the Seychelles were similar to each other and to those known in Asia, we performed whole-genome sequencing and phylogenetic analyses to trace their origin and explore some genetic features.

Methods

Origin of samples

Biological samples from Reunion Island were originated from two different collections. The CARBO collection consisted of a prospective cohort of patients with arbovirus infections, registered on clinicaltrials.gov (NCT01099852). The CARBO was started by the University Hospital of La Martinique in 2010 and extended to include patients from Reunion in 2018. The other collection, DEMARE, was established as part of an epidemiological cross-sectional study conducted in 2019–2020. Both collections were approved by The French Committee for the Protection of Individuals. Written consent was obtained from all participants. Blood samples from the Seychelles were collected by the Ministry of health of the Seychelles during outbreaks between January 2015 and December 2016.

Bloods were collected from serologically confirmed patients presenting one or several dengue symptoms including fever, myalgia, headache, asthenia and

Table 1 Circulation of DENV from 2017 to mid-2022 in Reunion

Year*	Number of confirmed cases	Number of serotyped cases (TotS)	DENV-1/TotS	DENV-2/TotS	DENV-3/TotS
2017	94	94	0	1	0
2018	6,759	950	0	1	0
2019	18,217	883	0.118	0.880	0.002
2020	16,414	838	0.85	0.12	0.03
2021	29,655	978	1	0	0
2022	1,129	329	1	0	0

*Data were obtained from [6] and Santé Publique France [7, 8].

thrombocytopenia and without recent travel history. To increase the probability of detecting the presence of viral RNA, the samples used in the study were from patients included in the cohort within the first seven days of the onset of symptoms. Collections were performed by accredited professionals in 10 mL EDTA tubes. Within the two hours after collection, samples were centrifuged at 2,000 g for 10 min at 20 °C. Supernatants were transferred into new tubes, homogenized by inversion, aliquoted into new 2 mL tubes, labeled, then frozen and stored at -80 °C until used.

Viral RNA extraction and amplification

To ascertain the presence and the amount of DENV-1 RNA in the selected samples, a reverse transcription quantitative real-time PCR (RT-qPCR) was performed. Briefly, total nucleic acids were extracted from the serum samples using the QIAamp® Mini Kit purification according to the manufacturer's recommendation (QIAGEN). For RT-qPCR, we used the QIAGEN OneStep kit following the manufacturer's instructions (QIAGEN). A mixed solution was prepared with RNA template (5 µl), a Taq-Man probe (FAM-ACACCTCAAGCTAA-TAMRA) and primers (Forward 5'-GAACATGGRACAAYTG-CAACYAT-3'; Reverse 5'-CCGTAGTCDGTCAGCT-GTATTTTC-3') specific to DENV-1. The thermocycler program consisted of a retrotranscription step of 45 min at 45 °C, denaturation for 5 min at 95 °C followed by 40 cycles of amplification (72 °C for 5 s and 56 °C for 60 s). Viral RNA copy number was estimated against a standard curve following the methodology published by the HAS (Haute Autorité de Santé, France). Plasmids containing targeted DENV-1 were synthesized by GeneCust (France) and used as the standard curve at concentrations of 10¹ to 10⁸ RNA copy per µl.

The E gene was amplified to confirm RT-qPCR-positive samples. cDNA was synthesized from extracted RNA using the ProtoScript® II Reverse Transcriptase Kit with random primers following the manufacturer's instructions (New England BioLab, USA) and amplified using a nested PCR protocol, as previously described [9]. Briefly, we designed degenerated primers targeting a fragment (~700–800 bp) of the E gene encoding the envelope protein. The first round of amplification reaction was performed with primers DNV1-E-F1 (CAC TGG TGG AAG AAC AAG ACG C) and DNV1-E-R2 (CMA CDG AYG TGA ACA CYC CTC C) generating an approximately 1100-bp fragment. The second round used the primers DNV1-E-F2 (ACG GAG CTC TYA CAT TGG ACT G) DNV1-E-R2 (CMA CDG AYG TGA ACA CYC CTC C) and generated a 750-bp fragment. PCR amplification was performed on a PCR System 2700 Thermocycler (ABI Applied Bio-system™). Amplification programs were as follows: 94 °C for 2 min; 3 cycles of 95 °C for 5 s, 60 °C for

30 s, 72 °C for 30 s; 3 cycles of 95 °C for 5 s, 55 °C for 30 s, 72 °C for 30 s; and 28 cycles of 95 °C for 5 s, 50 °C for 30 s, 72 °C for 1 min 30 s; 72 °C for 7 min. Amplified products were checked first on electrophoresis gel (Additional file) then samples showing amplicons of expected sizes were Sanger sequenced (Genoscreen, Lille, France).

Virus isolation

Viral isolation assays were conducted by inoculating the DENV-1 PCR-positive sera from viremic patients onto Vero E6 cell monolayers. Cultures were checked every day. When cytopathic effects were observed, supernatants (passage 1) were collected by centrifugation and stored at -80 °C. The presence of viral RNA was confirmed by RT-qPCR, as above, and by titration of viral infectious particles using plaque-forming unit assay [10].

Sequencing and genomic analysis

Genomic data were generated using either Oxford Nanopore Technologies (MinION) sequencing based on the amplicon tiling protocol, or Illumina shotgun sequencing, or by combining data from both sequencing platforms [15].

For Illumina sequencing, libraries were generated from 10 ng of cDNA using the Celero™ PCR Workflow with Enzymatic fragmentation (DNA-Seq) following the manufacturer's instructions. Sequencing was performed on the MiSeq platform, with 1 × 170 bp single-end reads. Demultiplexed sequences were provided by the sequencing company (Biofidal, Lyon, France). Sequences were quality trimmed and adapters removed using Trimmomatic v0.39 [16]. Trimmed reads were mapped to reference sequence NC_001477.1 using bowtie2 [17]. Geneious v9.1.8 [18] was used to inspect and curate mapped sequence data.

For Oxford Nanopore Technology's MinION sequencing, an amplicon tiling protocol was used in conjunction with DENV1 primers from the Oxford Centre for Arbovirus Discovery, Diagnostics, Genomics and Epidemiology (<https://www.caddecentre.org/protocols/>). Briefly, cDNA was amplified in two independent PCR reactions. PCR products were pooled, dA-tailed using the NEBNext® Ultra™ II End Repair/dA-Tailing Module then barcoded using the Nanopore Native Barcoding Expansion kit (EXP-NBD104). Barcoded amplicons were then purified using a 0.4x volume of AMPure-XP SPRI beads, washing the beads with an excess volume of Nanopore's small fragment buffer (SFB) to ensure that un-ligated barcode molecules were removed. Purified amplicons were then pooled in equimolar proportions before adapter ligation and sequencing, following the manufacturer's instructions. The sequencing run was left for 24 h and stopped when predicted coverage exceeded 1000-fold for each genome. Base-calling and demultiplexing were

performed using guppy (v4.0.11). Base-calling used default parameters in accurate mode, and demultiplexing was performed using the "require_barcodes_both_ends" parameter to minimize sample crosstalk. Reads were then assembled using Medaka v1.0.3 (<https://nanoporetech.github.io/medaka/>) mimicking the ARTIC network bioinformatics standard operating procedure for SARS-CoV-2 sequencing (<https://artic.network/ncov-2019/ncov2019-bioinformatics-sop.html>), subsampling amplicon coverage to 400x and using genome accession NC_001477.1 for read mapping. Geneious v9.1.8 was used to inspect and curate mapped sequence data. Consensus base-calling required a minimum of 30-fold coverage.

For samples from the Seychelles, only partial genomic assemblies were obtained by this method, as not all primer pairs produced amplified products. The partial assembly data were thus combined with Sanger sequence data from the envelope region of the genome to produce contiguous consensus sequences that spanned a 4,838 bp region at the 5' end of the DENV genome. This region corresponded to base positions 156–4,994 of the reference genome NC_001477.1 and contained genetic data from the capsid, membrane glycoprotein, envelope, NS1, NS2a and NS2b regions of the genome.

Phylogenetic analysis

Global phylogenetic comparisons were carried out using the E-gene, which is typically used for identifying dengue genotypes [5]. The E-gene region was extracted from all reference sequences in the NCBI database using custom scripts. Alignments were generated using MAFFT [19], and the alignment was curated by eye in Geneious v9.1.8. IQTree2 (v2.1.3) was used to identify optimal

substitution model parameters and to generate a bootstrapped maximum-likelihood phylogeny with 1000 replicates. Phylogenetic tree representations were generated in R, using the "ggtree" package [20].

Results

General characteristics

Among the 30 blood samples from Reunion and 14 from Seychelles collected within the first seven days of the onset of symptoms, only nine (PR1583, PR1615, PR1914, PR4443, PR4453, PR4463, PR4483, PR6594, P0409) of 2019 to 2021 outbreaks from Reunion and four (DS16177, DS16229, DS16232, DS16233) of 2016 outbreak from Seychelles tested positive for DENV-1 by RT-qPCR were further analyzed (Table 2). A higher viral titer was detected for the two samples PR1583 and PR6594 with approximately 10^7 RNA copies/ μ L. These two samples were used to infect VERO cells allowing the isolation of DENV-1 strains named RUN1-1583 and RUN1-6594, respectively, isolates which were also further analyzed (Table 2). Sequencing with Illumina generated complete genome sequences for 5 blood samples (PR4443, PR4453, PR4463, PR4483, PR6594) and the isolate RUN1-6594 (one passage) from Reunion. MinION technology allowed sequencing of almost complete genomes for 3 sera (PR1615, PR1914, P0409) and the isolate RUN1-1583 (one passage) from Reunion. The sequence of each isolated strain was identical to that obtained from the corresponding blood sample. Using MinION technology, only partial sequences were obtained from the four Seychelles samples. Upon sequence inspection, mismatches were identified in the DENV1_3, DENV1_11 and DENV1_12 primer pairs which may explain inefficient amplification of DENV1 genotype V. Further optimization of the

Table 2 Biological samples and DENV isolates used in this study

Sample	Origin	Biological material	Sequencing technology	Coverage	Viral isolation	Genbank Accession
RUN1-1583	Reunion, 2019	Supernatant, cDNA	MinION, Illumina	Complete	Yes	ON631277
PR1615	Reunion, 2019	Serum, cDNA	MinION	Complete*	No	ON631275
PR1914	Reunion, 2019	Serum, cDNA	MinION	Complete*	No	ON631274
PR4443	Reunion, 2020	Serum, cDNA	Illumina	Complete	No	ON631283
PR4453	Reunion, 2020	Serum, cDNA	Illumina	Complete	No	ON631282
PR4463	Reunion, 2020	Serum, cDNA	Illumina	Complete	No	ON631281
PR4483	Reunion, 2020	Serum, cDNA	Illumina	Complete	No	ON631280
PR6594	Reunion, 2021	Plasma, cDNA	Illumina	Complete	No	ON631278
RUN1-6594	Reunion, 2021	Supernatant, cDNA	Illumina	Complete	Yes	ON631279
P0409	Reunion, 2020	Plasma, cDNA	MinION, Illumina	Complete	No	ON631276
DS16177	Seychelles, 2016	Serum, cDNA	MinION	Incomplete, 71.2%	No	ON631270
DS16229	Seychelles, 2016	Serum, cDNA	MinION	Incomplete, 88.4%	No	ON631271
DS16232	Seychelles, 2016	Serum, cDNA	MinION	Incomplete, 76.9%	No	ON631272
DS16233	Seychelles, 2016	Serum, cDNA	MinION	Incomplete, 76.9%	No	ON631273

*Genome coverage is considered complete for Illumina data if the sequence obtained mapped to more than 98% of the DENV-1 reference sequence (Genbank accession number NC_001477.1). The amplicon tiling protocol for MinION used primer positions that did not amplify 155 bp at the 5' extremity, nor 575 bp at the 3' extremity of each genome, thus complete contiguous sequences using MinION map to 93% of the same reference sequence.

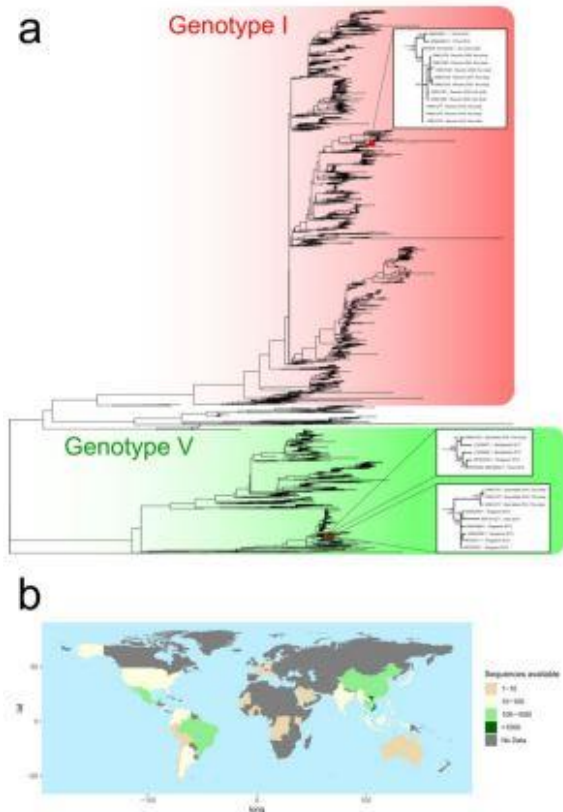


Fig. 1 Phylogenetic analyses of DENV. (a) Phylogenetic tree generated using a 4,838-bp region of the DENV genome (positions 156 to 4,994 of NC_001477.1). Inset are zoomed representations of the tree topology and bootstrap values for regions relevant to sequences generated as part of this study. (b) A graphical representation of the country of origin data for sequences included in the phylogenetic analysis

amplicon tiling protocol would be required to allow efficient amplification and sequencing of all DENV-1 genotypes by this method.

Phylogeny

Phylogenetic analyses of the approximately 5000-bp genomic region of 3,891 publicly available sequences

showed that all sequences from Reunion Island found in 2019, 2020 and 2021 belonged to genotype I of DENV-1 (Fig. 1a), and possessed nearly identical sequences, forming a monophyletic cluster in the phylogeny. These sequences showed greatest similarity (99.5% identity) to one isolate from Sri Lanka in 2020 (Accession number OL752439.1) and two sequences from China in 2016 (Accession numbers MN933661 and MN933663). Sequences from the Seychelles belonged to the same major phylogenetic branch of DENV-1 genotype V, but fell into two paraphyletic clusters (Fig. 1a). DS16177 showed greatest similarity (>99% identity) to sequences from 2016 to 2017 isolated in Bangladesh, Singapore and China, whereas DS16229, DS16232, DS16233 and DS16243 showed greatest similarity to numerous ancestral isolates from Singapore, dating back to 2012. However, interpretations of isolate origins should consider the strong sampling bias that exists in available DENV sequence data (Fig. 1b).

Further extending the phylogenetic comparison to include whole genomic data (Additional file) provided no strong evidence for the introduction of separate lineages in Reunion, as all whole genome sequences showed >99.8% identity (between 0 and 19 SNPs).

Major non-synonymous mutations

Fifteen non-synonymous amino acid changes were specifically observed in all Reunion DENV-1 full genome sequences compared with other DENV-1 genotype I sequences publicly available (Fig. 2). Mutations consisted of N90S in the C gene, R989K, N1068S and K1116R in NS1, I1389M, V1451I and E1487K in NS2B, A2271T in NS4B. Seven mutations H2620Y, K2881R, V2906I, E3052D, S3059A, V3179I and I3322V were observed in NS5. As only partial sequences were generated from the Seychelles samples, reconstruction of full polyprotein was not possible, thus precluding the analysis of amino acid changes.

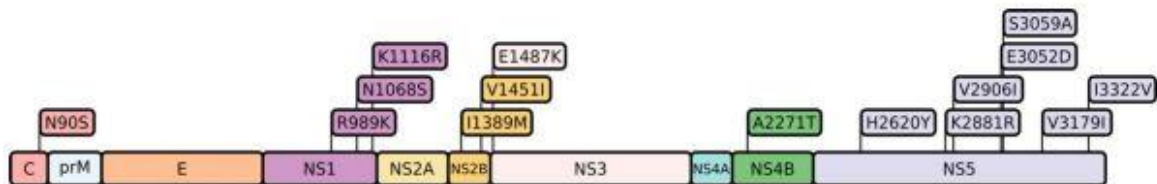


Fig. 2 Schematic representation of conserved mutations identified within isolates from Reunion. Position numbers are expressed relative to the first conserved start methionine of all polyprotein sequences belonging to DENV1 as defined in [28]. Mutations are expressed relative to the majority amino acid sequence of DENV1 genotype I sequences from the same dataset. Positions are colored by mature protein: C, Capsid; prM, pre-membrane; E, envelope; NS, Nonstructural. A: Alanine; D: Aspartic acid; E: Glutamic acid; H: Histidine; I: Isoleucine; K: Lysine; M: Methionine; N: Asparagine; R: Arginine; S: Serine; T: Threonine; V: Valine; Y: Tyrosine

Discussion

Since the recent dengue outbreak started in late 2017, uninterrupted transmission has occurred in Reunion Island. From 2017 to mid 2022, more than 70,000 confirmed cases were reported [6–8]. Circulation of DENV-2 in 2018 was followed by a major co-circulation of DENV-2 and DENV-1 at the end of 2019 and during 2020, with few cases of DENV-3. In 2021 and 2022, DENV-1 was the only serotype identified in Reunion Island [6]. The Seychelles were also confronted to dengue outbreaks years before (2015–2016) with a similar trend starting with DENV-2 which was replaced by DENV-1 [6].

Using phylogenetic analyses, we showed that the DENV-1 sequences from Reunion were closely related to a 2020 strain circulating in Sri Lanka, whereas those from the Seychelles showed a greatest similarity for isolates from Bangladesh, Singapore and China. This result suggested that the DENV-1 epidemic strains in Reunion and Seychelles were probably imported from Asian countries. We identified a single DENV-1 lineage belonging to genotype I that circulated in Reunion since its emergences at the end of 2019 and subsequently in 2020 and 2021. On the contrary, two lineages of DENV-1 of genotype V occurred in the Seychelles in 2016. It should be noted that these are likely partial descriptions of the total genetic diversity of DENV on the two islands due to the limited sample availability for our study. Even so, our results were distinct to previous observations in the region where similar viral epidemic strains usually occurred in contemporary outbreaks. For instance, between 2003 and 2004 an outbreak caused by DENV-1 serotype started in the Seychelles [21] and then spread in Reunion [22]. The same DENV-1 serotype was involved in an outbreak in Toamasina, eastern Madagascar, two years later in 2006 [23], but a regional link could not be established as no sequence was found. More recently, dengue outbreaks due to DENV-2 occurred in 2016 in the Seychelles and then in 2018 in Reunion, and it was shown to involve the same cosmopolitan sub-lineage I [9, 10]. Moreover, the link of dengue outbreaks between these two islands has been demonstrated by the presence of dengue cases in Reunion imported from the Seychelles whether in 2016 [7] or in 2017 [8].

The presence of two distinct sub-lineages of DENV-1 in the Seychelles suggests at least two introduction events. Conversely, the full-genome phylogenetic analysis (Additional file) strongly suggests that sequenced DENV-1 genotype I strains in Reunion originated from a single introduction event. In these two countries, DENV-1 displaced the previously circulating DENV-2. The displacement phenomenon was also reported among genotypes of the same dengue serotype. For instance, during the DENV-2 outbreaks in several South American countries,

the American genotype was replaced by the Asian genotype [24]. In some cases, lineage replacements were associated with an increased clinical severity [24, 25]. In Reunion, the introduction and subsequent dominance of DENV-1 was accompanied by severe cases with peculiar symptoms, notably the maculopathy. Ophthalmic complications associated with dengue, with certain dependence to serotype, have been increasingly described in recent times [13, 26]. The possible link between maculopathy observed in Reunion dengue patients and the emerging lineage DENV-1 genotype I needs to be further investigated.

Moreover, the probable single introduction of the DENV-1 genotype I in Reunion may have been accompanied by a discrete micro-evolution event since fifteen exclusive non-synonymous mutations were found in different proteins, including capsid, NS1, NS2B, NS3, NS4B, and NS5. Such local micro-evolution events have been observed in Asia, as shown for instance in the Hunan province where several mutations were seen in DENV-2 outbreak (2018) after importation from neighboring areas having higher incidence of dengue [27]. The micro-evolution event in either structural or non-structural proteins can be neutral or under positive or negative selection with an impact in disease epidemiology [27, 28]. For instance, mutations in NS1 have been shown to influence production and secretion with impact on NS1 ELISA-based dengue detection in clinical samples [29]. In addition, it was shown that mutations involving changes from basic to acidic residues or vice versa tend to affect NS1 surface expression and secretion patterns of flavivirus with impact in host immunity [29, 30]. Here, mutations were observed also in both NS2B and NS5, two proteins that play a pivotal role in the activity of NS3 protease, the latter being also a therapeutic target against flaviviruses [31]. Strikingly, when studying mutations in NS2B of the flavivirus Zika, researchers found one particular mutation able to enhance transmission potential and to confer escape from pre-existing DENV immunity [32]. When analyzing several natural dengue variants from dengue severe patients, from mild to fatal cases, a link was established between mutations in NS5 and the virulent DENV phenotypes [33]. Whether the mutations found in DENV-1 epidemic strain played a role in dengue severe cases observed in Reunion needs to be further investigated. Particular attention should be paid in the three mutations (R989K, N1068S and K1116R) located in NS1, since expression of this nonstructural glycoprotein has been linked to dengue disease severity [14, 34, 35]. In addition, as NS1 antigen is a marker for routine diagnosis in rapid detection of dengue virus infection [36], the impact of these mutations on the efficiency of the tests may require further control.

Conclusion

The southwestern Indian ocean region is usually subject to dengue outbreaks with co-occurrence of a given dengue serotype alternating intense circulation and inter-epidemic periods. In contrast to what was observed previously, uninterrupted dengue circulation is occurring and we showed that recent DENV-1 outbreaks in Reunion and the Seychelles were caused by distinct genotypes, all probably originating from Asia. Strikingly, the unique DENV-1 genotype I lineage circulating during three consecutive years in Reunion harbored non-synonymous mutations whose biological significance need to be further investigated.

Abbreviations

DENV	Dengue virus
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
NS	Nonstructural protein
CARBO	Cohorte arbovirusés
DEMARE	Observational cross-sectional epidemiological study conducted in Madagascar and La Réunion

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12879-023-08125-y>.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4

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Author contributions

S.H and T.B: molecular analyses, investigation, writing-review and editing; D.A.W: molecular analyses, investigation, methodology, visualization, writing-original draft; C.A: supervision, writing-review and editing; J.B, M.L and J.G: supervision; L.B: molecular analyses, sample acquisition, investigation; O.D.S: sample acquisition, investigation; A.F and A.C: funding acquisition; A.B: conceptualization, investigation, writing-original draft, funding acquisition; P.M: conceptualization, formal analysis, investigation, writing-original draft, funding acquisition, writing-review and editing. All authors reviewed and approved the final manuscript.

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Availability of data and material

The datasets generated and analysed during the current study are available in the Genbank repository under the accession numbers ON631270, ON631271, ON631272, ON631273, ON631274, ON631275, ON631276, ON631277, ON631278, ON631279, ON631280, ON631281, ON631282, ON631283. Data from the CARBO study are not publicly available to protect patient privacy. They can be requested at the Department of research and innovation, Martinique University Hospital 97261 Fort-de-France.

Declarations

Ethics approval and consent to participate

The study was approved by the French National Agency for the Safety of Medicines and Health Products (ANSM) (n°IDRCB 2010-A00282-37) and by the committee for the protection of individuals. All procedures were performed in accordance with relevant guidelines and regulations and written and signed informed consent was obtained from all participants included. Blood samples from the Seychelles were collected by the Ministry of Health of the Seychelles during outbreaks between January 2015 and December 2016.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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III.1.C.3. Perspectives

Despite Seychelles and La Réunion experiencing introductions of DENV-2 from the same source in recent years, the DENV-1 outbreaks in 2016 and 2019 on both islands were instigated by distinct genotypes that traced back to Asia. Moreover, the non-synonymous mutations identified in the circulating DENV-1 strains hint at the potential for genetic evolution, leading to the emergence of new variants within these regions. This phenomenon highlights the risk of novel DENV variants developing increased virulence in future epidemics and their capability to disperse across both proximate and remote areas. Consequently, further research is imperative to examine these mutations and their impacts on variant selection and virulence within specific regions.

Aedes albopictus populations circulating across the island during 2017-2021 developed specific competence towards the DENV-1, and DENV-2 serotypes/genotypes emerging. Highly competent vectors are behind the transmission of the virus to humans and the incidence burden. Therefore, conducting a comprehensive study on the influence of various factors and conditions on vector competence is crucial. It helps in understanding the adaptation of vectors, as well as the development of vector competence and the virus virulence in the circulating vector.

In the subsequent chapter, we will delve into an examination of various parameters associated with vector competence and the factors influencing them. Specifically, in the upcoming study (article 2), we aim to conduct a comprehensive analysis to improve our understanding of the vector competence exhibited by several *Aedes albopictus* populations in Réunion, particularly in the local DENV epidemic. We will investigate the impact of the geographical zones of different populations of mosquitoes, population generations, kinetics, and the natural presence of *Wolbachia* on the different aspects of vector competence.

Part III. Chapter II:
Vector competence of
Aedes albopictus from
La Réunion Island

Part III. Chapter 2: Vector competence of *Aedes albopictus* from La Réunion Island

“How does *Aedes albopictus* develop their competence in La Réunion Island?”

In this chapter, we will focus on the interaction between *Aedes albopictus* vector and Dengue virus.

First, we will explain the transmission of DENV by mosquito vector to the host

Second, we will define the different parameters of vector competence

Third, we will present extrinsic and intrinsic factors that can affect vector competence.

In this axis of the study, we will show how we explored the socio-environmental factors that might affect the vector competence toward DENV-1 and DENV-2 of different *Aedes albopictus* populations emerging in different regions of La Réunion Island.

Part III. Chapter 2: *Aedes albopictus* vector competence in La Réunion Island

III.2.A. Dengue virus transmission cycles

Two distinct DENV transmission cycles are recognized:

- Sylvatic/Zoonotic Cycle: This cycle involves mostly non-human primates and several different *Aedes* mosquitoes found in natural forest habitats of Asia and Africa (173,174). In this cycle, arboviruses are transmitted from infected to naive animals by arboreal mosquitoes (175).
- Endemic/Epidemic Cycle: In this cycle, the inter-human transmission of the viruses is facilitated by urban mosquito species *Aedes aegypti* and *Aedes albopictus* in urban areas (174,176).

The virus can be transmitted from animals to humans, contributing to a cycle switch that occurs when people encroach on forest habitats and are bitten by mosquitoes carrying the arbovirus, or when infected mosquitoes move from the forest into areas of human habitation. This transition from the sylvatic to urban transmission cycle is referred to as "spillover" (176). This transmission occurs through (i) Vector switching from arboreal *Aedes* mosquitoes to anthropophilic mosquitoes like *Aedes albopictus* and *Aedes aegypti* and (ii) reservoir host switching from non-human primates to humans (177) (Figure 18).

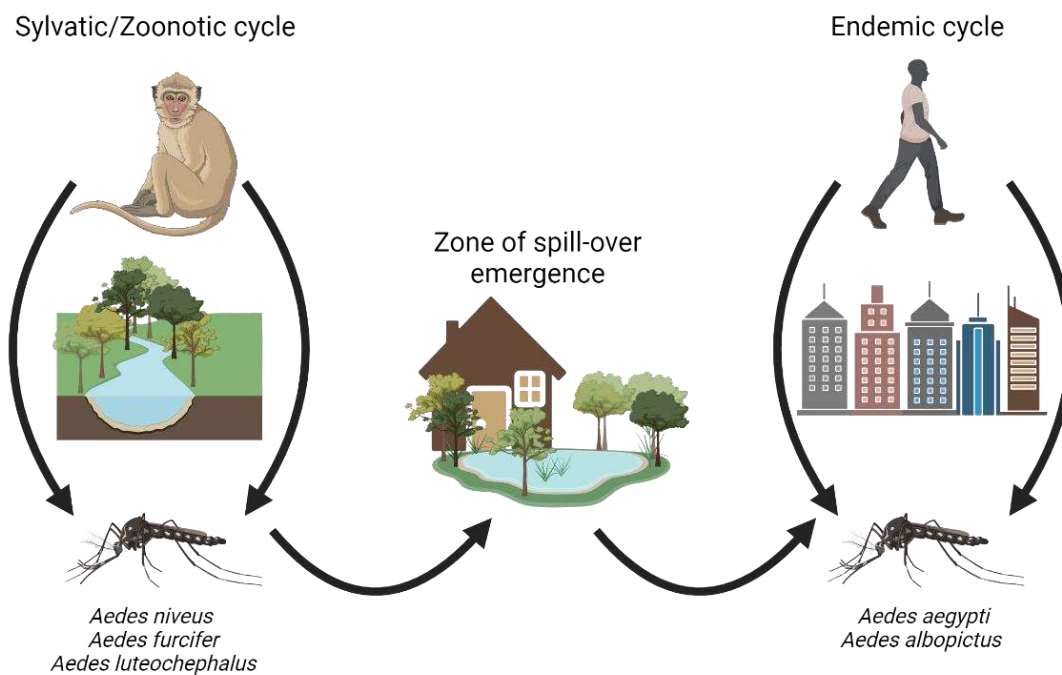


Figure 19: The transmission cycles of DENV in sylvatic/zoonotic cycle and the endemic cycle. The "Zone of emergence" is where the spill-over of both cycles happens.

III.2.B. Dengue virus-human transmission cycle

The Dengue virus maintains its life cycle through successful replication in both the human host and the mosquito vector. The hematophagous mosquito's species require a blood meal, crucial for their reproductive cycle, particularly in the development of eggs, as blood proteins and lipids are converted into yolk proteins in developing oocytes essential for egg maturation (178).

The febrile viremia period of an infected human is when the virus begins circulating in the host blood. Meanwhile, if an uninfected mosquito bites this infected person, the virus will be ingested in the mosquito's intestine. Afterward, the mosquito will become infectious after entering the Extrinsic Incubation Period (EIP), which can last from up to 21 days (15). During the EIP, the virus first infects midgut cells, which can take up to 14 days for some arboviruses like DENV. Then, on the 14th day, it spreads through the mosquito's body, reaching the salivary glands where it replicates. By day 21, the mosquito becomes infectious and can transmit the virus to another person during a blood meal. The mosquito remains infectious throughout its life and can infect every person it feeds on (179).

When an individual is bitten by the infected mosquito, the virus will be incubated in the human body for an average of 4 to 7 days before the onset of symptoms (acute fever, headaches, fatigue, other) and entering the febrile viremia period. Even if individuals do not show symptoms, they remain viremic during this period (161,180) (Figure 18).

To complete and maintain this vector-human transmission cycle, the *Aedes albopictus* vector must exhibit competence for DENV. Vector competence comprises 3 essential parameters that the virus must fulfill: Infection, Dissemination, and Transmission. In the following section, we will present the concept of vector competence and the factors influencing it.

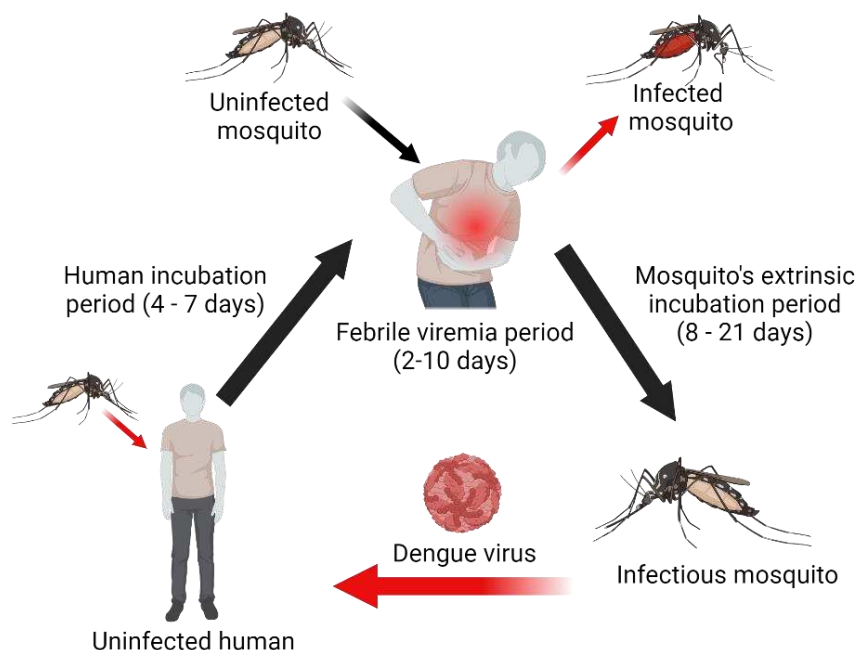


Figure 20 : Transmission cycle of Dengue virus to human by *Aedes albopictus* vector

III.2.C. Vectoral competence parameters

Vector competence has three parameters: Infection, Dissemination, and transmission. It is defined as the intrinsic permissiveness and ability of the vector to be infected by a pathogen, allow its dissemination in the body, and, subsequently, its transmission to another host (Figure 21).

Infection: Following a blood meal from a host during the viremic febrile period, DENV (or other arboviruses) are ingested into the mosquito's intestine. During the EIP, the intestine is the first tissue to become infected. This organ serves as the primary entry point for pathogens into the mosquito's body, acting as the initial barrier against microbes and representing the mosquito's defense mechanism against enemies ingested from the external environment. Within the midgut, DENV infects cells and undergoes replication, leading to the production of more viral particles. The viral titer typically peaks in the midgut between 7 and 10 days post-blood meal (181).

Dissemination: After infection, degenerated and translucent cells with vesicular nuclei are detected in the epithelium of the anterior and posterior midgut. Around these cells, the basal lamina (BL) forming an extracellular matrix surrounding and supporting the intestinal structure, is often disrupted. This suggests the potential dissemination of DENV virions, through the BL to the hemocoel, considered as a continuum for viral dissemination to the rest of the body (182,183). Viral dissemination to secondary tissues depends on various factors, most importantly the virus and viral titer (184,185). Normally, dissemination occurs to adipose tissue, hemocytes, and nervous tissues, while only certain viruses, like WNV and Venezuelan Equine Encephalomyelitis Virus, have been found in the muscle tissue of the midgut (182,186–188). The latter has never been detected in the Malpighian tubules or ovaries of infected mosquitoes even after 21 days following EIP (188). However, DENV is detected in the ovaries of *Aedes albopictus* starting 5 days post-infection but interestingly it was not detected in the intestinal muscular structure (181). Viral titer peaks in the mosquito's body between 7 and 17 days post-blood meal (189).

Transmission: Via the hemocoel, DENV will reach the salivary glands and the head. The dissemination and the replication of the virus in the salivary glands will allow its passage to the saliva. From there, it will be transmitted to another host during the next mosquito's bite, 12 to 18-days post-blood meal (15,46,152).

The efficiency of vector competence and the incubation periods of every parameter vary based on different interactions between vectors and infectious agents. These interactions can be influenced by many factors that depend on the environment, as well as on factors related to the virus and the vector biology and genetics (15,16,46,58,189).

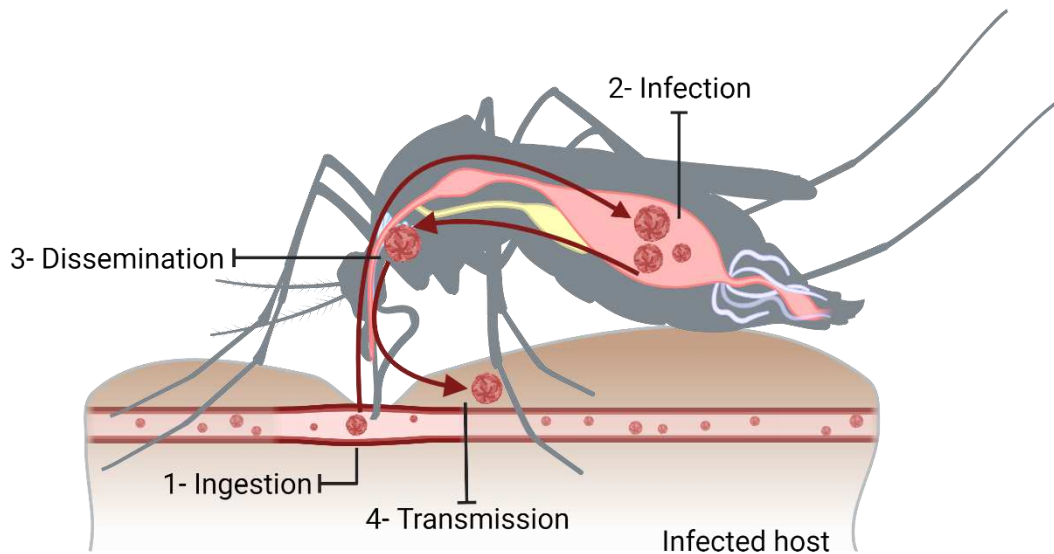


Figure 21 : The various parameters of vector competence

III.2.D. Extrinsic and intrinsic factors affecting vector competence.

In the same population of the same species, the level of vector competence can vary significantly based on several extrinsic and intrinsic factors (189–191).

Variable extrinsic ecological factors affect the interaction between viruses and their vectors and their ability to transmit them. Among these factors, we can mention insecticide resistance, climatic changes, the rainy season, mosquito population density, and the presence of suitable habitats, such as wet areas, small water bodies, or damp building basements (192–194). A study demonstrated that *Aedes albopictus* exhibited higher vector competence for ZIKV, DENV-2, and CHIKV when its resistance to insecticides was well-developed (195). Other studies showed that the increasing temperature with climatic changes shorten the EIP of the virus within the vectors. (189,193). This explains the risk of new epidemic waves with time and season (Figure 20).

As extrinsic factors, intrinsic factors play a major role in modulating the vector's competence for viral transmission. Population genetics or mosquito gene coding show a swift evolution rate, offering insights into population relationships. The genetic background of mosquito populations influences the vector's susceptibility to viral infection and its ability to replicate the virus. Studies analyzing the genetic mapping of genes responsible for regulating viral infection and maintaining barriers against it have unveiled a genetic pattern suggesting variations among genotypes in : i) the density of virus receptors on midgut cells, ii) the abundance of intracellular factors required for viral replication, or iii) the abundance of intracellular inhibitors that mitigate viral replication (191,196,197). Furthermore, intrinsic factors often manifest as inherited traits. An intriguing discovery in population genetics is the inferred correlation between genetic markers and vector competence, such as the susceptibility of subpopulations of *Aedes* mosquitoes to viral infection. It has been demonstrated to influence the mosquito's resistance and susceptibility to viral infections, particularly DENV infection, among different geographical populations (197). Studies have revealed that the susceptible phenotype of *Aedes aegypti* can be transformed into a refractory

phenotype, and crossing experiments between susceptible and resistant *Aedes albopictus* to DENV-2 strains have resulted in hybrid progeny with intermediate susceptibility (190,198,199).

Intraspecific variability in vector competence appears to be associated with multiple barrier systems that hinder the infection of different tissues in mosquitoes. These barrier systems, regulated by genetic factors, vary in their presence and strength across individuals within a mosquito species. Consequently, they influence the epidemiological dynamics of arboviral diseases. These findings indicate that the susceptibility to DENV in *Aedes albopictus* and *Aedes aegypti* is genetically determined.

Another crucial intrinsic factor affecting vector competence parameters is the mosquito's microbiome. This can influence vector competence in multiple ways, directly i) by suppressing or activating the immune system against viral infection, ii) by producing factors that impair a pathogen's viability or infectivity, iii) reactive oxygen species (ROS) production, and iv) other physiological mechanisms (200–203). Indirect inhibition occurs by competition between symbionts and pathogens for limited resources that are essential for the life cycle of both microorganisms (204–206). The multifactorial role of the microbiome in vector competence and viability is very large. More detailed explanations will be presented in the next chapter.

It is crucial to note interconnectedness of extrinsic and intrinsic factors. For instance, the viral titer ingested during blood feeding, which depend on the circulating virus load in the host's bloodstream, the presence of antibodies, proteins, and microbes in the blood, and the type of the blood ingested. These elements can influence specific intrinsic factors by affecting mosquito physiology, potentially influencing viral replication and the expression of genetically determined factors related to vector competence (190,207–209).

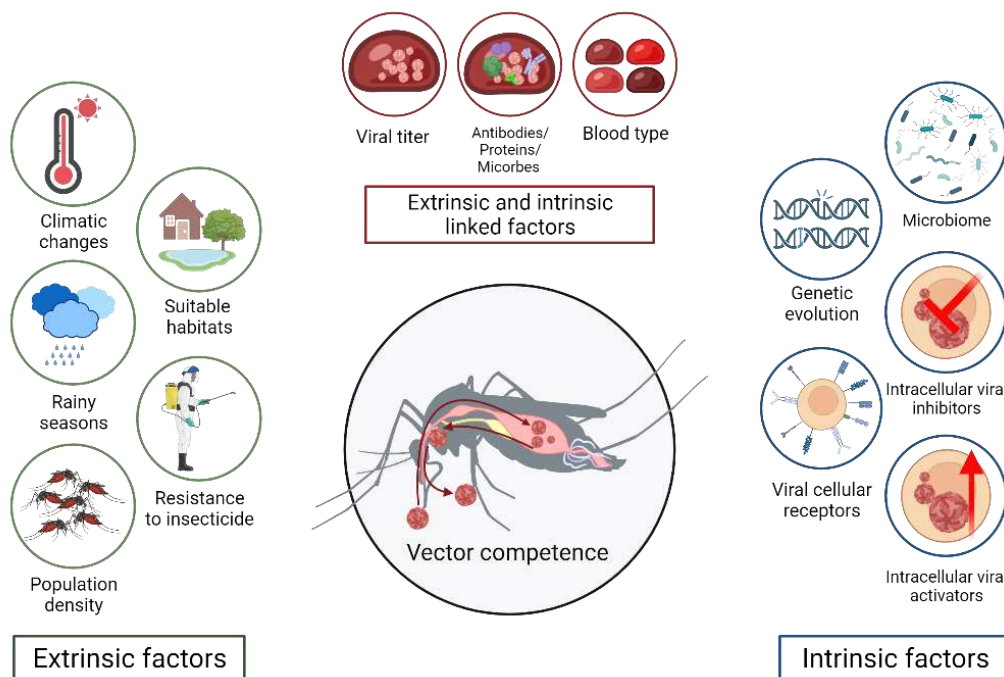


Figure 22: Schema representing the extrinsic and intrinsic factors affecting the vector competence parameters

III.2.E. Thesis second publication: Vector competence of *Aedes albopictus* field populations from La Réunion Island exposed to local epidemic Dengue viruses

III.2.E.1. Article's background and objectives

Reunionese populations of *Aedes albopictus* found on the island were responsible for the significant DENV outbreak that occurred between 2017 and 2021. The interplay between extrinsic factors present on the island and intrinsic factors specific to each population contributed to the development of distinct vector competence toward the emerging DENV-1 and DENV-2 serotypes during the outbreak. As depicted in Figure 17, the distribution of DENV cases across the island in 2021 was not uniform (147). This observation suggests that socio-environmental conditions in each region may have had an impact on the population-specific characteristics influencing the transmission of DENV-1 and DENV-2 by vectors.

Therefore, in the upcoming article, we aim to investigate the impact of socio-environmental conditions and the presence of the *Wolbachia* bacterium, naturally occurring in *Aedes albopictus*, on the vector competence. We will assess parameters such as infection rate, dissemination efficiency, and transmission efficiency, using one strain of DENV-1 and two strains of DENV-2 isolated from local patients. The study involved collecting wild mosquito populations in 2020 and 2021 from 10 regions across the Island. We will consider the natural infection status of mosquitoes by *Wolbachia*, known for its potential to modulate vector competence.

III.2.E.2. Thesis second publication

Vector competence of *Aedes albopictus* field populations from Reunion Island exposed to local epidemic dengue viruses

Article under submission

1 **Vector competence of *Aedes albopictus* field populations**
2 **from Reunion Island exposed to local epidemic dengue**
3 **viruses**

4

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23

24 **Short title:** Dengue epidemics sustained by *Aedes albopictus* in Reunion Island.

25

27 **Abstract**

28 Dengue virus (DENV) is the most prevalent mosquito-borne *Flavivirus* that affects humans
29 worldwide. *Aedes albopictus*, which is naturally infected with the bacteria *Wolbachia*, is
30 considered to be a secondary vector of DENV. However, it was responsible for a recent DENV
31 outbreak of unprecedented magnitude in Reunion Island, a French island in the South West
32 Indian Ocean. Moreover, the distribution of the cases during this epidemic showed a spatially
33 heterogeneous pattern across the island, leading to questions about the differential vector
34 competence of mosquito populations from different geographic areas. The aim of this study
35 was to gain a better understanding of the vector competence of the *Ae. albopictus* populations
36 from Reunion Island for local DENV epidemic strains, while considering their infection by
37 *Wolbachia*. Experimental infections were conducted using ten populations of *Ae. albopictus*
38 sampled across Reunion Island and exposed to three DENV strains: one strain of DENV
39 serotype 1 (DENV-1) and two strains of DENV serotype 2 (DENV-2). We analyzed three vector
40 competence parameters including infection rate, dissemination efficiency and transmission
41 efficiency, at different days post-exposition (dpe). We also assessed whether there was a
42 correlation between the density of *Wolbachia* and viral load/vector competence parameters.
43 Our results show that the *Ae. albopictus* populations tested were not able to transmit the two
44 DENV-2 strains, while transmission efficiencies up to 40.79% were observed for the DENV-1
45 strain, probably due to difference in viral titres. Statistical analyses showed that the
46 parameters mosquito population, generation, dpe and area of sampling significantly affect the
47 transmission efficiencies of DENV-1. Although the density of *Wolbachia* varied according to
48 mosquito population, no significant correlation was found between *Wolbachia* density and

49 either viral load or vector competence parameters for DENV-1. Our results highlight the
50 importance of using natural mosquito populations for a better understanding of transmission
51 patterns of dengue.

52

53 **Keywords:** dengue virus, *Aedes albopictus*, vector competence, Reunion Island,
54 epidemiological patterns.

55

56 Introduction

57 Dengue is the most widespread mosquito-borne disease affecting humans, with half of the
58 global population located in 128 tropical and subtropical countries, at risk of infection [1,2].
59 Most of the 96 million symptomatic cases per year are dengue fevers, which are characterized
60 by a panel of mild symptoms [3]. However, some cases demonstrate much more severe
61 syndromes called severe dengue (which can include severe plasma leakage, severe
62 hemorrhagic syndrome, with or without shock, and various organ dysfunctions). Severe
63 dengue is responsible for 20,000 deaths each year [4,5]. The etiological agent of the disease,
64 the dengue virus (DENV), is a positive single-stranded linear RNA genome (11 kilobases),
65 belonging to the genus *Flavivirus* (*Flaviviridae* family). Four different DENV serotypes exist,
66 each of which is divided into several genotypes based on molecular analyses [6–8]. Long term
67 serotype-specific immunity follows a DENV infection and lasts for several decades [9], whereas
68 secondary infection with a heterologous serotype can lead to severe disease manifestations
69 through the antibody-dependent enhancement effect [10,11]. The DENV is transmitted to
70 humans through the bite of infected female mosquitoes, mostly of the *Aedes* genus. Globally,
71 the mosquito species *Aedes aegypti* is considered as the primary DENV vector, with *Aedes*
72 *albopictus* recognized as a secondary vector [5,12–14]. However, outbreaks of dengue
73 involving *Ae. albopictus* have been reported in some countries, such as in Japan (in 1942 and
74 in 2014) [15,16], Hawaii (in 2001) [17], Madagascar (in 2006) [18], Gabon (in 2007) [19], China
75 (in 2004 and 2010) [20,21] and more recently in Reunion Island (in 2017) [22].

76

77 Reunion Island is an overseas French department located in South-Western Indian Ocean
78 (SWIO), about 700 km east of Madagascar. Several dengue outbreaks have been documented

79 on the island, with the first officially recorded one dating back to 1977 [23]. Subsequently,
80 low-intensity outbreaks have been reported for several years, with less than 230 cases per
81 year and no death related to dengue. However, Reunion Island has faced unprecedented
82 epidemiological patterns from 2017 to 2021. During this period, 71,636 confirmed cases, 542
83 severe forms and 78 deaths were reported [22]. All four DENV serotypes have been detected
84 in Reunion Island since 1977: the three serotypes DENV-1, DENV-2 and DENV-3 in
85 autochthonous cases, and all four serotypes in imported cases [22]. A strong seasonal pattern
86 in dengue incidence has been reported on the island, with peaks occurring between March
87 and June (i.e., during the hot and rainy season) [22]. Besides this seasonality, the geographic
88 distribution of dengue cases is often heterogeneous across the island, with the western and
89 the southern parts being the most affected compared to the northern, eastern and central
90 parts [22]. Although factors related to human populations (demography, geographic mobility,
91 immunity) and DENV genetics may explain the transmission pattern of DENV in Reunion Island,
92 the role of mosquito populations should also be taken into account [22].

93

94 Among the 12 species of mosquitoes encountered in Reunion Island, both *Ae. aegypti* and *Ae.*
95 *albopictus* are present [24–26]. However, *Ae. albopictus* is the most abundant and is
96 commonly found everywhere on the island, including at altitudes higher than 1,000 m [24,27].
97 This mosquito species was identified as the main vector responsible for a large chikungunya
98 outbreak that hit Reunion Island in 2005-2006 [28], but also as the major vector involved in
99 epidemics of dengue on the island [23,29–31]. The role of *Ae. albopictus* in the transmission
100 of DENV in Reunion Island is supported by its large distribution across the island and the
101 occurrence of peaks of DENV transmission that coincide with periods of high population

102 densities of this vector [32,33]. DENV-1 was detected in pooled samples of *Ae. albopictus*
103 collected in Reunion Island during the 2004 epidemic [30]. Other studies have shown that *Ae.*
104 *albopictus* from Reunion Island are capable of being infected with both DENV-1 [34] and
105 DENV-2 [35,36]. However, these data do not provide a clear picture of the vector competence
106 variability (the ability of a vector to be infected and to transmit a pathogen) among *Ae.*
107 *albopictus* populations from Reunion Island and their potential role in the geographical
108 contrasts of dengue cases.

109

110 The aim of this study was to examine vector competence of natural populations of *Ae.*
111 *albopictus* from Reunion Island, collected in areas of high and low DENV transmission. A
112 laboratory line of *Ae. aegypti* from Reunion Island was used as a control. Experimental
113 infections were performed using local epidemic DENV-1 and DENV-2 strains. Since no
114 geographic structure of the genetic diversity of *Ae. albopictus* populations has been observed
115 in Reunion Island [37,38], we also assessed the influence of the density of endosymbiotic
116 bacteria *Wolbachia* on vector competence phenotypes. *Aedes albopictus* is naturally infected
117 with two *Wolbachia* strains, namely *wAlbA* and *wAlbB* [39], which can interfere with the
118 replication and transmission of DENV [35,40,41]. The results of this investigation may help to
119 better understand the role of *Ae. albopictus* populations in the epidemiological patterns of
120 dengue in Reunion Island.

121

122

123

124 **Materials and Methods**

125 **Mosquitoes**

126 *Aedes albopictus* specimens were collected as eggs, larvae and pupae in 10 localities across
127 Reunion Island in 2020 and 2021: Sainte-Marie (F0_SM), Saint-André (F0_SA), Saint-Gilles les
128 Hauts (F0_SG), Saint-Philippe (F0_SPh), Saint-Paul (F1_SPa), Saint-Louis (F1_SL), Ligne Paradis
129 (F1_LP), Sainte-Clotilde (F2_SC), Bras-Panon (F2_BP) and Trois Bassins (F2_TB) (Fig 1 and S1
130 Table). Field samples (F₀ generation) were reared in the laboratory under standard conditions
131 [26 ± 1°C, 80% relative humidity (RH), 12 h light/12 h dark photoperiod]. Larvae were fed with
132 yeast tablets, and adults were provided with 10% sucrose solution. Samples collected as eggs
133 were amplified for one generation (F₁) or two generations (F₂) before vector competence
134 experiments. To achieve this, adult females were artificially fed with bovine blood using the
135 Hemotek feeding system (Hemotek Limited, Great Harwood, UK) covered by pig intestine.
136 Experimental infections were conducted using four *Ae. albopictus* populations of F₀ generation
137 (F0_SM, F0_SA, F0_SG and F0_SPh), three populations of F₁ generation (F1_SPa, F1_SL and
138 F1_LP), and three populations of F₂ generation (F2_SC, F2_BP and F2_TB) (S1 Table). A
139 laboratory colony of *Ae. aegypti* (F₃₁ and F₃₇ generations), established from mosquitoes
140 collected in the Trois Bassins locality in 2014 (F31_Aeg and F37_Aeg), was also used (Fig 1 and
141 S1 Table).

142

143 **Viral strains**

144 Three clinical DENV strains isolated from autochthonous human cases from Reunion Island
145 were used: one DENV-1 strain of genotype 1, (GenBank accession number: ON631277), and

146 two DENV-2 strains of the same lineage inside the cosmopolitan genotype, DENV-2_JUL
147 (GenBank accession number: MN272404) and DENV-2_EVAg (EVAg reference: UVE/DENV-
148 2/2018/RE/47099). The DENV-1 strain was isolated on Vero E6 cells (ATCC, ref. CRL-1586) from
149 a serum sampled in 2019 [42]. The DENV-2_JUL was isolated on Vero E6 cells from a blood
150 sample collected from a patient in 2018 [43]. The DENV-2_EVAg was purchased as lyophilizate
151 from the European Virus Archive goes global (EVAg) at passage 4. This latest DENV strain was
152 isolated from a traveler returning from Reunion Island to mainland France in 2018. Before viral
153 production, the lyophilized DENV-2_EVAg was resuspended into 200 µl distilled water. Viral
154 stocks of the three DENV strains used in experimental infections were amplified on Vero E6
155 cells at a MOI of 0.1 in an Eagle's minimum essential medium (MEM) supplemented with 2%
156 heat-inactivated fetal bovine serum (FBS), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate,
157 10 U/ml of penicillin, 0.1 mg/ml of streptomycin and 0.5 µg/ml of fungizone (PAN Biotech,
158 Aidenbach, Germany). Vero cells were maintained at 37°C with a 5% CO₂ atmosphere. For all
159 virus stocks, supernatants were harvested three to five days post-infection after the onset of
160 cytopathic effects and then frozen at -80°C until use. The final titers of DENV stocks were 1×10⁷
161 PFU/ml, 4.5×10⁵ PFU/ml and 1.02×10⁶ PFU/ml for DENV-1 (passage 3), DENV-2_JUL (passage
162 6) and DENV-2_EVAg (passage 6), respectively.

163

164 **Experimental infections**

165 Seven to fifteen-day-old female mosquitoes were isolated in small cages (16×16×16 cm) and
166 starved for 24 to 30 hours. After this starvation period, they were allowed to feed for 45
167 minutes on infectious blood meals consisting of 1 ml of washed rabbit erythrocytes, 1 ml of
168 DENV suspension and 5 mM (21 µl) of adenosine triphosphate used as a phagostimulant. The

169 infectious blood meal was delivered to mosquitoes using the Hemotek feeding system
170 (Hemotek Limited, Great Harwood, UK) covered with pig intestine. Because we were unable
171 to increase the titers of viral stocks of the two DENV-2 strains for experimental infections,
172 infectious blood meals were performed with the maximum possible virus titer which differed
173 between DENV strains with 7×10^6 PFU/ml, 6.8×10^4 PFU/ml and 3.2×10^5 PFU/ml for DENV-1,
174 DENV-2_JUL and DENV-2_EVAg, respectively. Then, mosquitoes were cold-anesthetized, and
175 engorged females were transferred into a climatic chamber ($26 \pm 1^\circ\text{C}$, 80% of RH and with a
176 photoperiod of 12 h light/12 h dark) where they were maintained with a 10% sucrose solution
177 for a maximum of 28 days. Seven *Ae. albopictus* populations were infected with DENV-1
178 (FO_SM, FO_SG, FO_SPh, FO_SA, F2_SC, F2_BP, F2_TB), three populations were infected with
179 DENV-2_JUL (F1_SPa, F1_SL, F1_LP), and four populations with DENV-2_EVAg (FO_SM, FO_SG,
180 FO_SPh, FO_SA) (S1 Table). The *Ae. aegypti* colony (F₃₁ and F₃₇ generations) was infected with
181 the three DENV strains (S1 Table).

182

183 **Vector competence analysis**

184 At 14, 21, and 28 days post-exposure (dpe) to infectious blood meals, legs and wings of
185 mosquitoes (N= 5 to 76, S1 Table) were removed before salivation [44]. Saliva from individual
186 mosquitoes was collected for 30 min by inserting the proboscis into a pipette tip containing 5
187 μl of FBS. Afterwards, the solution contained in the tip was transferred to 45 μl of complete
188 MEM medium (i.e. MEM supplemented with l-glutamine, sodium pyruvate, penicillin,
189 streptomycin and fungizone as described above). Then the head and the body (thorax and
190 abdomen) were separated and ground in 200 μl of complete MEM medium supplemented
191 with 2% FBS. After a centrifugation at $10,000 \times g$ for 5 minutes to pellet tissue debris, 150 μl of

192 the supernatant of each sample was stored at -80°C until detection and titration of DENV. For
193 bodies, pellets were stored in -80°C for the measurement of *Wolbachia* density (see below).
194 The detection of DENV in bodies, heads and saliva was performed by plaque forming unit (PFU)
195 assays on Vero cells. For bodies and heads, 48-well culture plates were seeded with 5×10^4
196 Vero E6 cells per well. For saliva, 12-well plates were seeded with 3×10^5 Vero E6 cells per well.
197 The following day, cells were incubated for 2 hours (37°C, 5% CO₂) with 100 µl of ten-fold
198 dilutions of body or head homogenates, or with 250 µl of ten-fold dilutions of the solution
199 containing saliva. All dilutions were performed with complete MEM medium supplemented
200 with 2% FBS. Then, 200 µl (for bodies and heads) or 1 ml (for saliva) of MEM medium
201 supplemented with 5% of FBS and 0.8% of carboxymethylcellulose sodium salt (CMC; Sigma-
202 Aldrich, Saint-Quentin-Fallavier, France) were added to each well. After 5 days of incubation
203 (37°C, 5% CO₂), supernatants were removed, cells were washed twice with PBS, fixed with
204 3.7% paraformaldehyde (Sigma-Aldrich), and stained with 0.5% crystal violet (Sigma-Aldrich)
205 dissolved in ethanol 20%. Vector competence of each population was evaluated based on
206 three parameters: the infection rate (IR), the dissemination efficiency (DE), and the
207 transmission efficiency (TE). IR, DE and TE correspond respectively to the proportion of
208 infected bodies, head and saliva among the total number of mosquitoes tested.

209

210 **Viral RNA extraction and amplification**

211 Viral loads in the bodies of infected F₀ *Ae. albopictus* mosquitoes (N=43) previously exposed
212 to DENV-1 and collected at 21 and 28 dpe were quantified using the reverse transcription
213 quantitative real-time PCR (RT-qPCR). These samples are sub-samples of those tested for
214 vector competence (infection rates). RNA from mosquito bodies was individually extracted

215 using the QIAcube HT robotic workstation and the associated Cador Pathogen 96 QIAcube HT
216 Kit (QIAGEN) following manufacturer's recommendations with slight modifications. Extracted
217 RNA was eluted in 100 µl of AVE buffer (QIAGEN). The RT-qPCR was then performed using the
218 QIAGEN OneStep RT-PCR Kit according to the manufacturer's recommendations. For this, a
219 mixed solution was prepared with RNA template (5 µl), a TaqMan probe (FAM-
220 ACACCTCAAGCTAA-TAMRA), and primers (Forward 5'-GAACATGGRACAAATGCAACYAT-3';
221 Reverse 5'-CCGTAGTCDGTCAGCTGTATTTTC-3') specific for the DENV-1 viral envelope gene. The
222 thermocycler program consisted of a reverse transcription step of 45 min at 45°C,
223 denaturation for 5 min at 95°C followed by 40 cycles of amplification (72°C for 5 s and 56°C
224 for 60 s). The number of viral RNA copies was estimated against a standard curve following
225 the methodology published by the HAS (Haute Autorité de Santé, France). Briefly, plasmids
226 containing targeted DENV-1 were synthesized by GeneCust (France) and used as the standard
227 curve at concentrations of 10¹ to 10⁸ RNA copies per µl.

228

229 ***Wolbachia* density**

230 *Wolbachia* were quantified in bodies of F₀ *Ae. albopictus* mosquitoes (N=75) previously
231 exposed to DENV-1 and collected at 21 and 28 dpe. DNA was extracted from individual body
232 carcasses, previously stored in -80°C from samples used to test vector competence (infection
233 rates), using the QIAcube HT robotic workstation and the associated Cador Pathogen 96
234 QIAcube HT Kit (QIAGEN) following the manufacturer's recommendations with slight
235 modifications. Afterwards, DNA was eluted in 100 µl of AVE buffer (QIAGEN) and stored at -
236 20°C until molecular investigations. Real-time quantitative PCRs were performed with the
237 CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to estimate the

238 number of *Wolbachia* genome copies in each sample. Two PCRs that specifically amplified the
239 *Wolbachia* surface protein gene *wsp* for the strains *wAlbA* and *wAlbB* were performed using
240 newly designed primers for *wAlbA* (*wspA*: *wspA_F* 5'-TAACAGCAATTTTCAGGACTAG-3' and
241 *wspA_R* 5'-CTGTTTTGATTATTTATAGCGG-3') and *wAlbB* (*wspB*: *wspB_F* 5'-
242 GTGGCAGTATTTTCAGGATTG-3' and *wspB_R* 5'-CTGCACTAGCTTCTGAAGG-3') that amplify 140
243 bp and a 130 bp fragments, respectively. *Wolbachia* genomes were quantified relative to
244 mosquito genomes. To this end, a fragment of the *Ae. albopictus* 40S ribosomal protein S7
245 (RSP7) gene of 140 bp was amplified with designed primers (*RSP7_F* 5'-
246 ATCGAGTTCAACAGCAAGAA-3' and *RSP7_R* 5'-CGACGTGCTTGCCGGAGAAC-3'). About 5 ng of
247 genomic DNA was mixed with 10 µl of QuantiNova Probe RT-PCR master mix (QIAGEN), 1 µl
248 (10 µM) of each primer and 3.6 µl of RNase-free water. PCRs were run with activation for 2
249 mins at 95°C followed by 45 cycles (95°C for 5s and 60°C for 5s). Each DNA template was
250 analyzed in triplicate for *wspA*, *wspB* and RSP7. A standard curve was generated for each qPCR
251 run to standardize the signals with the RSP7 reference. The relative mean genome number of
252 *wAlbA* and *wAlbB* strains was obtained per RSP7 copy number.

253

254 **Statistical analysis**

255 *Vector competence for DENV*

256 A first analysis was performed on all data using the proportion test to compare IR, DE and TE
257 parameters between mosquito populations for each dpe and each DENV strain separately. A
258 Bonferroni correction was applied for multiple comparisons [45]. A second analysis was
259 performed to study the effects of four explanatory parameters on the vector competence of
260 *Ae. albopictus* populations to DENV-1 using generalized linear models (GLM) with a binomial

261 error structure (or quasi-binomial in case of over-dispersed data). The explanatory parameters
262 tested were all categorical: '*population*' (seven modalities: F0_SM, F0_SA, F0_SG, F0_SPh,
263 F2_SC, F2_BP and F2_TB), '*generation*' (two modalities: F₀ and F₂), '*dpe*' the day post-exposure
264 (three modalities: 14 dpe, 21 dpe, and 28 dpe), and '*area*' (four modalities: North, East, South
265 and West). The GLM analyses were made independently for each of the three vector
266 competence parameters (i.e. IR, DE, and TE) used as binary response variables (DENV infected
267 and non-infected). As each population had only one generation, the effect of the two
268 parameters '*population*' (of F₀ or F₂ generation) and '*generation*' were also analyzed
269 independently to avoid any confounding effect. Therefore, three distinct models were used:
270 GLM1 with the following maximal model on F₀ generation only "*population * dpe*", GLM2 with
271 on F₂ generation only "*population * dpe*", and GLM3 on all populations with "*(generation +*
272 *dpe + area)^2*". Selection of the minimal model was assessed using the likelihood ratio test
273 (LRT), and the significance of the selected parameters addressed by Anova from the *car* R
274 package [46]. Based on the minimal model selected, the *emmeans* R package [47] was used
275 to assess the statistical difference between the modalities.

276

277 *Wolbachia density*

278 We first compared *Wolbachia* density (*wAlbA*, *wAlbB*, or *wAlbTot*) according to dpe for each
279 population using the Mann_Whitney test for unpaired samples and no significant difference
280 was noted between 21 and 28 dpe either for *wAlbA*, *wAlbB*, or *wAlbTot* (Mann_Whitney tests;
281 all p-values > 0.0789). Therefore, subsequent analyses were carried out by combining samples
282 from both dpe (N=75, 41 samples from 21 dpe and 34 samples from 28 dpe). The
283 Mann_Whitney test was also used to compare the densities of the two *Wolbachia* strains for

284 each mosquito population. The effect of population on *Wolbachia* density ($wAlbA$, $wAlbB$, or
285 $wAlbTot$) was explored using the non-parametric Kruskal-Wallis analysis followed by pairwise
286 post-hoc comparisons of medians with a Dunn's test. To assess the influence of *Wolbachia* on
287 vector competence parameters, mosquitoes were first classified according to four IDT
288 (Infection, Dissemination, Transmission) scores (0, 1, 2 or 3). These IDT scores were defined
289 as follows: the IDT score 0 for mosquitoes with no infectious DENV-1 particles either in the
290 body, head or saliva; the IDT score 1 for samples with only infected bodies; the IDT score 2 for
291 mosquitoes with infectious particles in the bodies and the heads; and the IDT score 3 for
292 mosquitoes with infectious DENV-1 particles in the bodies, heads and saliva (S2 Table). Then
293 *Wolbachia* density medians were compared between different combinations of these IDT
294 scores using Mann_Whitney tests for unpaired samples. The correlation between *Wolbachia*
295 densities and DENV-1 viral load in the bodies of infected mosquitoes was examined using a
296 Pearson correlation coefficient test in a sub-sample of DENV-1 infected *Ae. albopictus* (N=43).
297 The effect of mosquito population on DENV-1 viral load was also explored using the non-
298 parametric Kruskal-Wallis analysis followed by pairwise post-hoc comparisons of medians
299 with a Dunn's test.

300

301 All the statistical analyses were performed in R software (v.3.6.2) (R Core Team 2019) with
302 also the following packages: PropCIs [48], stats (R Core Team 2019), ggplot2 [49], rstatix [50],
303 ggbreak [51], and glm2 [52].

304

305

306

307 **Results**

308 *Aedes albopictus* populations of three generations (F₀, F₁ and F₂) collected in different
309 geographic areas (North, East, South and West) in Reunion Island, and a laboratory colony of
310 *Ae. aegypti* (F₃₁ and F₃₇ generations) used as control, were exposed to three DENV strains: one
311 DENV-1 and two DENV-2 that have circulated on the island between 2018-2020 (S1 Table).

312

313 **No transmission of DENV-2 strains by *Aedes albopictus* populations**

314 Three *Ae. albopictus* populations of F₁ generation (F1_SPa, F1_SL and F1_LP) were exposed to
315 the DENV-2_JUL strain and vector competence parameters were examined at 14 dpe. Low IR
316 were observed with values ranging between 5.88% (95% Confidence Interval = 1.05 - 26.98%)
317 and 11.76% (3.29 - 34.34%) (S3 Table) but no significant differences between populations
318 were observed in pairwise comparisons (all *p-values* > 0.99). No dissemination or transmission
319 of DENV-2_JUL was observed for the three *Ae. albopictus* populations (S3 Table). In
320 comparison, a laboratory colony of *Ae. aegypti* exposed to DENV-2_JUL was examined at 14
321 and 21 dpe. Similar to *Ae. albopictus*, low IRs were observed: 9.38% (3.24 - 24.22%) and 8.82%
322 (3.05 - 22.96%) at 14 and 21 dpe, respectively, and no dissemination or transmission of the
323 DENV-2_JUL strain at either dpe (S3 Table).

324

325 Four *Ae. albopictus* populations of F₀ generation (F0_SM, F0_SA, F0_SG, and F0_SPh) were
326 exposed to the DENV-2_EVAg strain and vector competence parameters were examined at
327 14, 21, and 28 dpe for F0_SM and F0_SA, and only at 28 dpe for F0_SG and F0_SPh. The *Ae.*
328 *albopictus* populations also showed very low vector competence to DENV-2_EVAg. In F0_SM

329 and F0_SA populations, only one infected body was observed at 14 dpe (IR=2.08%; 0.37 -
330 10.90%; for both populations), and no infection was observed later at 21 or 28 dpe (S4 Table).
331 At 28 dpe, no infected body was observed in the F0_SG population (S4 Table), while IR in the
332 F0_SPh population was estimated at 25% (10.18 - 49.50%), and was significantly higher than
333 in F0_SM and F0_SA population (IR=0.00%; 0.00 - 7.41% for both populations; and *p-values* =
334 0.009 for both comparisons). No dissemination or transmission was observed for the different
335 populations and dpe considered (S4 Table). For *Ae. aegypti*, IR was 8.57% (2.96 - 22.38%) at
336 14 dpe, and, unlike *Ae. albopictus* populations, virus dissemination was observed (DE=2.86%;
337 0.51 - 14.53%) for the DENV-2_EVAg strain. However, as with the *Ae. albopictus* populations,
338 no transmission was observed at 14 dpe for *Ae. aegypti* (S4 Table).

339

340 **Transmission of the DENV-1 strain by *Aedes albopictus* populations**

341 We examined the vector competence of four *Ae. albopictus* populations of F₀ generation
342 (F0_SM, F0_SA, F0_SG and F0_SPh) and three populations of F₂ generation (F2_SC, F2_BP and
343 F2_TB), as well as a laboratory colony of *Ae. aegypti*, at 14, 21, and 28 dpe after exposure to
344 the DENV-1 strain (Fig 2 and S5 Table). All populations were susceptible to DENV-1 infection
345 (Fig 2A and S5 Table): IRs values ranged from 9.38% (3.24 - 24.22%) to 64.58% (50.44 - 76.57%)
346 at 14 dpe, from 18.75% (10.19 - 31.94%) to 68.75% (54.67 - 80.05%) at 21 dpe, and from 9.38%
347 (3.24 - 24.22%) to 68.42% (57.30 - 77.77%) at 28 dpe (Fig 2A and S5 Table). Statistical analysis
348 of IR showed that the explanatory parameters "dpe", and "area" were not retained in the
349 minimal models of the three GLMs, and therefore had no effect on the IR values (Table 1).
350 Only the "generation" parameter showed a significant influence on IR (GLM3, X² = 51.024, df
351 = 1, *p-value* < 0.0001), with a higher IR for populations of F₂ generation (IR=58.09%; 53.25 -

352 62.78%) than for populations of F₀ generation (IR=24.19%; 20.63 - 28.15%; *p-value* < 0.0001,
353 S6 Table) but no significant difference was noted between populations of the same generation
354 (all *p-values* > 0.05).

355

356 All *Ae. albopictus* populations, as well as the *Ae. aegypti* colony, were able to disseminate
357 DENV-1 (Fig 2B and S5 Table). Similar to IR, pairwise comparisons revealed significantly higher
358 DE for populations of F₂ generation compared to those of F₀ generation, but only at 21 and 28
359 dpe (S6 Table), and no significant difference was noted between populations of the same
360 generation (all *p-values* *P* > 0.05). Analyzing both generations separately, DE were not
361 different between populations ("population" parameters not retained in the GLM1 and GLM2
362 minimal models; Table 1), whereas the "generation" parameter showed a significant influence
363 (GLM3, $X^2 = 61.320$, $df = 1$, *p-value* < 0.0001) with higher mean DE for populations of F₂
364 generation (DE=44.61%; 39.86 - 49.46%) than for populations of F₀ generation (DE=12.90%;
365 10.24 - 16.14%; *p-value* < 0.0001). In contrast to IR, statistical analyses showed a significant
366 effect of dpe, with DE increasing over time for populations of F₂ generation (GLM2, $X^2=30.485$,
367 $df = 2$, *p-value* < 0.0001) from 17.97% (12.28 - 25.52%) at 14 dpe to 51.49% (43.11 - 59.79%)
368 at 21 dpe or to 61.64% (53.55 - 69.14%) at 28 dpe (pairwise comparison, *p-value* = 0.0003 and
369 *p-value* < 0.0001, respectively). For populations of F₀ generation, no effect of "dpe" was
370 observed (GLM1, Table 1), with mean DEs ranging from 3.13% (0.55 - 15.74%) to 15.63% (6.86
371 - 31.75%) at 14 dpe, from 12.50% (5.86 - 24.70%) to 25.00% (14.92 - 38.78%) at 21 dpe, and
372 from 6.25% (1.73 - 20.15%) to 20.83% (11.73- 34.26%) at 28 dpe (S5 Table). When both
373 generations are analyzed together, "dpe" significantly influenced DE (GLM3, $X^2 =29.334$, $df =$
374 2 , *p-value* < 0.0001) with DE significantly increasing between 14 dpe (DE=12.11%; 8.66 -

375 16.68%) and 21 dpe (DE=30.98%; 26.21 - 36.20%) or 28 dpe (DE=35.40%; 30.38 - 40.77%)
376 (pairwise comparison, p -value = 0.0001 and p -value < 0.0001, respectively). Finally, and as for
377 the IR, the “area” parameter was not retained in the GLM3 minimal model, demonstrating no
378 influence on the DE values (Table 1).

379

380 The *Ae. albopictus* populations and the *Ae. aegypti* colony tested were all able to transmit the
381 DENV-1 strain tested at 21 dpe, with F0_SM, F0_SA and F2_BP populations containing
382 infectious virus particles in the saliva as soon as 14 dpe (Fig 2C and S5 Table). As with the DE,
383 significantly higher TE was found for populations of F₂ generation compared to populations of
384 F₀ generation at both 21 and 28 dpe (S6 Table), but no significant difference was noted
385 between populations of the same generation (all p -values > 0.05). Only the “dpe” parameter
386 was retained in the GLM2 minimal model (GLM2, $X^2 = 60.324$, $df = 2$, p -value < 0.0001), with
387 a significantly lower TE for the populations of F₂ generation at 14 dpe (TE=0.78%; 0.14 - 4.29%)
388 than at 21 dpe (26.12%; 19.42 - 34.15%, p -value = 0.002) or than at 28 dpe (36.99%; 29.58 -
389 45.06%, p -value = 0.0003). TE were not significantly different between the populations of F₀
390 generation (GLM1) and ranged from 3.13% (0.55 - 15.74%) to 9.38% (3.24 - 24.22%) at 14 dpe,
391 from 2.08% (0.37 - 10.90%) to 14.58% (7.25 - 27.17%) at 21 dpe, and from 3.13% (0.55 -
392 15.74%) to 12.50% (5.86 - 24.70%) at 28 dpe (Fig 2C and S5 Table). For the GLM3, all the
393 explanatory parameters and their two-by-two interactions were retained in the minimal
394 model (Table 1). Post-hoc analyses showed differences in TE between generations within
395 certain areas. In the West, the population of F₂ generation had a significantly higher TE
396 (TE=23.84%; 18.09 - 30.73%) than that of F₀ generation (TE=3.91%; 1.68 - 8.82%; p -value <
397 0.0001). In the East, the population of F₂ generation had a significantly higher TE (TE=25.90%;

398 19.84 - 33.06%) than that of F_0 generation (TE=8.04%; 4.29 - 14.57%; p -value < 0.0001).
 399 Moreover, TE increased over time according to the area with, for example, a mean TE for the
 400 northern populations increasing significantly between 14 dpe (TE=1.56%; 0.28 - 8.33%) and 21
 401 dpe (TE=17.44%; 10.86 - 26.80%; p -value < 0.0001) and between 14 and 28 dpe (TE=12.50%;
 402 5.86 - 24.70%; p -value < 0.0001). The *Ae. aegypti* colony presented similar TEs to *Ae.*
 403 *albopictus* with 12.50% (5.86 - 24.70%) and 16.67% (7.34 - 33.56%) at 21 and 28 dpe,
 404 respectively (Fig 2C and S5 Table).

405

406 **Table 1.** Statistical analyses of vector competence parameters of *Ae. albopictus* populations infected
 407 with the DENV-1 strain. Mosquitoes were examined at 14, 21 and 28 days post-exposure (dpe). In these
 408 analyses, the influence of mosquito population, dpe, generation and area were tested. d.f. is the
 409 degree of freedom and χ^2 is the Chi-square value.

	Maximal model	Parameters retained in the minimal model	Generation	IR			DE			TE		
				χ^2	d.f.	p-value	χ^2	d.f.	p-value	χ^2	d.f.	p-value
GLM1	population of F_0 generation + dpe	population	F0	-	-	-	-	-	-	-	-	-
		dpe		-	-	-	-	-	-	-	-	-
GLM2	population of F_2 generation + dpe	population	F2	-	-	-	-	-	-	-	-	-
		dpe		-	-	-	30.485	2	<0.0001	60.324	2	0.0001
GLM3	(generation + dpe + area) ²	generation x dpe	All	-	-	-	-	-	-	154.34	2	<0.0001
		generation x area		-	-	-	-	-	-	38.09	2	<0.0001
		dpe x area		-	-	-	-	-	-	185.61	6	<0.0001
		generation		51.024	1	<0.0001	61.320	1	<0.0001	461.31	1	<0.0001
		dpe		-	-	-	29.334	2	<0.0001	811.59	2	<0.0001
		area		-	-	-	-	-	-	47.41	3	<0.0001

410

411 We also tested the influence of the sampling period on vector competence to the DENV-1
 412 strain by comparing IRs, DEs and TEs at 14, 21 and 28 dpe between two populations collected

413 in the same location (F0_SM) a month apart. No significant difference was observed between
414 the two populations for either IRs (p -values > 0.760, for all the dpe tested), DEs (p -values >
415 0.356), or TEs (p -values > 0.425) (see S7 Table for all proportion data).

416

417 **No effect of *Wolbachia* on the replication of DENV-1 in *Aedes albopictus***

418 We measured the density of *Wolbachia* strains *wAlbA*, *wAlbB* and of both strains (*wAlbTot*,
419 i.e. *wAlbA* + *wAlbB*) in individual *Ae. albopictus* mosquitoes (N=75) from the four populations
420 of F₀ generation (F0_SM, F0_SG, F0_SPh, F0_SA) previously exposed to DENV-1 infectious
421 blood meals and collected at 21 and 28 dpe. These two dpe were selected because they
422 showed higher values of vector competence parameters in particular DE and TE (see Fig 2),
423 thus allowing testing the correlation between *Wolbachia* densities and vector competence
424 referred as IDT scores (S2 Table). The *wAlbA* strain presented a significantly higher density
425 than the *wAlbB* strain in the four populations (Mann_Whitney tests; p -value = 0.002 for F0_SM
426 and for F0_SA; p -value < 0.001 for F0_SG and for F0_SPh), with median densities per
427 population ranging from 2.20 (0.70 - 2.20) to 9.00 (3.90 - 24.80) bacteria/cell for *wAlbA* and
428 from 0.40 (0.23 - 0.60) to 1.95 (1.40 - 2.30) bacteria/cell for *wAlbB* (S8 Table). The density of
429 both *Wolbachia* strains varied according to mosquito populations (S8 Table), with the lowest
430 densities observed in the F0_SG population, followed by the F0_SPh population and higher
431 densities in the F0_SM and F0_SA populations (S1 Fig and S8 Table).

432

433 To examine the influence of *Wolbachia* density on *Ae. albopictus* vector competence, the
434 samples from the four populations and from both 21 and 28 dpe were gathered (since no

435 significant difference was found between dpe and between populations for F₀ generation, i.e.
436 GLM1, see Table 1). They were then classified according to their IDT score (N = 12 to 19
437 mosquitoes per IDT score, S9 Table). No significant difference in *Wolbachia* density was
438 observed between the IDT score 0 (i.e. no infection) and all three other IDT groups (1, 2 and
439 3) gathered (i.e. with at least one infected tissue) either for *wAlbA* (Mann_Whitney test, *p*-
440 *value* = 0.609), *wAlbB* (*p-value* = 0.613), or *wAlbTot* (*p-value* = 0.696), suggesting that
441 *Wolbachia* density did not affect the ability of mosquitoes to become infected after exposure
442 to DENV-1 (S2 Fig, S9 Table). Similarly, no significant difference was found by comparing the
443 *Wolbachia* density between the score IDT 1 on one hand, and the scores 2 and 3 gathered on
444 the other hand (S9 Table) (Mann_Whitney tests; *p-value* = 0.434 for *wAlbA*; *p-value* = 0.066
445 for *wAlbB*; *p-value* = 0.494 for *wAlbTot*), suggesting that *Wolbachia* density did not affect the
446 ability of mosquitoes to disseminate the DENV-1 after being infected (S2 Fig). *Wolbachia*
447 density had also no impact on the ability of mosquitoes to transmit DENV-1 after
448 dissemination in the heads, a result highlighted by the absence of any significant difference
449 between the *Wolbachia* density of IDT scores 2 and 3 (S9 Table) (Mann_Whitney tests; *p-value*
450 = 0.151 for *wAlbA*; *p-value* = 0.238 for *wAlbB*; *p-value* = 0.113 for *wAlbTot*) (S2 Fig).

451

452 Finally, we examined the correlation between *Wolbachia* density (*wAlbTot* i.e. *wAlbA*+*wAlbB*)
453 and the number of DENV-1 RNA copies in the bodies of the infected mosquitoes (N = 43
454 positive samples for DENV-1). Firstly, we compared the *Wolbachia* density (Fig 3A) or the
455 DENV-1 viral load (Fig 3B) between the four populations. The mosquito population showed a
456 significant effect on the median *Wolbachia* density (Kruskal-Wallis test, $X^2 = 16.86$, d.f. = 3, *p*-
457 *value* < 0.001) and on the median number of DENV-1 RNA copies (Kruskal-Wallis test, $X^2 =$

458 13.48, d.f. = 3, *p*-value = 0.004). Although both *Wolbachia* density and DENV-1 viral load vary
459 significantly according to mosquito populations (Fig 3A and Fig 3B), no significant correlation
460 between the two parameters was noted (Pearson correlation coefficient test, *cor* = -0.190,
461 95% CI = [-0.464;0.117], *t* = -1.236, d.f. = 41, *p*-value = 0.224) (Fig 3C), suggesting a lack of
462 association between *Wolbachia* density and DENV-1 viral load.

463

464 **Discussion**

465 In this study, we examined the vector competence of *Ae. albopictus* populations from Reunion
466 Island exposed to local epidemic DENV-1 and DENV-2 strains that had been previously isolated
467 and genetically characterized [42,43]. The mosquito populations used for experimental
468 infections were collected in different geographic areas across Reunion Island and were of F₀,
469 F₁ and F₂ generations. The use of F₀ populations allowed experimental conditions to be as
470 close as possible to those of natural mosquito populations. We also assessed the vector
471 competence of a laboratory colony of *Ae. aegypti* as control because this species is considered
472 as the primary DENV vector [5,7,53–57]. In general, no significant difference between vector
473 competence of *Ae. albopictus* populations and the *Ae. aegypti* colony was observed, in
474 agreement with the results of Florida mosquito populations exposed to a DENV-1 strain [58],
475 or of China vector populations exposed with DENV-2 strains [59].

476

477 Contrasting results were observed when *Ae. albopictus* populations were exposed to DENV-1
478 and DENV-2 strains. None of the tested populations was able to transmit the DENV-2 strains,
479 while the DENV-1 strain was transmitted by all populations with TEs reaching 40.8% (in the

480 F2_TB population at 28 dpe). Our results are different from those observed with *Ae. albopictus*
481 populations from other geographic regions which generally show higher levels of transmission
482 with DENV-2 strains compared to DENV-1 strains [60]. For instance, higher viral loads were
483 described in saliva of an *Ae. albopictus* population from Vietnam exposed to DENV-2
484 compared to specimens infected with DENV-1 [57]. The results observed in *Ae. aegypti* are
485 quite similar to those described in *Ae. albopictus* with no transmission of DENV-2 strains, while
486 TEs of 12.5% and 16.7% were observed with DENV-1 at 21 and 28 dpe, respectively (the means
487 TEs for *Ae. albopictus* being 15.5% and 17.3% at 21 and 28 dpe, respectively). The observed
488 difference in the transmission of the two DENV serotypes by *Ae. albopictus* and *Ae. aegypti*
489 mosquitoes could be explained by two non-exclusive hypotheses. Firstly, the viral titers in
490 infectious blood meals were higher for the DENV-1 strain (7×10^6 PFU/ml) than for the DENV-
491 2 strains (between 6.8×10^4 PFU/ml and 3.2×10^5 PFU/ml), and viral titers in blood meals are
492 known as a factor affecting the vector competence of mosquitoes [57,61–63]. Secondly, the
493 difference in vector competence between DENV-1 and DENV-2 strains could be linked to a
494 greater replicative fitness or affinity of the DENV-1 local strain with the *Ae. albopictus* and *Ae.*
495 *aegypti* from Reunion Island compared to the two DENV-2 strains. It is recognized that vector
496 competence for a given virus is the result of interactions between a viral strain, a mosquito
497 population and a given environment [41,55,59,62,64–66]. The hypothesis of a better
498 replicative fitness is reinforced by a previous investigation with Colombian DENV strains
499 showing a greater replicative fitness of DENV-1 compared to DENV-2 in the human hepatocyte
500 cell line (Huh-7) [67]. It would be interesting in future investigations to compare replicative
501 fitness of DENV-1 and DENV-2 strains from Reunion Island in human cell lines, *Ae. albopictus*
502 cell lines as well as in *Ae. albopictus* populations using identical viral titers for both DENV
503 serotypes.

504

505 Among the four explanatory parameters including “population”, “dpe”, “generation”, and
506 “area” that can affect the three vector competence parameters (IR, DE, and TE), three
507 parameters (“dpe”, “generation”, and “area”) showed a significant effect. Concerning the
508 “dpe”, a global increase of IRs, DEs and TEs occurred over time, higher values being observed
509 in older mosquitoes in particular at 28 dpe compared to 21 and 14 dpe. This result reflects the
510 kinetics of replication of arboviruses inside mosquitoes, from the initial midgut infection to
511 the release of infectious viral particles in saliva following a dissemination phase in all the
512 tissues [68]. The other significant parameter was the “generation”, with higher vector
513 competence parameters observed in mosquito populations of F₂ generation than that of F₀
514 generation. The effect of generation on vector competence could be explained by a reduced
515 genetic diversity in mosquitoes reared in the laboratory due to a founder effect. The rearing
516 of mosquitoes in the laboratory for two generations could alter the mosquito genetic diversity
517 as well as their microbiota [62,69–72]. Indeed, mosquito microbiota, in particular midgut
518 bacteria, have been shown to modulate vector competence in several mosquito species
519 [56,62,73–78] including *Ae. albopictus* [79]. This result shows the importance of working with
520 mosquito populations of F₀ generation to better understand the transmission patterns of
521 arboviruses in the field. Finally, as the geographic distribution of dengue cases is often
522 heterogeneous across the island, with the western and the southern parts being the most
523 affected compared to the northern, eastern and central parts [22], mosquito populations used
524 in this study were collected in areas of high (West and South) and low (North and East) DENV
525 transmission. Our data indicated a significant influence of the area of collection on TEs alone,
526 but also in interaction either with the “dpe” parameter or with the “generation” parameter.
527 However, the number of mosquito populations from each area was too low (between one and

528 three populations) to conclude whether the geographic origin of mosquitoes affects the vector
529 competence.

530

531 We examined whether the density of the *Wolbachia* strains *wAlbA* and *wAlbB*, naturally
532 occurring in the *Ae. albopictus* from Reunion Island, affect vector competence and DENV-1
533 replication inside the mosquitoes, since *Wolbachia* have been shown to affect the replication
534 and transmission of pathogens such as DENV, Chikungunya virus, or Zika virus in mosquito
535 vectors [80–86]. For all examined populations, the density of the strain *wAlbA* was higher than
536 that of the strain *wAlbB* in accordance with results described in populations from Thailand
537 [87] and Madagascar [88]. However, other studies have reported a higher density of *wAlbB*
538 strain than *wAlbA* in *Ae. albopictus* populations from China [41,89], Taiwan [40], Greece and
539 Corsica [90], and Thailand [87]. We observed variations in *Wolbachia* density between
540 populations, the FO_SG population (from the West) exhibited the lowest *Wolbachia* density,
541 followed by the FO_SPh population (from the South), the FO_SM population (from the North),
542 and the FO_SA population (from the East) with the highest *Wolbachia* density. We also
543 described a variation in the quantity of DENV-1 viral RNA copies in the bodies of infected
544 mosquitoes according to their population of origin. However, no significant correlation was
545 observed between *Wolbachia* density and DENV-1 viral load, and no difference in *Wolbachia*
546 density was found between IDT scores, suggesting that *Wolbachia* did not influence vector
547 competence parameters (i.e. infection, dissemination or transmission) of *Ae. albopictus*
548 populations exposed to DENV-1. Although it has been previously shown that the *Wolbachia*
549 strains infecting *Ae. albopictus* can affect the vector competence towards DENV [35,40,41],
550 this interference could depend on the mosquito populations and DENV strains.

551

552 **Conclusion**

553 Our study shows that *Ae. albopictus* populations from Reunion Island are experimentally
554 competent for the transmission of the local DENV-1 epidemic strain, but not the DENV-2
555 strains. However, the low viral titres of DENV-2 used means that these results must be taken
556 with caution. We observed a significant effect of generation on vector competence
557 parameters: mosquitoes of F₀ generation showing significantly lower infection rates,
558 dissemination efficiencies and transmission efficiencies than those of F₂ generation. No
559 significant correlation was found between *Wolbachia* density and either vector competence
560 parameters or viral loads of DENV-1 in infected *Ae. albopictus* mosquitoes. Taken together,
561 our results highlight the importance of using natural mosquito populations and considering
562 various parameters for a better understanding of DENV transmission by mosquito vectors in
563 the field.

564

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569

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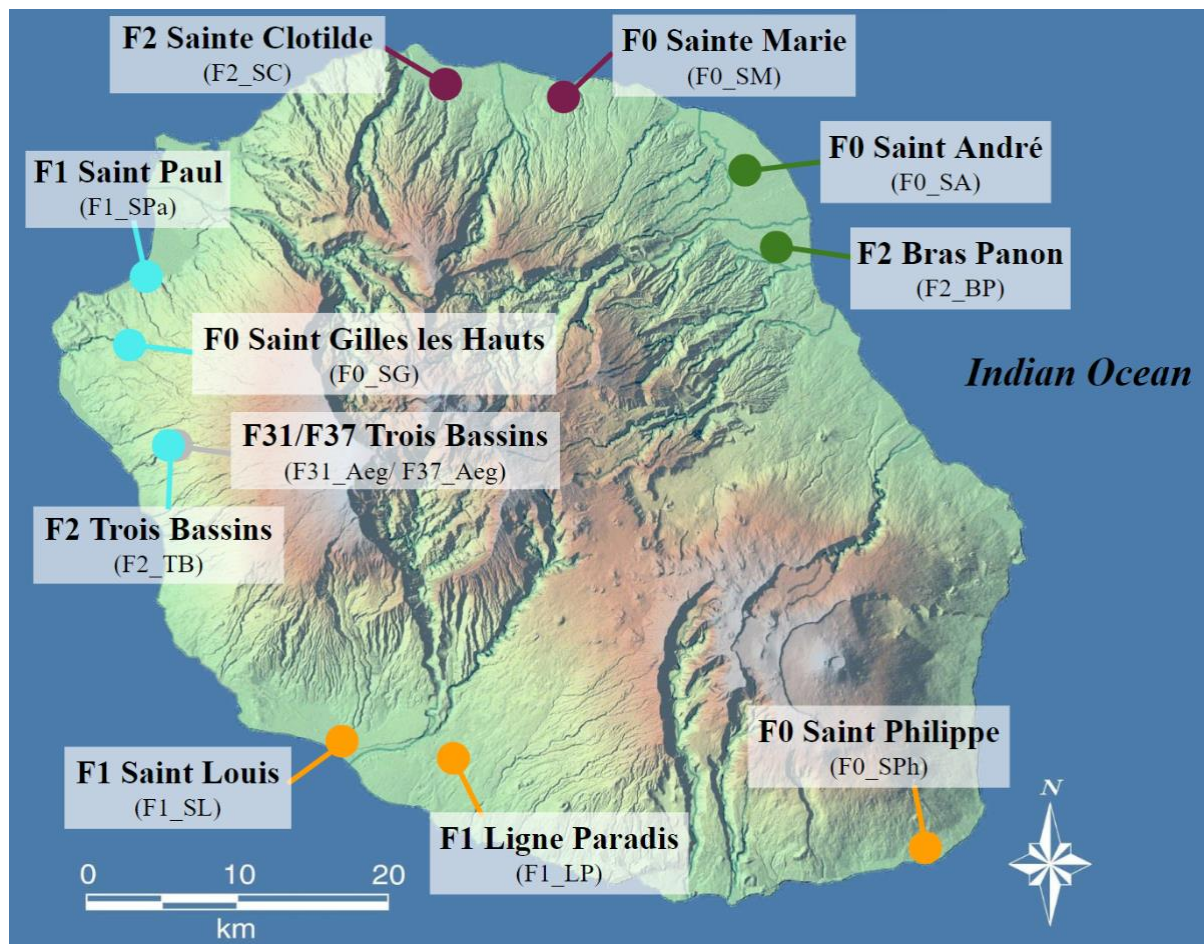
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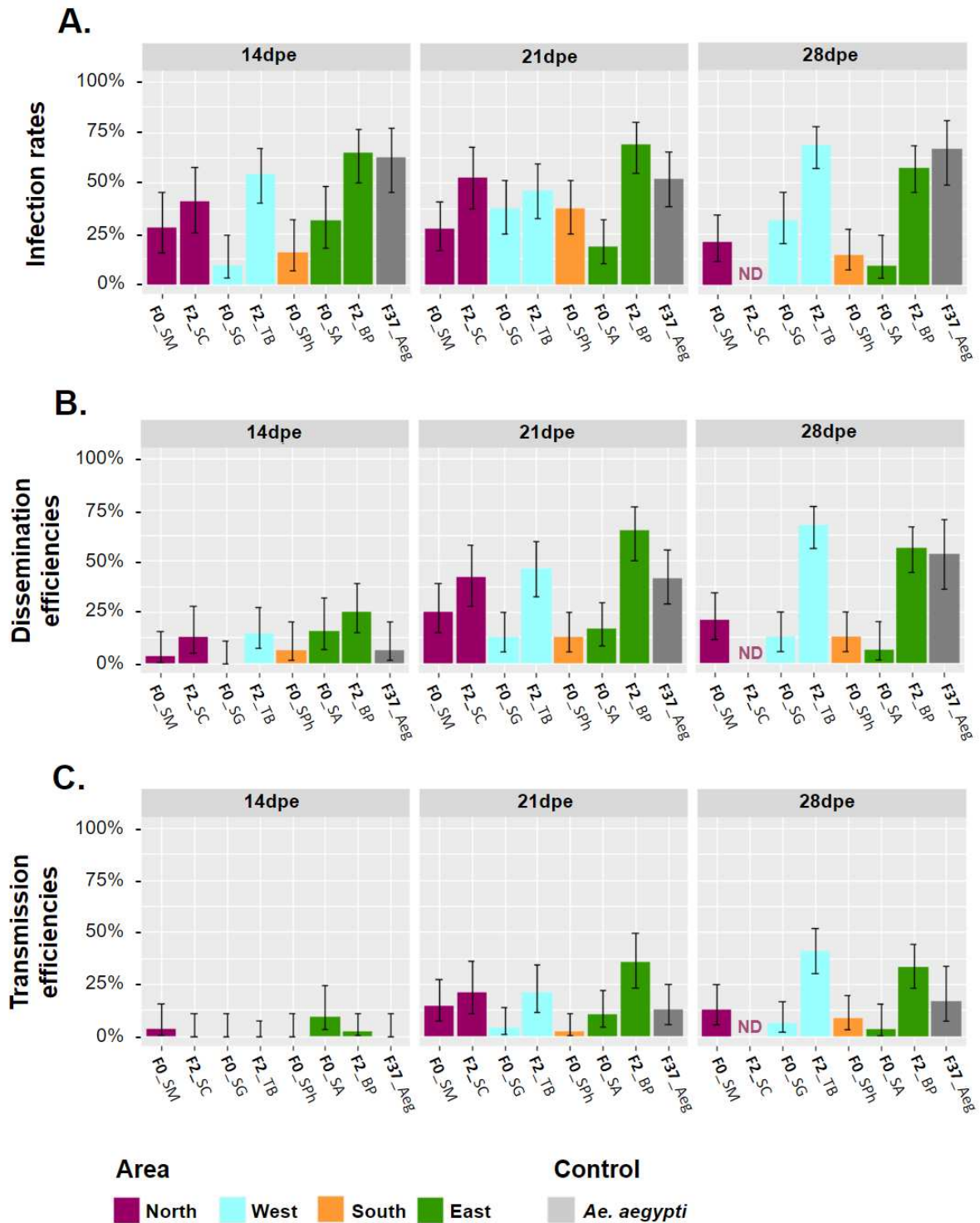
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806 **List of Figures**



808 **Fig 1. Map of sampling sites of *Aedes albopictus* and *Aedes aegypti* in Reunion Island.** For *Ae.*
809 *albopictus*, the sampling sites are colored according to geographic regions: orange, South; blue, West;
810 purple, North; and green, East. The unique *Ae. aegypti* population is colored in gray. Population codes
811 are given in brackets. The generation (i.e. F₀, F₁, F₂, F₃₁, or F₃₇ generation) at which mosquitoes were
812 used for the vector competence experiments is noted before the locality. Map was extracted and
813 modified from Conseil Régional de La Réunion website (Conseil Régional de La Réunion, 2006).

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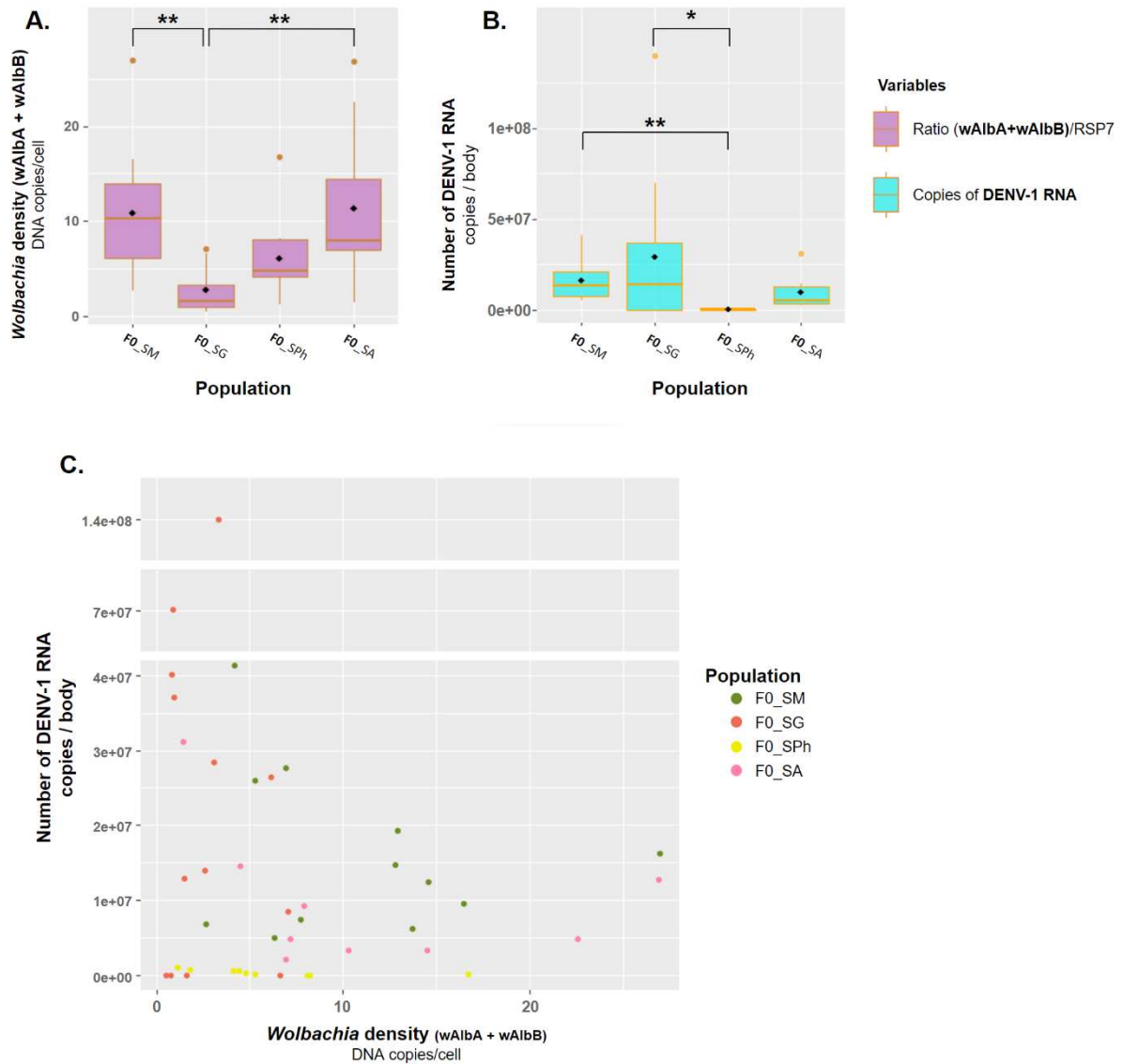


815

816 **Fig 2. Vector competence parameters of *Aedes albopictus* and *Aedes aegypti* populations from**
 817 **Reunion Island exposed to the DENV-1 strain. (A.) Infection rates (IR), (B.) dissemination efficiencies**
 818 **(DE), and (C.) transmission efficiencies (TE) of mosquito populations according to geographic areas**
 819 **(orange: South; blue: West; purple: North; and green: East). Data obtained with *Ae. aegypti* are shown**

820 in gray. Vector competence parameters were examined at 14, 21, and 28 days post-exposure (dpe) to
 821 the DENV-1 strain via an infectious blood meal. Error bars correspond to the 95% confidence intervals.
 822 ND= not determined.

823



824

825 **Fig 3. Correlation between *Wolbachia* density and viral load of the DENV-1 strain in *Aedes albopictus***
 826 **mosquitoes from Reunion Island.** The mosquitoes tested belong to the populations of F₀ generation
 827 from Sainte-Marie (F0_SM), Saint-Gilles les Hauts (F0_SG), Saint-Philippe (F0_SPh), or Saint-André

828 (F0_SA), and were collected 21 or 28 days post-exposure (dpe) to the DENV-1 local strain. **(A.)** The total
829 *Wolbachia* density ($wAlbTot$ i.e. $wAlbA+wAlbB$) is given based on the ratio between the *Wolbachia* and
830 RSP7 concentrations (DNA copies/cell) in the body of the mosquitoes according to their population of
831 origin, ratio which provided the number of *Wolbachia* genomes relative to the *Ae. albopictus* genomes,
832 in other words the *Wolbachia* density per mosquito cell. $**p < 0.01$, Dunn's tests. **(B.)** Number of DENV-
833 1 RNA copies in the body of the mosquitoes according to their population of origin. In graphs A and B,
834 the line inside each boxplot represents the median and the diamond corresponds to the mean of each
835 population. $*p < 0.05$, $**p < 0.01$, Dunn's tests. **(C.)** Number of DENV-1 RNA copies according to the
836 total *Wolbachia* density in the body of each mosquito tested (N=43).

837

838 Supporting information

Mosquitoes					DENV strain	Number of samples examined		
Species	Location of collection (Population code)	Area of collection	Generation	Date of collection		14 dpe	21 dpe	28 dpe
<i>Aedes albopictus</i>	Sainte-Marie (F0_SM)	North	F ₀	April 2021	DENV-1	32	48	48
				May 2021	DENV-2_EVAg	48	48	48
	Sainte-Clotilde (F2_SC)	North	F ₂	February to March 2020	DENV-1	32	38	0
	Saint-Gilles les Hauts (F0_SG)	West	F ₀	April 2021	DENV-1	32	48	48
					DENV-2_EVAg	0	0	5
	Saint Paul (F1_SPa)	West	F ₁	February to March 2020	DENV-2_JUL	17	0	0
	Trois Bassins (F2_TB)	West	F ₂	February to March 2020	DENV-1	48	48	76
	Saint-Philippe (F0_SPh)	South	F ₀	April 2021	DENV-1	32	48	48
					DENV-2_EVAg	0	0	16
	Saint-Louis (F1_SL)	South	F ₁	February to March 2020	DENV-2_JUL	22	0	0
	Ligne Paradis (F1_LP)	South	F ₁	February to March 2020	DENV-2_JUL	17	0	0
Saint-André (F0_SA)	East	F ₀	April 2021	DENV-1	32	48	32	
				DENV-2_EVAg	48	48	48	
Bras-Panon (F2_BP)	East	F ₂	February to March 2020	DENV-1	48	48	70	
<i>Aedes aegypti</i>	Trois Bassins (F31_Aeg or F37_Aeg)	Laboratory population	F ₃₁	2014	DENV-2_EVAg	35	0	0
					DENV-2_JUL	32	0	0
			F ₃₇		DENV-1	32	48	30

839

840 **S1 Table. General information about mosquito populations and DENV strains used in this study.** For

841 each population and condition, the number of samples examined after DENV exposure are indicated.

842 dpe = days post-exposure.

IDT score	21 dpe				28 dpe				Total
	F0_SM	F0_SG	F0_SPh	F0_SA	F0_SM	F0_SG	F0_SP h	F0_SA	
0	3	3	3	3	2	3	3	3	23
1	1	2	3	1	0	3	1	1	12
2	3	4	3	3	3	3	2	0	21
3	3	2	1	3	3	3	3	1	19
Total	10	11	10	10	8	12	9	5	75

843

844 **S2 Table. Number of mosquitoes tested for *Wolbachia* density by categories studied.** The categories
845 are defined by the day post-exposure studied (21 or 28 dpe), the vector competence score (IDT score),
846 and the population (F0_SM: Sainte-Marie, F0_SG: Saint-Gilles les Hauts, F0_SP: Saint-Philippe, or
847 F0_SA: Saint-André). The IDT score (0, 1, 2 or 3) was defined as follows: the IDT score 0 for
848 mosquitoes with no infectious DENV-1 particles either in the body, head or saliva; the IDT
849 score 1 for samples with only infected bodies; the IDT score 2 for mosquitoes with infectious
850 particles in the bodies and the heads; and the IDT score 3 for mosquitoes with infectious
851 DENV-1 particles in the bodies, heads and saliva.

Population	14 dpe			21 dpe		
	IR	DE	TE	IR	DE	TE
F1_SPa	11.76% [3.29 - 34.34%] (2/17)	0.00% [0.00 - 18.43%] (0/17)	0.00% [0.00 - 18.43%] (0/17)	ND	ND	ND
F1_SL	9.09% [2.53 - 27.81%] (2/22)	0.00% [0.00 - 14.87%] (0/22)	0.00% [0.00 - 14.87%] (0/22)	ND	ND	ND
F1_LP	5.88% [1.05 - 26.98%] (1/17)	0.00% [0.00 - 18.43%] (0/17)	0.00% [0.00 - 18.43%] (0/17)	ND	ND	ND
F31_Aeg	9.38% [3.24 - 24.22%] (3/32)	0.00% [0.00 - 10.72%] (0/32)	0.00% [0.00 - 10.72%] (0/32)	8.82% [3.05 - 22.96%] (3/34)	0.00% [0.00 - 10.15%] (0/34)	0.00% [0.00 - 10.15%] (0/34)

852

853 **S3 Table. Vector competence parameters of *Aedes albopictus* and *Aedes aegypti* populations**
854 **exposed to the DENV-2_JUL strain.** Infection rates (IR), dissemination efficiencies (DE), and

855 transmission efficiencies (TE) were examined at 14 and 21 days post-exposure to an infectious blood
 856 meal (dpe). IR = number of infected bodies among the mosquitoes tested (%); DE = number of infected
 857 heads among the mosquitoes tested (%); TE = number of infected saliva among the mosquitoes tested
 858 (%). The numbers in square brackets correspond to the 95% confidence interval, and the fraction in
 859 brackets represents the number of positive samples out of the total number of samples tested. ND =
 860 not done. F1_SPa, F1_SL and F1_LP correspond to *Ae. albopictus* populations and F31_Aeg is the *Ae.*
 861 *aegypti* population.

Population	14 dpe			21 dpe			28 dpe		
	IR	DE	TE	IR	DE	TE	IR	DE	TE
F0_SM	2.08% [0.37 - 10.90%] (1/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)
F0_SA	2.08% [0.37 - 10.90%] (1/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)	0.00 % [0.00 - 7.41%] (0/48)
F0_SG	ND	ND	ND	ND	ND	ND	0.00 % [0.00 - 43.45%] (0/5)	0.00 % [0.00 - 43.45%] (0/5)	0.00 % [0.00 - 43.45%] (0/5)
F0_SPh	ND	ND	ND	ND	ND	ND	25.00% [10.18 - 49.50%] (4/16)	0.00 % [0.00 - 19.36%] (0/16)	0.00 % [0.00 - 19.36%] (0/16)
F31_Aeg	8.57% [2.96 - 22.38%] (3/35)	2.86% [0.51 - 14.53%] (1/35)	0.00% [0.00 - 9.89%] (0/35)	ND	ND	ND	ND	ND	ND

862

863 **S4 Table. Vector competence parameters of *Aedes albopictus* and *Aedes aegypti* populations**
 864 **exposed to the DENV-2_EVAg strain.** Infection rates (IR), dissemination efficiencies (DE), and
 865 transmission efficiencies (TE) were examined at 14, 21, and 28 days post-exposure to an infectious
 866 blood meal (dpe). IR = number of infected bodies among the mosquitoes tested (%); DE = number of
 867 infected heads among the mosquitoes tested (%); TE = number of infected saliva among the
 868 mosquitoes tested (%). The numbers in square brackets correspond to the 95% confidence interval,
 869 and the fraction in brackets represents the number of positive samples out of the total number of
 870 samples tested. ND = not done. F0_SM, F0_SA, F0_SG and F0_SPh correspond to *Ae. albopictus*
 871 populations and F31_Aeg is the *Ae. aegypti* population.

Population	14 dpe			21 dpe			28 dpe		
	IR	DE	TE	IR	DE	TE	IR	DE	TE
F0_SM	28.13% [15.56 - 45.37%] (9/32)	3.13% [0.55 - 15.74%] (1/32)	3.13% [0.55 - 15.74%] (1/32)	27.08% [16.57 - 41.00%] (13/48)	25.00% [14.92 - 38.78%] (12/48)	14.58% [7.25 - 27.17%] (7/48)	20.83% [11.73- 34.26%] (10/48)	20.83% [11.73- 34.26%] (10/48)	12.50% [5.86 - 24.70%] (6/48)
F0_SA	31.25% [17.95 - 48.57%] (10/32)	15.63% [6.86 - 31.75%] (5/32)	9.38% [3.24 - 24.22%] (3/32)	18.75% [10.19 - 31.94%] (9/48)	16.67% [8.70 - 29.58%] (8/48)	10.42% [4.53 - 22.17%] (5/48)	9.38% [3.24 - 24.22%] (3/32)	6.25% [1.73 - 20.15%] (2/32)	3.13% [0.55 - 15.74%] (1/32)
F0_SG	9.38% [3.24 - 24.22%] (3/32)	0.00% [0.00 - 10.72%] (0/32)	0.00% [0.00 - 10.72%] (0/32)	37.50% [25.22 - 51.64%] (18/48)	12.50% [5.86 - 24.70%] (6/48)	4.17% [1.15 - 13.98%] (2/48)	31.25% [19.95- 45.33%] (15/48)	12.50% [5.86 - 24.70%] (6/48)	6.25% [2.15- 16.84%] (3/48)
F0_SPh	15.63% [6.86 - 31.75%] (5/32)	6.25% [1.73 - 20.15%] (2/32)	0.00% [0.00 - 10.72%] (0/32)	37.50% [25.22 - 51.64%] (18/48)	12.50% [5.86 - 24.70%] (6/48)	2.08% [0.37 - 10.90%] (1/48)	14.58% [7.25 - 27.17%] (7/48)	12.50% [5.86 - 24.70%] (6/48)	8.33% [3.29 - 19.55%] (4/48)
F2_SC	40.63% [25.52 - 57.74%] (13/32)	12.50% [4.97 - 28.07%] (4/32)	0.00% [0.00 - 10.72%] (0/32)	52.63% [37.26 - 67.52%] (20/38)	42.11% [27.85 - 57.81%] (16/38)	21.05% [11.07 - 36.35%] (8/38)	ND	ND	ND
F2_BP	64.58% [50.44 - 76.57%] (31/48)	25.00% [14.92 - 38.78%] (12/48)	2.08% [0.37 - 10.90%] (1/48)	68.75% [54.67 - 80.05%] (33/48)	64.58% [50.44 - 76.57%] (31/48)	35.42% [23.43 - 49.56%] (17/48)	57.14% [45.48- 68.06%] (40/70)	55.71% [44.08- 66.75%] (39/70)	32.86% [23.00- 44.50%] (23/70)
F2_TB	54.17% [40.29 - 67.42%] (26/48)	14.58% [7.25 - 27.17%] (7/48)	0.00% [0.00 - 7.41%] (0/48)	45.83% [32.58 - 59.71%] (22/48)	45.83% [32.58 - 59.71%] (22/48)	20.83% [11.73- 34.26%] (10/48)	68.42% [57.30- 77.77%] (52/76)	67.11% [55.94- 76.62%] (51/76)	40.79% [30.44- 52.02%] (31/76)
F37_Aeg	62.50% [45.25 - 77.07%] (20/32)	6.25% [1.73 - 20.15%] (2/32)	0.00% [0.00 - 10.72%] (0/32)	52.08% [38.33 - 65.53%] (25/48)	41.67% [28.85 - 55.72%] (20/48)	12.50% [5.86 - 24.70%] (6/48)	66.67% [48.78 - 80.77%] (20/30)	53.33% [36.14 - 69.77%] (16/30)	16.67% [7.34 - 33.56%] (5/30)

872

873 **S5 Table. Vector competence parameters of *Aedes albopictus* and *Aedes aegypti* populations**
874 **exposed to the DENV-1 strain.** Infection rates (IR), dissemination efficiencies (DE), and transmission
875 efficiencies (TE) were examined at 14, 21, and 28 days post-exposure to an infectious blood meal (dpe).
876 IR = number of infected bodies among the mosquitoes tested (%); DE = number of infected heads
877 among the mosquitoes tested (%); TE = number of infected saliva among the mosquitoes tested (%).
878 The numbers in square brackets correspond to the 95% confidence interval, and the fraction in
879 brackets represents the number of positive samples out of the total number of samples tested. ND =
880 not done. F0_SM, F0_SA, F0_SG, F0_SPh, F2_SC, F2_BP and F2_TB correspond to *Ae. albopictus*
881 populations and F37_Aeg is the *Ae. aegypti* population.

Variable	Day post-exposure	Population 1		Population 2		p-value<0.05 pairwise proportion test
		Population code	Variable value	Population code	Variable value	
IR	14 dpe	F2_BP	64.58% [50.44 - 76.57%] (31/48)	F0_SG	9.38% [3.24 - 24.22%] (3/32)	6.55E-05
		F0_SPh	15.63% [6.86 - 31.75%] (5/32)			9.35E-04
	F2_TB	F0_SG	9.38% [3.24 - 24.22%] (3/32)			2.53E-03
		F0_SPh	15.63% [6.86 - 31.75%] (5/32)			2.58E-02
21 dpe	F2_BP	68.75%	F0_SA	18.75% [10.19 - 31.94%] (9/48)	4.67E-05	

			[54.67 - 80.05%] (33/48)	F0_SM	27.08% [16.57 - 41.00%] (13/48)	2.18E-03			
		F2_SC	52.63% [37.26 - 67.52%] (20/38)	F0_SA	18.75% [10.19 - 31.94%] (9/48)	4.48E-02			
	28 dpe	F2_BP	57.14% [45.48 - 68.06%] (40/70)	F0_SA	9.38% [3.24 - 24.22%] (3/32)	2.37E-04			
F0_SM				20.83% [11.73 - 34.26%] (10/48)	2.86E-03				
F0_SPh				14.58% [7.25 - 27.17%] (7/48)	1.30E-04				
		F2_TB	68.42% [57.30 - 77.77%] (52/76)	F0_SA	9.38% [3.24 - 24.22%] (3/32)	1.03E-06			
F0_SM				20.83% [11.73 - 34.26%] (10/48)	9.64E-06				
F0_SPh				14.58% [7.25 - 27.17%] (7/48)	2.24E-07				
F0_SG				31.25% [19.95 - 45.33%] (15/48)	1.70E-03				
DE		21 dpe	F2_BP	64.58% [50.44 - 76.57%] (31/48)	F0_SA	16.67% [8.70 - 29.58%] (8/48)	1.01E-04		
	F0_SM				25.00% [14.92 - 38.78%] (12/48)	4.63E-03			
	F0_SPh				12.50% [5.86 - 24.70%] (6/48)	1.01E-05			
	F0_SG				12.50% [5.86 - 24.70%] (6/48)	1.01E-05			
	F2_TB		45.83% [32.58 - 59.71%] (22/48)	F0_SPh	12.50% [5.86 - 24.70%] (6/48)	1.59E-02			
				F0_SG	12.50% [5.86 - 24.70%] (6/48)	1.59E-02			
				28 dpe	F2_BP	55.71% [44.08 - 66.75%] (39/70)	F0_SA	6.25% [1.73 - 20.15%] (2/32)	9.72E-05
							F0_SM	20.83% [11.73 - 34.26%] (10/48)	5.02E-03
	F0_SPh	12.50% [5.86 - 24.70%] (6/48)	7.87E-05						
	F0_SG	12.50% [5.86 - 24.70%] (6/48)	7.87E-05						
	F2_TB	67.11% [55.94 - 76.62%] (51/76)	F0_SA	6.25% [1.73 - 20.15%] (2/32)	3.91E-07				
			F0_SM	20.83% [11.73 - 34.26%] (10/48)	1.99E-05				
			F0_SPh	12.50% [5.86 - 24.70%] (6/48)	1.28E-07				
			F0_SG	12.50% [5.86 - 24.70%] (6/48)	1.28E-07				
	TE	21 dpe	F2_BP	35.42% [23.43 - 49.56%] (17/48)	F0_SPh	2.08% [0.37 - 10.90%] (1/48)	1.84E-03		
					F0_SG	4.17% [1.15 - 13.98%] (2/48)	7.04E-03		
28 dpe		F2_BP	32.86% [23.00 - 44.50%] (23/70)	F0_SA	3.13% [0.55 - 15.74%] (1/32)	3.63E-02			
				F0_SG	6.25% [2.15 - 16.84%] (3/48)	2.06E-02			
		F2_TB	40.79% [30.44 - 52.02%] (31/76)	F0_SA	3.13% [0.55 - 15.74%] (1/32)	3.45E-03			
				F0_SM	12.50% [5.86 - 24.70%] (6/48)	2.43E-02			
				F0_SPh	8.33% [3.29 - 19.55%] (4/48)	3.15E-03			
				F0_SG	6.25% [2.15 - 16.84%] (3/48)	9.79E-04			

883 **S6 Table. Pairwise proportion tests showing significant differences between the vectorial**
 884 **competence parameters of *Aedes albopictus* populations after exposure to an infectious blood meal**
 885 **containing the DENV-1 strain.** All dpe were tested independently. The populations are referenced by
 886 their generation and the initial of the collection location on the field (generation_location). The
 887 comparison of the vectorial competence parameters (IR, DE, or TE) were realized with pairwise
 888 proportion comparison tests for each parameter and each dpe independently. Only comparisons with
 889 a significant difference ($P < 0.05$) are presented in the table.

Population	14 dpe			21 dpe			28 dpe		
	IR	DE	TE	IR	DE	TE	IR	DE	TE
F0_SM	28.13% [15.56 - 45.37%] (9/32)	3.13% [0.55 - 15.74%] (1/32)	3.13% [0.55 - 15.74%] (1/32)	27.08% [16.57 - 41.00%] (13/48)	25.00% [14.92 - 38.78%] (12/48)	14.58% [7.25 - 27.17%] (7/48)	20.83% [11.73 - 34.26%] (10/48)	20.83% [11.73 - 34.26%] (10/48)	12.50% [5.86 - 24.70%] (6/48)
F0_SM-bis	25.00% [12.00 - 44.90%] (6/24)	0.00% [0.00 - 13.80%] (0/24)	0.00% [0.00 - 13.80%] (0/24)	25.00% [12.00 - 44.90%] (6/24)	12.50% [4.34 - 31.00%] (3/24)	8.33% [2.32 - 25.85%] (2/24)	26.92% [13.70 - 46.08%] (7/26)	15.38% [6.15 - 33.53%] (4/26)	3.85% [0.68 - 18.89%] (1/26)

890

891 **S7 Table. Vector competence parameters of two *Aedes albopictus* populations exposed to the DENV-**
 892 **1 strain and previously collected at the same location in Sainte-Marie at two different times of the**
 893 **year.** Infection rates (IR), dissemination efficiencies (DE), and transmission efficiencies (TE) were
 894 examined at 14, 21, and 28 days post-exposure to an infectious blood meal (dpe). IR = number of
 895 infected bodies among the mosquitoes tested (%); DE = number of infected heads among the
 896 mosquitoes tested (%); TE = number of infected saliva among the mosquitoes tested (%). The numbers
 897 in square brackets correspond to the 95% confidence interval, and the fraction in brackets represents
 898 the number of positive samples out of the total number of samples tested. ND = not done. F0_SM and
 899 F0_SM-bis correspond to *Ae. albopictus* populations of Sainte-Marie collected on the field in April and
 900 May 2021, respectively.

901

902 **S1 Fig. Densities of *Wolbachia* strains *wAlbA* and/or *wAlbB* in *Aedes albopictus* populations from**
 903 **Reunion Island.** The mosquitoes of F₀ generation from four populations (N=75) namely Sainte-Marie

904 (F0_SM), Saint-Gilles les Hauts (F0_SG), Saint-Philippe (F0_SPh) and Saint-André (F0_SA) were tested
 905 for their *Wolbachia* densities after being exposed to infectious blood meals containing the DENV-1
 906 strain. For each sample, the value provided corresponds to the mean of a triplicate measure. The
 907 densities of *Wolbachia* wAlbA (orange), or wAlbB (green), or both (violet), are given based on the ratio
 908 between the *Wolbachia* and RSP7 concentrations which provided the number of *Wolbachia* genomes
 909 relative to the *Ae. albopictus* genomes. dpe = days post-exposure. The “population” parameter showed
 910 a significant effect on the median of all the *Wolbachia* densities (Kruskal-Wallis tests; $\chi^2 = 17.72$, d.f. =
 911 3, *p-value* < 0.001 for wAlbA; $\chi^2 = 35.42$, d.f. = 3, *p-value* < 0.001 for wAlbB; $\chi^2 = 20.85$, d.f. = 3, *p-value*
 912 < 0.001 for wAlbTot). For the wAlbA strain, a significantly lower median density of 2.20 (0.70 - 2.70)
 913 wAlbA/cell was observed for the F0_SG population compared to the medians of 4.75 (2.20 - 11.22) and
 914 9.00 (3.90 - 24.80) wAlbA/cell for the F0_SM (Dunn test, *p-value* = 0.047) and the F0_SA (Dunn test, *p-*
 915 *value* < 0.001) populations, respectively. For the wAlbB strain, both F0_SG and F0_SPh populations had
 916 significantly lower median densities, of 0.45 (0.27 - 0.80) and 0.40 (0.23 - 0.60) wAlbB/cell respectively,
 917 compared with medians of 1.95 (1.40 - 2.30) and 1.80 (1.00 - 4.70) wAlbB/cell for the F0_SM and F0_SA
 918 populations, respectively (Dunn tests; all *p-values* < 0.001). Regarding the overall *Wolbachia* density,
 919 the results are equivalent to those for wAlbA, with the F0_SG population presenting a significantly
 920 lower median density of 2.60 (1.22 - 3.30) wAlbTot/cell, compared with the medians of 6.60 (4.20 -
 921 12.94) and 12.80 (6.91 - 26.90) wAlbTot/cell for the F0_SM (Dunn test, *p-value* = 0.012) and F0_SA (
 922 Dunn test, *p-value* < 0.001) populations, respectively.

Population	wAlbA		wAlbB		wAlbTot	
	median	95% CI median	median	95% CI median	median	95% CI median
F0_SM (N=18)	4.75	2.20 - 11.22	1.95	1.40 - 2.30	6.60	4.20 - 12.94
F0_SG (N=23)	2.20	0.70 - 2.70	0.45	0.27 - 0.80	2.60	1.22 - 3.30
F0_SPh (N=19)	4.00	2.50 - 7.79	0.40	0.23 - 0.60	4.81	2.80 - 8.11
F0_SA (N=15)	9.00	3.90 - 24.80	1.80	1.00 - 4.70	12.80	6.91 - 26.90

923

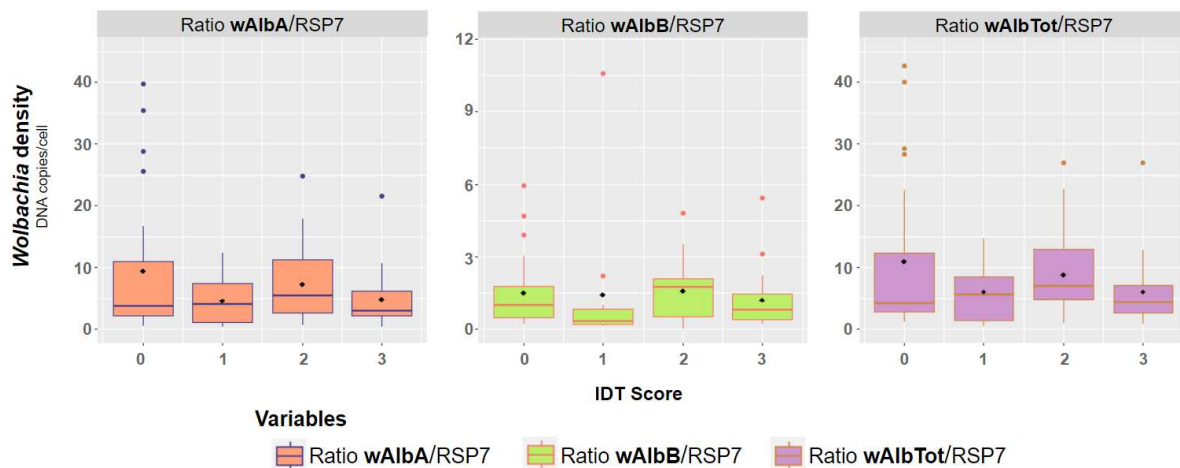
924 **S8 Table. Parameters of the *Wolbachia* wAlbA, wAlbB and wAlbTot (wAlbA + wAlbB) densities in**
 925 ***Aedes albopictus* from Reunion Island according to their populations of origin and after exposure to**
 926 **a DENV-1 local strain. The mosquitoes of F₀ generation, belonging to the populations of Sainte-Marie**
 927 **(F0_SM), Saint-Gilles les Hauts (F0_SG), Saint-Philippe (F0_SPh), or Saint-André (F0_SA), were**
 928 **examined at 21 and 28 days after being exposed to infectious blood meals containing the DENV-1 local**
 929 **strain. In this table N = number of mosquitoes tested; 95% CI, 95% confidence interval.**

Score IDT	wAlbA		wAlbB		wAlbTot	
	median	95% CI median	median	95% CI median	median	95% CI median
IDT 0 (N=23)	3.70	2.30 - 10.04	1.00	0.48 - 1.75	4.18	2.80 - 11.88
IDT 1 (N=12)	3.95	0.60 - 7.79	0.31	0.20 - 0.97	5.61	0.74 - 9.90
IDT 2 (N=21)	5.48	2.60 - 11.22	1.72	0.50 - 2.10	6.90	4.81 - 12.94
IDT 3 (N=19)	2.95	2.07 - 6.26	0.80	0.40 - 1.54	4.40	2.60 - 7.20
IDT 1+2+3 (N=52)	4.20	2.60 - 6.11	0.80	0.50 - 1.40	5.72	4.20 - 7.50
IDT 2+3 (N=40)	4.41	2.60 - 6.20	0.86	0.60 - 1.80	5.72	4.20 - 7.70

930

931 **S9 Table. Parameters of the *Wolbachia* wAlbA, wAlbB, and wAlbTot (wAlbA + wAlbB) densities in**
 932 **the *Aedes albopictus* bodies according to their IDT score groups and after exposure to a DENV-1 local**
 933 **strain. The mosquitoes of F₀ generation, belonging to the populations of Sainte-Marie (F0_SM), Saint-**
 934 **Gilles les Hauts (F0_SG), Saint-Philippe (F0_SPh), or Saint-André (F0_SA) and examined at 21 and 28**
 935 **days after being exposed to infectious blood meals containing the DENV-1 local strain, were pooled**
 936 **according to their IDT score as follows: IDT score 0 for mosquitoes with no infectious DENV-1 particles**
 937 **either in the body, head or saliva; the IDT score 1 for samples with only infected bodies; the IDT score**
 938 **2 for mosquitoes with infectious particles in the bodies and the heads; and the IDT score 3 for**
 939 **mosquitoes with infectious DENV-1 particles in the bodies, heads and saliva. In this table: N, number**
 940 **of mosquitoes tested; 95% CI, 95% confidence interval.**

941



942

943 **S2 Fig. Densities of *Wolbachia* strains *wAlbA* and *wAlbB* in *Aedes albopictus* mosquitoes according**

944 **to vector competence parameters represented by IDT scores.** These analyses were performed using

945 individual mosquito of F₀ generation, belonging to the populations of Sainte-Marie (FO_SM), Saint-

946 Gilles les Hauts (FO_SG), Saint-Philippe (FO_SPh), or Saint-André (FO_SA) and examined at 21 and 28

947 days after being exposed to infectious blood meals containing the DENV-1 strain. The densities of

948 *Wolbachia wAlbA* (orange), or *wAlbB* (green), or both (violet) are given based on the ratio between

949 the *Wolbachia* and RSP7 concentrations which provided the number of *Wolbachia* genomes relative

950 to the *Ae. albopictus* genomes. We measured the density of *Wolbachia* in individuals (N=75) classified

951 according to their IDT scores (0, 1, 2 or 3) that were defined as follows: the IDT score 0 for mosquitoes

952 with no infectious DENV-1 particles either in the body, head or saliva; the IDT score 1 for samples with

953 only infected bodies; the IDT score 2 for mosquitoes with infectious particles in the bodies and the

954 heads; and the IDT score 3 for mosquitoes with infectious DENV-1 particles in the bodies, heads and

955 saliva. dpe = days post-exposure.

III.2.E.3. Perspectives

In summary, our comprehensive analysis revealed that the *Aedes albopictus* local mosquitoes are competent to transmit of DENV virus. Moreover, it has unveiled a multitude of interactions and factors influencing every aspect of vector competence, IR, DE, and TE. The conditions of the regions where mosquito populations emerge, encompassing factors such as temperature, altitude, host types, and urbanization, in addition to intrinsic factors related to the vector, such as age and generation, were identified as crucial determinants affecting the transmission of the DENV. Our study underscores also the probable diverse effects of the natural environment conditions and the adaptation to laboratory conditions.

Another pivotal aspect investigated was the *Wolbachia* bacterium, naturally presented in various organs of *Aedes albopictus*. Recognized for its ability to limit viral infection and transmission through various physiological mechanisms (152,210). *Wolbachia's* role was assessed and did not exhibit an effect on DENV-1 IR, DE, and TE in our populations. Despite variations in *Wolbachia* density between populations, our study did not reveal any significant correlation between *Wolbachia* density differences and its effects on competence parameters on the macro scale of *Aedes albopictus* Reunionese populations.

However, we decided to go further and explore at the micro-scale of an *Aedes albopictus* laboratory population, the ability of *Wolbachia* to maintain intestinal homeostasis in basal conditions and after DENV infection. We believe that *Wolbachia* might play a role in preserving the integrity of the intestinal epithelium and restricting DENV proliferation within intestinal cells. Thus, it might indirectly assist in limiting viral infection and dissemination throughout the intestinal barrier to the vector's body.

In the next chapter, we will explore various physiological antiviral mechanisms employed by *Wolbachia* and focus on its impact on intestinal homeostasis, along with the molecular mechanisms involved in intestinal cellular proliferation and apoptosis.

Part III. Chapter 3:
Wolbachia impact on
Aedes albopictus intestinal
homeostasis

Part III. Chapter 3:
Wolbachia impact on
Aedes albopictus intestinal homeostasis

“Is the midgut microbiota a major contributor to the defense system of *Aedes albopictus*?”

In this chapter, we will study *Wolbachia*'s implication in maintaining *Aedes albopictus* intestinal homeostasis.

First, we will describe structural aspects of the midgut tissue, blood digestion and homeostatic self-renewal of the adult mosquito midgut.

Second, we will delve into the bibliography of mosquitoes' microbiota in general, the effect of blood on microbes' population composition, and its protective role.

Third, we will focus on the presence of *Wolbachia* in the midgut and its impact on the vector defense system, especially against DENV in *Aedes* vectors.

In this context, we will present our findings on the role of *Wolbachia* in preserving the intestinal homeostasis of *Aedes albopictus*, particularly after consuming both normal and DENV-infected blood meal.

Part III. Chapter 3: *Wolbachia* as a biological factor involved in midgut protection against viral infections

III.3.A. The mosquito midgut

III.3.A.1. Midgut structure and function

The midgut is the entry point of the blood meal into the mosquito's body and plays a vital role as the initial site for digestion and absorption of essential nutrients, crucial for the mosquito's physiology and longevity (211–213). In addition, ingesting vertebrate blood is essential for mosquito eggs' production by providing essential proteins, digested into amino acids, required for vitellogenin synthesis (214–216).

The midgut is delimited by two ectodermal sections, the foregut and hindgut, each playing a crucial role in essential processes for blood digestion, reproduction, and viability (Figure 23)

The anterior section, the foregut, comprises the buccal cavity, pharynx, esophagus, dorsal diverticula, and crop (217). From the pharynx, food proceeds to the proventriculus before being gradually released into the midgut for digestion and absorption (215,218,219).

The posterior section, the hindgut is associated with the Malpighian tubules, after which the ileum extends to the rectum. In the posterior digestive tract, wastes are transformed into urine and undergo numerous processes of absorption and secretion before being expelled from the rectum (215,220).

The midgut has a sac-like structure composed of a simple columnar epithelium resting on a basal lamina surrounded by a well-organized structure of circular and longitudinal fibers forming a complex muscular network (221,222). The apical membrane features a microvillous-associated network formed by the membrane strands of epithelial cells. The basolateral membrane exhibits deep folds and invaginations constituting the basal labyrinth without intercellular junctions (211). The microvillous-associated network renders the midgut lumen complex and stratified, aids in protecting the epithelium from pathogens ingested with the blood meal, and increases the surface for enzyme secretion and nutrient absorption (223).

III.3.A.2. Midgut epithelium cellular composition

As previously mentioned, the midgut acts as the primary site where the mosquito's body receives the blood meal. After its ingestion, a cascade of reactions occurs along the entire digestive tract. Midgut epithelium contributes to the digestion and absorption of proteins and essential nutrients crucial for the mosquito's physiology and egg production (211–213).

The midgut epithelium consists of four cell types (Figure 23). The two most abundant cell types are the columnar epithelial cells, also known as **Enterocytes (EC)**, constituting up to 80% of epithelial cells, and the **Enteroendocrine cells (EE)** (224).

Enterocytes present microvilli on the apical surface and extensive invaginations on the cell's basal membrane. These features, along with the presence of rough endoplasmic reticulum, a complex Golgi network, and secretory vesicles, explain their involvement in enzyme secretion and nutrient absorption from the blood meal (222,225–228).

Enteroendocrine cells are chemo-sensitive cells. They play a crucial role in hormonal stimulation as well as the production and secretion of digestive enzymes, such as proteinase and trypsin(225,229).Two other types of cells have been described in the midgut of hematophagous insects: **Intestinal Stem Cells (ISC)** and **Enteroblasts (EB)** (224). ISCs are involved in the epithelium renewal dynamic, a process typically occurring every 3-7 days as known in *Drosophila* (230,231), and in the repair mechanism activated in response to bacterial, chemical, or injury-induced midgut damages (224,232–235).

Intestinal Stem Cells or regenerative cells are the only cell type in the midgut undergoing mitosis (230). The ISCs play a crucial role in tissue regeneration following damage in the midgut (236) (discussed later). In *Drosophila*, ISCs derive from adult midgut precursors known as AMPs. These AMPs, originating from endodermal cells, are specified during the early stages of embryonic development and contribute to the formation of the embryonic midgut (237). In the adult female midgut, they are located in the basal intestinal epithelium, in direct contact with the basal lamina, and are evenly distributed beneath the ECs (238). The mitotic asymmetrical division of these stem cells gives rise to two cells, one retaining its stem cell properties and the other becomes an **Enteroblast** or pre-EE (239) (Figure 25).

Enteroblasts, are undifferentiated progenitor cells adjacent to ISCs. They respond to maturation signals and differentiate mainly into ECs (or sometime to EEs) (230). Moreover,

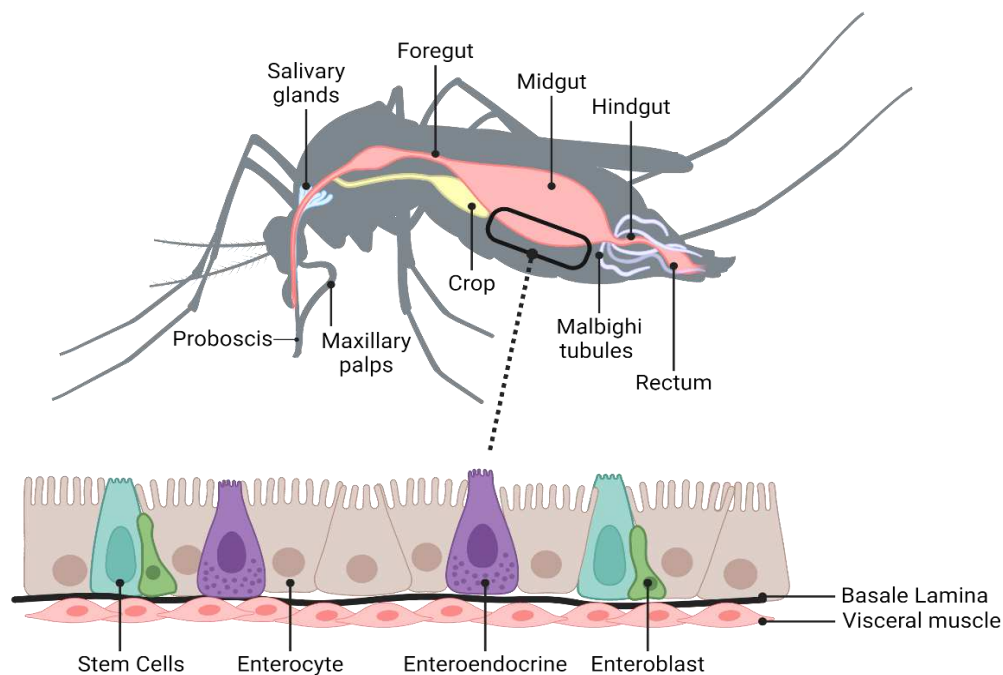


Figure 23 : Digestive tract anatomy and intestinal cellular

EBs play a role in secreting cytokines and growth factors that influence the proliferation and differentiation of ISCs (240,241).

III.3.A.3. Blood digestion

In addition to digesting nutrients from sources such as nectar or sugar-based foods, the gut epithelium also processes blood meals to support reproductive functions.

Transcriptomic analysis showed that, in *Aedes aegypti*, after a standard blood meal, various mechanisms involved in the expression of antimicrobial peptides, blood digestion and absorption exhibit distinct regulation in midgut. These include the repression or depletion of multiple cohorts of peptidases crucial for protein digestion (218). Vertebrate blood, rich in proteins, requires the action of proteinase enzymes, such as trypsin, specialized in breaking down proteins, for digestion in mosquito gut (267). This process involves also upregulating peptidase ferritin to sequester iron, along with activating immune effectors and modulators (210,268,269). The nutrients derived from the blood meal can be stored as nutritional reserves for use in other metabolic processes(242). As an example, a significant portion of the digested amino acids from the blood meal is utilized in synthesizing yolk protein precursor, crucial for vitellogenesis and essential for mosquito reproduction and egg production (171).

III.3.A.4. Microbe infections by oral route

Besides being the primary organ for digesting blood meals, the midgut acts as the initial point of contact between the mosquito and pathogens that might be ingested during feeding. It plays a significant role in the mosquito's defense against accidentally ingested microbes, limiting their potential infection and transmission.

The gut epithelium is actively involved in these processes and deploys numerous mechanisms. After a DENV-infected blood meal, viral particles could be prevented to enter or replicate into the intestinal epithelium cells, which represent the Midgut Infection Barrier (MIB). A second barrier, the Midgut Escape Barrier (MEB), could limit the virus's dissemination from the midgut to the rest of the mosquito's body. When altered, the MEB allows the virus to bypass and spread to the hemocoel and eventually to another part of the mosquito body, including the salivary glands and saliva (190,243,244). In *Aedes* vectors, the MIB and MEB are major determinants of the vector competence for *Flaviviruses* (245), influencing the permissiveness of epithelial cells for viral infection and transmission (246,247) (Figure 23). Additionally, DENV ingestion triggers immune responses by activating antiviral genes and innate immunity pathways such as Toll, Jak/Stat, and IMD, which effectively defend against viral infections (248–251) (figure 24). Additional research has concentrated on examining the midgut transcriptomic reactions to DENV infection within *Aedes* mosquitoes. These studies have identified genes that are either upregulated or downregulated, primarily also linked to the vector's immune response. Furthermore, these genes were observed to be expressed at different time points following blood feeding in each respective study (252–254) .

Together, these studies highlighted that the refractoriness displayed by most *Aedes* strains against DENV infection may be linked to reactions occurring in the midgut, underscoring the importance of maintaining homeostatic balance in this tissue.

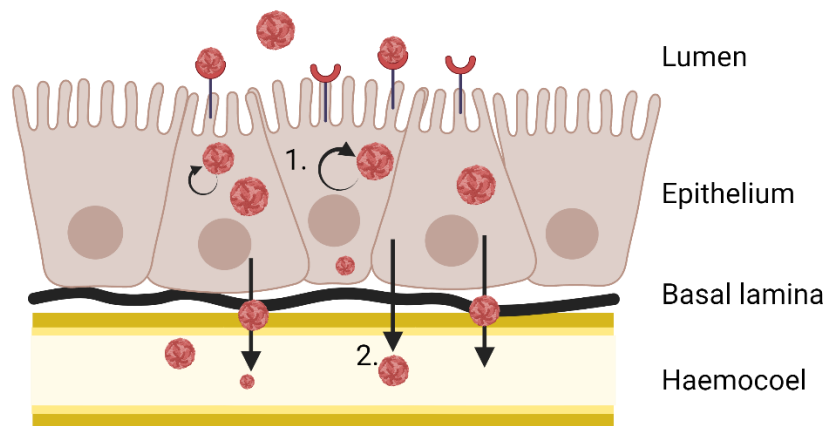


Figure 24 : Schematic Overview of Mosquito Infection and Escape Barriers to DENV Infection. 1. Multiplication of DENV within epithelial cells while traversing the midgut infection barrier (MIB). 2. DENV passage through the midgut escape barrier (MEB) and subsequent dissemination throughout the mosquito's body

III.3.A.5. Homeostatic self-renewal of the adult mosquito midgut

Maintaining healthy midgut epithelial tissue throughout life relies on the fundamental balance between eliminating old or damaged cells and generating new ones. Typically, these functions are orchestrated by the intestinal stem cells localized within particular sites or niches (236,255,256). Thus, a series of studies have focused on exploring the ISCs turnover in insects to maintain integrity in normal conditions or after induced damage.

The discovery of stem cells in the adult midgut of *Drosophila melanogaster* has profoundly advanced our understanding of the ISCs role in maintaining physiological intestinal balance in Diptera (238,256,257). The adult *Drosophila* midgut serves as a robust model system for studying host-microbial interactions as well and exploring diverse cellular and molecular dynamics associated with the ISC behavior (256). Normal self-renewal of the intestinal epithelium in *Drosophila* relies on tightly regulated activity in multiple conserved signaling pathways, including Delta, Notch, JAK/STAT, and EGFR (256). In response to various stressors and factors compromising epithelial integrity (231,238,258), such as chemical and microbial damage, the adult posterior *Drosophila* midgut exhibits remarkable rapid and effective regeneration of the midgut epithelium. This regeneration involves the rapid division and differentiation of ISCs to restore the midgut homeostasis, mediated by conserved signaling pathways such as JNK, Hippo, JAK/Stat, Hippo, EGFR, and Dpp/BMP signaling (230,241,259–264) (Figure 25).

In *Aedes aegypti*, the examination of the cellular homeostatic response and the expression of cellular markers following blood feeding, conducted through RNA sequencing, revealed significant changes in genes involved the overall cellular structure of the intestinal epithelium. This led to the identification of 20 distinct cell-type clusters post-blood meal ingestion, along with an increase in the proportions of EC/EC-like cells and ISCs (224). However, the response of ISCs after damages in the *Aedes* mosquito remains largely unexplored. Limited research has explored these physiological reactions, revealing that in response to different stressors such as chemical and bacterial damage, divided ISCs are observed in the *Aedes* intestine (219,232,255). This response is often accompanied by a

noticeable disruption of the intestinal muscular structure (219,232,255). After DENV-2 infection in the *Aedes aegypti*, DNA synthesis was detected in the midgut, suggesting the presence of divided ISCs (235,265). These observations underscore the adaptability of the midgut epithelium in mosquitoes' physiological responses to various challenges (Figure 25).

Another response induced by viral stress in the midgut is cellular apoptosis (Figure 25). Essential components of the apoptotic machinery include caspases, inhibitors of apoptosis (IAP proteins), IAP antagonists, Bcl-2 family members, and hid/grim/reaper (263,266–268). Depending on specific viruses and vector combinations, the apoptotic response can positively or negatively correlate with the vector competence. Some viruses actively exploit apoptosis or caspases as integral components of their replication cycle in the vector mosquito. This exploitation may occur either because they require host cell lysis for release or because caspase-mediated cleavage of viral proteins is essential for specific steps in viral replication (269). However, apoptosis can also serve as a significant cellular defense response against various viruses. These reactions may correlate with vector refractoriness, as they facilitate the removal of both infected cells and pathogens from the host and negatively impact the virus's ability to replicate and spread to new hosts (270). Previous studies have demonstrated an increase in the expression of apoptotic genes, apoptotic protein production, and apoptotic cell rates in the refractory strain of *Aedes aegypti* refractory following DENV infection (268,271–275). Thus, midgut epithelium cellular apoptosis and ISC division could have major impact on influencing barrier permissiveness.

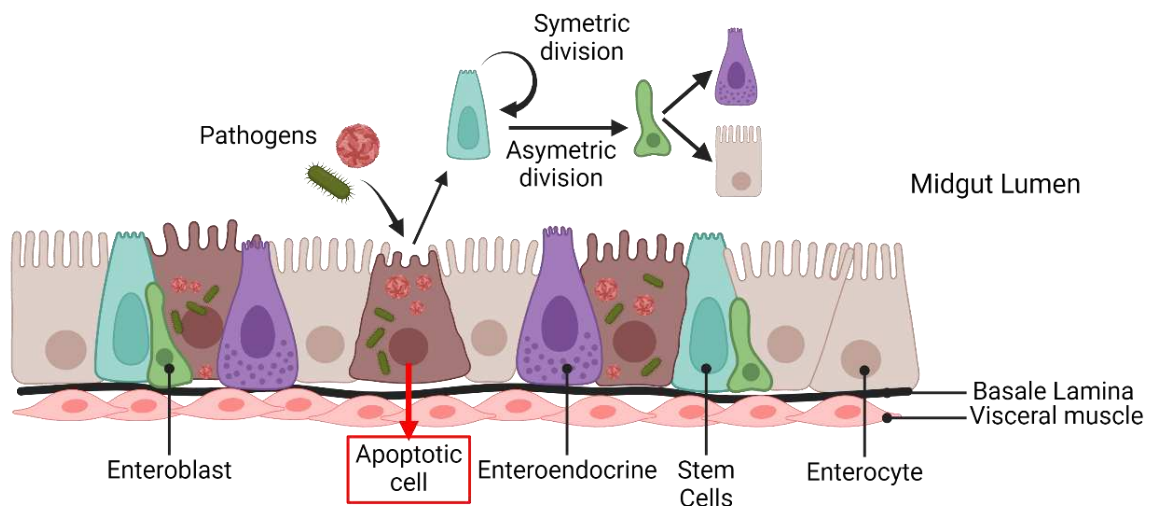


Figure 25 : Illustration of Stress Impact on Midgut Epithelium and Cellular Turnover. Exposure to bacterial, or viral stress triggers cellular apoptosis within the midgut epithelium. This apoptotic process prompts intestinal stem cells (ISCs) to undergo turnover through symmetric or asymmetric division. This turnover mechanism leads to cellular differentiation and maturation, ultimately leading to the regeneration of the midgut epithelium and compensating for the cellular loss resulting from apoptosis.

Similarly to extrinsic factors discussed earlier, intrinsic factors within the midgut can impact intestinal integrity and vector longevity. One of the most important factors in the midgut is the microbiota. The microbiota profoundly influences nutrition, reproduction, development, and defense against adversaries, thus affecting vector longevity, survival, reproduction, and vector competence (276–280). In essence, they form a vital mutualistic partnership, further enhancing their significance in insect biology. Therefore, examining the microbiota composition and understanding its implication in intestinal homeostasis could provide novel biological strategies potentially altering vector competence.

III.3.B.2. The Microbiota

III.3.B.1. Definition and composition

The microbiota, consisting of host-associated microorganisms, resides in various organs within the mosquito's body, including ovaries, salivary glands, and midgut (281,282). It consists of a diverse, dynamic, and variable population comprising both intra and extracellular microorganisms such as fungi, viruses, archaea, bacteria, and protozoans (190). Insects acquire their gut microbiota, through various modes, with some inheriting it directly from parents or other individuals, while others primarily acquire it from the environment. Additionally, the composition and density of the intestinal microbiota are complex and vary among mosquito genera and species. They depend on factors such as the mosquito's developmental stage, gender, ecological reproductive site, and are influenced by blood-feeding and ingested pathogens (203,283–286).

The majority of bacteria identified in the mosquito microbiota are gram-negative bacteria, belonging to the phyla of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (280,287). A study conducted on both species of *Aedes aegypti* and *Aedes albopictus* originating from Sri Lanka revealed that the phyla *Proteobacteria*, *Actinobacteria*, and numerous species of *Firmicutes* (constituting 99% of the microbial population) belonging to 14 genera, such as *Terribacillus*, *Lysinibacillus*, *Agromyces*, and *Kocuria* are present in the gut of both adult mosquitoes (288). The main bacterial families found in *Aedes albopictus* populations studied from Virginia, include *Enterobacteriaceae* (making up half of the bacterial population), *Enterococcaceae*, *Pseudomonadaceae*, *Bacillaceae*, *Flavobacteriaceae*, *Acetobacteraceae*, and *Sphingobacteriaceae* (289). One of the most important bacterial genera naturally residing in the *Aedes albopictus* mosquitoes' intestines is *Wolbachia*, which is implicated in numerous biological and physiological mechanisms essential to mosquito survival. A comprehensive explication of these mechanisms will follow subsequently.

III.3.B.2. Blood feeding and microbiota interaction

The microbiota affects midgut function and intricately influences the blood's digestion mechanisms (290,291). The reduction of bacteria impacts red blood cell lysis, thereby slowing protein digestion, hindering oocyte maturation, and reducing the production of vial eggs (290). Studies have shown that the gut of *Aedes aegypti* devoid of microbiota produces fewer eggs and exhibits extended lifespan with lower metabolic rates (292). Conversely, blood-feeding can alter the microbial community's homogeneity in the intestine without affecting the microbial richness and regularity (293,294). However, the influence of the host blood meal

source on microbial diversity and density can vary (209,295). In hematophagous mosquitoes, the number of bacteria increases 24 hours after blood ingestion, peaking 48 hours post-meal (287). This shift leads to an abundance of *Proteobacteria* (*Pseudomonas*, *Comamonadaceae*, *Ralstonia*, and *Delftia*). In *Aedes albopictus*, six hours after a blood meal, there is an increase in *Anaplasmatacea*, *Actinobacteria*, and *Rhodospirillaceae* families (209). *Wolbachia* bacterium belonging to *Anaplasmatacea* showed an increase following the blood meal (209). Interestingly, another study showed that the density of *Wolbachia* in the midgut does not change after feeding (296).

III.3.B.3. Microbiota effect on ingested pathogens

Various pathogens can be ingested through blood-feeding, leading to different forms of stress and damage in the midgut. The microbiota plays a significant role in maintaining stable physiological conditions following ingestion of these pathogens. In addition to the mosquito's innate immunity, reciprocal interactions occur between pathogens, the microbiota, and the immune system. The gut microbiota triggers/primes immune activity that can combat pathogens infections and influence vector competence, particularly towards DENV (201,297–299).

Research has shown that altering the microbiome through antibiotic treatment increases viral titer in mosquitoes by modulating immune responses to DENV infection (249). Similarly, reintroducing endosymbiotic bacteria in the vector microbiome limits the pathogen infectivity for DENV and *Plasmodium* by altering the expression of immune genes (200,276,300). Additionally, in *Anopheles* mosquito, *Serratia marcescens*, and *Enterobacter* bacteria can render the vector 99% resistant to *Plasmodium* infection by disrupting the parasite development before it invades the midgut epithelium (301–303).

Among microbiota bacteria, *Wolbachia* is one of the most prominent symbiotic microbes. Extensive research has been conducted to investigate this bacterium and understand its ability to influence host physiology and manipulate various biological mechanisms crucial for mosquito survival and longevity. This research will contribute to the development of effective strategies to understand and adapt better biological mechanisms to limit arboviral infections in vectors and subsequently their transmission to humans.

III.3.C. *Wolbachia* endosymbiont

III.3.C.1. Definition

Wolbachia was first discovered in the reproductive tissues of the mosquito *Culex pipiens* by Hertig and Wolbach in 1924. Later named *Wolbachia pipientis*, it belongs to *Anaplasmatacea* family. Natural colonization of *Wolbachia* has been demonstrated in up to 66% of insect species today (304,305). As an obligatory intracellular proteobacterium (306), *Wolbachia* resides in the cell cytoplasm and is present in several tissues with different densities, including ovaries, midgut, and head (307–309). It is vertically transmitted from one generation to another and manipulates host reproduction through mechanisms, such as feminization, male killing, parthenogenesis, and cytoplasmic incompatibility (277,310–313).

III.3.C.2. Protective and antiviral role of *Wolbachia*

A significant aspect of *Wolbachia* biology lies in its ability to influence the replication and transmission of pathogens across various mosquito vectors and insects, coupled with its widespread distribution and reproductive parasitism (314–318). Consequently, researchers are increasingly exploring its potential use as a biological control against numerous arboviruses.

In *Drosophila melanogaster* infected by the wMelpop Teixeira et al. (2008) demonstrated a significant beneficial effect of *Wolbachia* infection with an increase in survival, reduce viral titers of *Drosophila C* virus, Nora virus, and Flock House virus after infection, and enhance resistance to viral infection (319,320). Additionally, Osborne et al. observed a positive correlation between pathogen blocking and *Wolbachia* wMel density in several organs, including the head, gut, and Malpighian tubules (321). This suggests that the significance of different tissues in pathogen blocking may vary, influenced by their *Wolbachia* densities or their specific functional roles in *Wolbachia*-mediated pathogen blocking.

In cell culture, it has been shown that high *Wolbachia* density in *Aedes aegypti* and *Aedes albopictus* cellular lines correlates with strong DENV blocking (322,323). *In vivo*, studies involving transinfection of wAlbB and wMel strains in *Aedes aegypti* have demonstrated their high density in the mosquitoes' body and their effectiveness in blocking DENV. This results in reduced viral infection, transmission, and dissemination, along with increase mosquito survival (210,324–326). The host response induced by *Wolbachia* against DENV typically involves the induction of ROS species and activation of the host's innate immunity, such as Toll and IMD pathways (202,327). Additionally, *Wolbachia* indirectly reduces DENV infection by competing for essential intracellular resources that both microorganisms need, as they are both intracellular dependent (204,205).

The *Aedes albopictus* mosquito, a vector of DENV, naturally harbors two *Wolbachia* strains, wAlbA and wAlbB (328), in several organs, including the midgut (45,329). Despite this natural association, *Aedes albopictus* remains competent for DENV transmission, as demonstrated previously. Few studies have shown that wAlbA and wAlbB strains increase the survival rate of *Aedes albopictus*, and modulate the vector competence towards DENV (203,210). Furthermore, transinfection with the *Drosophila* wMel strain confers high resistance to DENV in *Aedes albopictus* (330). This challenges the belief that *Aedes albopictus* lack the necessary genetic makeup for *Wolbachia*-induced viral interference, suggesting the potential for natural induction of resistance. However, the low density of *Wolbachia* in somatic tissues of *Aedes albopictus* may limit its effectiveness compared to transinfected *Aedes aegypti*. These findings suggest that *Wolbachia* may contribute to viral resistance in *Aedes albopictus*.

Investigations conducted on *Aedes aegypti* have revealed a shift in the bacterial makeup prompted by *Wolbachia*, with a prevalence of *Serratia* sp. in mosquitoes harboring *Wolbachia* compared to higher abundance of *Pseudomonas* sp. and *Acinetobacter* sp. in those devoid of *Wolbachia* (331). This underscores *Wolbachia*'s influence, suggesting a role in modulating the colonization of specific bacterial taxa through competitive interactions. In

Drosophila, *Wolbachia* significantly shapes the composition of the midgut microbiome throughout developmental stages, although its impact is not direct (306). These findings highlight the need to consider gut microbiome composition background in studies investigating *Wolbachia*-induced phenotypic changes.

Despite *Wolbachia*'s significant impact on mosquito biology, its role in the midgut remains poorly understood. Further investigations are needed to elucidate *Wolbachia*'s role in the midgut, particularly its effect on epithelial barriers, which are crucial for modulating vector competence. Consequently, further investigations are imperative to understand the role of this bacterium within the midgut, particularly exploring its effect on the epithelial barriers, which play a crucial role in modulating vector competence.

III.3.D. Third thesis publication: *Wolbachia* maintains intestinal homeostasis in *Aedes albopictus* after blood-meal

III.3.D.1. Article's background and objectives

Insects depend on mutualistic relationships with microorganisms residing in various organs to sustain their survival and physiology. The microbiota in the mosquito's midgut plays a crucial role in modulating basic physiological responses, particularly in fighting pathogens that could induce considerable cellular damage and turnover in the midgut tissue (201,297). This symbiotic relationship is also crucial under normal conditions, such as during blood meals (290,292).

Studies have underscored *Wolbachia*'s significance in *Aedes* mosquitoes for its capability to manipulate the host's defense system against pathogens. This manipulation involves biological mechanisms like activating the host's immunity (310,332). Intriguingly, *Wolbachia* may potentially restrict the spread of DENV from the midgut epithelium to the rest of the body, thereby impacting vector competence (210,324–326).

Despite the midgut pivotal role as the primary site for blood meal intake and pathogens entry, along with its involvement in various aspects of the vector's defense system, research on the natural occurrence of *Wolbachia* in *Aedes albopictus*' midgut remains limited. Previous studies have elucidated cellular turnover mechanisms crucial for maintaining epithelium renewal and stability following various types of damage. We aim to investigate *Wolbachia*'s role in maintaining intestinal homeostasis in vector mosquitoes. Our objectives are based on studies demonstrating *Wolbachia*'s crucial involvement in regulating cellular turnover and homeostasis across different organisms. For instance, it regulates stem cell division in *Filarial nematodes* and *Drosophila* ovaries (333,334), as well as cellular apoptosis in *Asobara tabida* wasp and *Culex quinquefasciatus* mosquito ovaries (335,336). These findings reveal the vital dependency organisms have developed on *Wolbachia*'s presence.

Recognizing the importance of effective and stable midgut barriers in limiting viral spread, along with *Wolbachia*'s ability to regulate cellular division and apoptosis in other organs and species, our upcoming study seeks to investigate *Wolbachia*'s potential in preserving midgut epithelium integrity in *Aedes albopictus* following both regular and DENV-infected blood meals. Our investigation will examine the cellular and molecular mechanisms

governing cell division and apoptosis, as well as the structural integrity of the midgut musculature. A comprehensive study employing RNAseq analysis will be briefly mentioned in the general discussion section, even though preliminary results from this study we conducted will not be included in the current version of the manuscript.

III.3.D.2. Thesis third publication

Wolbachia* plays a major role in the maintenance of midgut homeostasis in *Aedes albopictus

Article under submission

1 ***Wolbachia* plays a major role in the maintenance of midgut homeostasis in**
2 ***Aedes albopictus***

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13 **ABSTRACT**

14 *Wolbachia*, a symbiotic bacterium residing within various tissues of *Aedes albopictus*
15 mosquito, affects various aspects of host biology and defense mechanisms against
16 arboviruses like Dengue virus (DENV). Although *Wolbachia* is prevalent in the mosquito
17 midgut—the portal of nutrient assimilation and first barrier against foreign invaders— little is
18 known about its influence on intestinal homeostasis. Our research addresses this gap by
19 analyzing the intestinal cellular dynamics in mosquitoes with and without *Wolbachia*, under
20 standard conditions and following DENV infection. In the absence of *Wolbachia*, we observed
21 a marked increase in both intestinal stem cell (ISC) division and in apoptosis one day after
22 blood meal consumption. Interestingly, DENV infection mildly decreases apoptosis without
23 influencing ISC division one day postinfection. Furthermore, the lack of *Wolbachia* leads to
24 midgut muscular contraction, a condition that DENV infection slightly intensifies. These
25 findings not only highlight the essential role of the *Wolbachia* endosymbiont in regulating
26 intestinal homeostasis and cell turnover, but also open new paths for exploring midgut-
27 endosymbiotic relationships as potential targets for innovative mosquito management
28 strategies.

29 **AUTHORS SUMMARY**

30 *Wolbachia* is an intracellular bacterium that resides within many insects, including the *Aedes*
31 *albopictus* mosquito, a known vector of dengue fever. This bacterium is recognized for
32 influencing the mosquito lifespan, reproduction, and virus transmission capabilities. Our study
33 unveils a critical effect of *Wolbachia* in maintaining the health of the mosquito midgut,
34 demonstrating the endosymbiont's involvement in regulating cell division and cell death to
35 preserve gut integrity. This regulatory function is vital for the mosquito ability to recover from
36 the stress of blood feeding and operates independently of dengue virus infection. Our findings
37 reinforce the importance of understanding symbiotic relationships between mosquitoes and
38 their microbial inhabitants, opening new avenues to disrupt disease transmission and enhance
39 public health protections.

40 INTRODUCTION

41 Mutualistic interactions between microbiota and their hosts are fundamental for maintaining
42 physiological equilibrium in a diverse array of life forms, spanning from plants and animals to
43 humans (1). Within the insect kingdom, relationships with endosymbiotic bacteria notably
44 influence host survival by regulating essential functions such as nutrition, reproduction,
45 development, and defense against pathogens (2).

46 *Wolbachia*, a prevalent gram-negative endosymbiont found in arthropods, infects an
47 estimated 66% of species (3,4). In *Aedes* mosquitoes, *Wolbachia* significantly alters their host
48 biology and vectorial capacity through various mechanisms. These include cytoplasmic
49 incompatibility, which promotes its spread by affecting host reproduction (5,6); pathogen
50 blocking, which diminishes the transmission of pathogens like the dengue virus (7–9); and
51 influences on host fitness, including changes in longevity and fecundity (10,11). Additionally,
52 *Wolbachia* can engage in horizontal gene transfer with its host genome and modulate the host
53 immune system, enhancing resistance to some pathogens while potentially increasing
54 susceptibility to others (9,12,13). These diverse interactions underscore *Wolbachia*' crucial role
55 in mosquito biology and its potential in vector-borne disease management strategies.

56 In the Asian mosquito *Aedes albopictus*, *Wolbachia* naturally inhabits various organs,
57 including the midgut (14,15) . Despite the critical roles of the midgut in nutrient assimilation,
58 pathogen defense, and preventing microbial dissemination within the host, research on
59 *Wolbachia*' influence on the structural integrity of the midgut epithelium was not investigated
60 yet. Previous studies have identified significant cellular processes within the midgut epithelium,
61 such as intestinal stem cell proliferation and regulated cell death, which are vital for its
62 maintenance and resilience under different conditions (16–19). However, *Wolbachia* potential
63 impact on these cellular activities and its ability to maintain midgut homeostasis, both under
64 normal conditions and in response to external threats, remains underexplored. This gap is
65 especially intriguing given the evidence of *Wolbachia* critical role in modulating stem cell

66 dynamics (20,21) and apoptosis in other arthropod tissues (22,23), suggesting its regulatory
67 influence might extend to midgut homeostasis.

68 This study examines the impact of *Wolbachia* on *Aedes albopictus* intestinal
69 homeostasis, focusing on cellular dynamics including division and apoptosis following a
70 standard blood meal or in Dengue virus (DENV) infection conditions. Our findings reveal that
71 *Wolbachia* plays a pivotal role in regulating normal intestinal stem cell proliferation rates, as its
72 absence leads to an accelerated regenerative state in the midgut at one day after blood meal
73 consumption. This increased cell division rate coincided with a higher proportion of apoptotic
74 cells in the midgut when *Wolbachia* was absent, indicating a disrupted balance within the
75 intestinal epithelium. Additionally, the overall structural organization of the visceral muscle was
76 significantly compromised in the absence of *Wolbachia*, leading to disordered tissue
77 architecture. Unexpectedly, the expression levels of Keren and DIAP, key regulatory proteins
78 in the midgut involved in the division (24) and apoptosis (25) pathways respectively—did not
79 account for the observed alterations. Interestingly, the effects mediated by *Wolbachia* were not
80 modulated by the presence of the DENV one day postinfection, highlighting a direct role of this
81 bacterium in maintaining gut integrity independent of external challenges.

82 METHODS

83 Mosquito rearing

84 Two *Aedes albopictus* lines were used for the experiments. The first line, called S.RUN, was a
85 local strain collected in La Réunion and naturally infected with *wAlbA* and *wAlbB*. The second
86 line, named S.RUN *Asymb*, was a *Wolbachia*-free line obtained from the S.RUN population
87 after treatment with a tetracycline antibiotic to eliminate *Wolbachia*. To confirm the
88 absence of *Wolbachia*, individuals were screened using PCR with primers 81F (5'-TGG TCC
89 AAT AAG TGA TGA AGA AAC) and 691R (5'-AAA AAT TAA ACG CTA CTC CA), which specifically
90 amplify the *Wolbachia* surface protein gene *wsp* for the *wAlbA* and *wAlbB* strains (26). Both

91 S.RUN (W+) and S.RUN Asymb (W-) populations were maintained in the insectary at 28 °C
92 ± 1°C, with 80% relative humidity and a 12-hour light/12-hour dark photoperiod. Larvae
93 were fed with yeast tablets, while adults were provided with a 10% sucrose solution. During
94 the experiments, the S-RUN population was reared from F30 to F52 generations, while the
95 S.RUN Asymb population was reared from F20 to F28 generations. To maintain and amplify
96 both populations, adult females were artificially fed with bovine blood using the Hemotek
97 feeding system (Hemotek Limited, Great Harwood, UK) covered with pig intestine, once or
98 twice a week.

99 **DENV production**

100 The DENV-1 strain used for the experimental mosquito infection was isolated from the serum
101 of a patient from La Reunion Island in 2018 (namely RUN1-1583; Genbank accession number:
102 ON63127). To produce viral stocks for experimental infections, the RUN1-1583 strain was
103 amplified on Vero E6 cells at a Multiplicity of Infection (MOI) of 0.1 in Eagle's Minimum
104 Essential Medium (MEM). The MEM was supplemented with 2% heat-inactivated fetal bovine
105 serum (FBS), 2 mmol/l l-glutamine, 1 mmol/l sodium pyruvate, 10 U/ml penicillin, 0.1 mg/ml
106 streptomycin, and 0.5 µg/ml fungizone (PAN Biotech, Aidenbach, Germany). The Vero cells
107 were maintained at 37°C with a 5% CO₂ atmosphere. Supernatants containing virus particles
108 were collected three to five days postinfection when cytopathic effects appeared and then
109 centrifuged and stored at -80°C until use. The final titers of the DENV stocks were determined
110 to be 3.3×10⁷ PFU/ml.

111 **Experimental infection**

112 Female mosquitoes, aged five to eight days, were confined in small cages (16×16×16 cm) and
113 subjected to a 24 to 30-hour starvation period. Following this fasting phase, they were allowed
114 a 45-minute feeding session on infectious blood meals, consisting of 1 ml of washed rabbit
115 erythrocytes, 1 ml of DENV suspension, and 5 mM (21 µl) of adenosine triphosphate as a

116 phagostimulant. The Hemotek feeding system was used to provide the infectious blood meal
117 to the mosquitoes. To assess the stability of viral titers during blood feeding, blood samples
118 were collected before and after the 45-minute feeding period and stored at -80°C for
119 subsequent titration. Then, mosquitoes were cold-anesthetized, and fully engorged females
120 were transferred to a climatic chamber (26±1°C, 80% RH, with a photoperiod of 12 h light/12
121 h dark), where they were maintained on a 10% sucrose solution for 24 hours before proceeding
122 with experiments. After feeding both mosquito populations, including *Wolbachia* and
123 *Wolbachia*-free individuals, on DENV-uninfected and infected blood meals, four categories of
124 mosquitoes were obtained: W+/D-, W+/D+, W-/D-, & W-/D+ (Fig. 1).

125 **Dissection of mosquito midguts**

126 One day post blood meal, mosquitoes were anesthetized by placing cages containing
127 engorged mosquitoes into a +4°C cold chamber. Mosquitos were then transferred one by one
128 onto a glass slide in a drop of 50% ethanol for the isolation of midguts under a light
129 stereomicroscope. Using fine forceps, the mosquito head was severed, and the abdomen was
130 pulled from the posterior end until the midgut detached. A small incision was made using the
131 forceps to allow blood bolus to be evacuated from the intestine. Once the midgut was
132 completely blood-free, it was then placed in a 1.5 ml Eppendorf tube containing 1X PBS and
133 kept on ice.

134 **Immunostaining**

135 Isolated guts were fixed in a 4% Paraformaldehyde (VWR, USA) solution in 1XPBS for 30
136 minutes. Then, the guts underwent three 15-minute washes in 0.1% PBS-Triton to allow
137 permeabilization. Blocking was then performed for 30 minutes by adding a solution of 1X PBS
138 -Triton 0.1%-BSA 1%. Following blocking, the primary rabbit α -Phosphorylated-histone-3 (α -
139 PH3) antibodies (ABCAM, UK) were added (1:1000 in 1X PBS-Triton 0.1%-BSA 1%), and
140 incubated overnight at 4 °C. α -PH3 it is a highly specific marker for condensed chromosomes
141 and is used to assess the number of mitotic ISC (27). After three 15-minute washes in PBS-

142 Triton 0.1%, the secondary antibodies Alexa Fluor® 555 (ABCAM, UK) were added (1:1000 in
143 PBS-Triton 0.1%-BSA 1%) and incubated overnight at 4 °C. Phalloidin coupled to Alexa Fluor®
144 647 (ABCAM, UK), was added for one hour at room temperature (1:1000 in PBS-Triton 0.1%-
145 BSA 1%). Once phalloidin was removed, DAPI stain was applied at a concentration of 1:1000
146 for 30 minutes, followed by three 15-minute washes in PBS-Triton 0.1%. The guts were
147 mounted on microscope slides in 50% glycerol:1xPBS mounting medium, and the coverslips
148 were sealed with colorless nail varnish.

149 **TUNEL staining**

150 Pools of 3-4 midguts were fixed in 4% paraformaldehyde-1XPBS for 30 minutes. They were
151 then dehydrated in a Tris-buffered saline (TBS) gradient of 25%–50%–75%–100% methanol,
152 with each step taking 5 minutes, and stored at 4 °C in 100% methanol until further processing.
153 To rehydrate the midguts, they were placed back in the same TBS methanol gradient, but in
154 reverse order. Next, the midguts were permeabilized in 20 µg/mL proteinase K for 5 minutes
155 at room temperature, rinsed twice, and washed twice for 5 minutes in TBS. Then they were
156 fixed in 4% paraformaldehyde in TBS for 10 minutes (28). TUNEL reactions were performed
157 using equilibration buffer and labeling mix from the Millipore FragEL™ DNA Fragmentation
158 Detection Kit, Fluorescent (FITC conjugated) (QIA39-1EA) following the manufacturer's
159 recommendations.

160 **Cell counting and statistical analysis**

161 The slides prepared were examined using a confocal microscope (Confocal NIKN C2si) for the
162 counting of targeted cells, and image acquisition was conducted using NIIS Software. Cell
163 counts were analyzed using GraphPad Prism software, and an unpaired t-test was carried out.

164 **Primers generation**

165 DIAP and Keren genes were identified on VectorBase for *Aedes albopictus* and *Aedes aegypti*
166 species. Multiple isoforms were found for each gene, exhibiting distinct loci and positions within

167 the genome. For DIAP, the isoforms include AALF001357, AALFPA_051841, AALF001458,
168 AALF019523, AALFPA_056496, and AALFPA_076435. The Keren isoforms comprise
169 AALFPA_047463, AALFPA_061379, AALFPA_070949, AALFPA_071273, AALFPA_075378,
170 AALFPA_054420, and one set of Keren primers is copied from (16). An alignment of isoforms
171 was performed using the online tool ClustalOmega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)
172 to identify shared consensus sequences. Five DIAP isoforms and three Keren isoforms were
173 identified, and primers were generated using the website [https://www.dkfz.de/signaling/e-](https://www.dkfz.de/signaling/e-rnai3/0)
174 [rnai3/0](https://www.dkfz.de/signaling/e-rnai3/0). The primers sets are named DIAP1, DIAP2, DIAP3, DIAP4, DIAP5, Keren1, Keren2,
175 and Keren3. (Table S.1) (Table 1).

176 We conducted several experiments to select the optimal sets of primers for our *Aedes*
177 *albopictus* populations. For each of the four mosquito modalities (W+/D-, W+/D+, W-/D-, and
178 W-/D+), we pooled 10 midguts in 1.5 mL Eppendorf tube. Three replicates for each modality
179 were used. Total RNA was extracted using TRIzol™ Reagent from Invitrogen (15596026).
180 Samples were treated with TURBO™ DNase (AM2238) to remove any DNA contamination.
181 The extracted RNAs were quantified using the QUBIT RNA HS ASSAY (Q32855), and 400 ng
182 of RNA were used for reverse transcription into cDNA using the LunaScript® RT SuperMix Kit
183 (NEB #E3010S/L). All experiments were performed according to the manufacturer's
184 instructions. A PCR was conducted to ascertain the specificity of the primer hybridization on
185 DIAP and Keren RNA sequences within the 4 modalities. Amplicons were purified and
186 sequenced (GenoScreen, France) to validate their sequences. Real-time quantitative PCRs
187 were performed using SYBR green (Qiagen) with the CFX96 Touch Real-Time PCR Detection
188 System (Bio-Rad, Hercules, CA, USA) to identify primers that exhibited optimal performance
189 across different modalities. Three independent experiments were conducted and the results
190 were averaged. Two-way ANOVA and unpaired t-tests were employed for statistical analysis.

191 RESULTS

192 Temporal effects of *Wolbachia* on intestinal cell division after blood meals in *Aedes*

193 *albopictus*

194 To determine the best time for evaluating *Wolbachia* influence on intestinal homeostasis of
195 *Aedes albopictus*, we performed a kinetic study, synchronizing our analysis with the sequential
196 stages of blood meal digestion. This investigation was structured around three distinct phases:
197 the early phase (1DPB), occurring on the first day post-blood meal and reflecting the initial
198 interaction with the gut lining; the middle phase (5DPB), corresponding to the completion of
199 blood digestion five days later; and the late phase (13DPB), taking place thirteen days after
200 digestion, during which the mosquitoes reverted to sugar consumption (Fig. 1A).

201 Our examinations focused on the midguts of *Aedes albopictus* with *Wolbachia* (W+) and without
202 *Wolbachia* (W-), dissected at the specified time points (1DPB, 5DPB, and 13DPB) after a standard rabbit blood meal. At 1DPB, to facilitate blood coagulation, dissected midguts
203 were treated with 50% ethanol, followed by the creation of a small incision in the epithelium to
204 extract the blood bolus (Fig. 1A). Subsequently, we applied a staining protocol utilizing an anti-
205 PH3 antibody to specifically highlight phosphorylated histone H3 within condensed chromatin,
206 enabling the quantification of mitotic cells.

208 Microscopic examination (Fig. 1B) revealed that midguts from W+ mosquitoes
209 displayed a limited distribution of small ISCs, intensely marked with PH3 labeling, indicative of
210 minimal cell division. In contrast, midguts from W- mosquitoes showed a significant increase
211 in ISC division, characterized by a high frequency of PH3-positive nuclei with a distinctive
212 coffee-bean shape, resulting from the recent division of a progenitor cell. Further analysis of
213 nuclear structure (Fig. 1B') showed that W+ mosquitoes maintained organized, uniform nuclei,
214 while W- specimens exhibited a loss of nuclear uniformity and nuclear membrane irregularities.

215 A statistical comparison of mitotic cell counts (Fig. 1C) revealed marked differences
216 between the W+ and W- strains at 1DPB, with the W- mosquitoes displaying a wider range of
217 mitotic cell counts (5-61 cells/midgut) compared to their W+ counterparts (0-20 cells/midgut).
218 This variance underscores *Wolbachia* crucial role in maintaining cellular division equilibrium
219 and intestinal homeostasis. At 5DPB, the mitotic cell counts between the two strains showed
220 no significant disparity, both presenting low levels of cell division. Conversely, at 13DPB, the
221 W- mosquitoes exhibited a higher frequency of mitotic cells than the W+ mosquitoes, albeit
222 with numbers reduced from those observed at 1DPB.

223 These observations emphasize the critical juncture at 1DPB for investigating *Wolbachia*
224 modulation of the *Aedes albopictus* intestinal epithelium. The absence of *Wolbachia* leads to
225 a disruption in nuclear structure and an increase in ISC proliferation, possibly reflecting an
226 enhanced apoptosis as a consequence to the endobacterium removal.

227 **Impact of *Wolbachia* removal on basal intestinal cell apoptosis**

228 To explore the role of *Wolbachia* in modulating apoptosis within the mosquito midgut, we
229 applied the Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling (TUNEL) assay
230 on *Aedes albopictus* specimens at 1DPB from both W+ and W- groups. This method enabled
231 the detection and quantification of apoptotic cells, facilitating a comparative analysis of
232 apoptosis rates in the midguts of the two mosquito populations (Fig. 2A). W+ mosquito midguts
233 exhibited fewer TUNEL-positive cells, indicating lower levels of apoptosis. In contrast, W-
234 mosquito midguts displayed a significantly higher count of TUNEL-positive cells, aligning with
235 the nuclear disruption observed in Figure 1B and B'. Quantification of TUNEL-positive cells
236 revealed a marked increase in apoptotic cell numbers following *Wolbachia* removal, with cell
237 counts reaching a maximum of 62 cells/midgut in W+ mosquitoes compared to 150
238 cells/midgut in W- mosquitoes (Fig. 2B). This difference was statistically significant (****p <
239 0.0001), denoting a substantial rise in apoptosis levels post-*Wolbachia* elimination.

240 In correlation with the our findings on mitotic cell variance, the apoptotic cell data further
241 emphasized the discrepancy between *Wolbachia*-presence and -absence scenarios. While the

242 W+ group showed a relatively stable range of apoptotic cells, from 0 to 45 cells/midgut (with
243 an exception of two midguts reaching 62 cells/midgut), the W- group exhibited a wider
244 variance, from 0 to 150 cells/midgut. This observation underlines *Wolbachia* critical role in
245 stabilizing intestinal epithelial homeostasis and maintaining controlled levels of cellular
246 apoptosis, which become disrupted upon the endosymbiont removal.

247 **Influence of *Wolbachia* on visceral muscular architecture**

248 To investigate the role of *Wolbachia* in the structural integrity of the midgut muscle layer, we
249 employed phalloidin, a fluorescent dye that binds specifically to actin filaments, to visualize the
250 muscular framework within the midguts of both W+ and W- *Aedes albopictus* strains. This
251 approach allowed us to assess the structural differences attributable to the presence or
252 absence of *Wolbachia* (Fig. 3).

253 Observations revealed that the midgut muscles of W+ mosquitoes exhibited a robust
254 and orderly arrangement, characterized by straight and uniformly aligned actin filaments,
255 reflective of a healthy and stable muscular structure. Conversely, in the midguts of W-
256 mosquitoes, a noticeable deviation was observed; the actin filaments appeared irregular and
257 wavy, suggesting a compromised structural integrity. This distortion was further corroborated
258 by the increased density and contraction of actin filaments in W- mosquitoes, hinting at an
259 induced stress response from the absence of *Wolbachia*.

260 These findings emphasize the significant impact of *Wolbachia* on maintaining the
261 muscular structure of the *Aedes albopictus* midgut, indicating that the symbiotic relationship
262 extends beyond cellular regulation to include physical aspects of tissue architecture, which
263 could have implications for gut function and overall mosquito health.

264 **Exploring the molecular footprint of *Wolbachia* on classical cell cycle and apoptosis** 265 **regulators**

266 To gain insights into the molecular determinants conveying *Wolbachia* effects within the *Aedes*
267 *albopictus* midgut, we examined the expression of two genes, *DIAP* (Hippo pathway) and
268 *Keren* (EGFR pathway), as prime candidates due to their known roles in regulating apoptosis
269 and cellular proliferation within the midgut, respectively (24,29–31). To probe their expressions
270 primer sets were synthesized (Table S1), targeting the RNA sequences of *DIAP* and *Keren*
271 across various isoforms and genes (Fig. 4A for *DIAP* primers), referencing the *Cytochrome*
272 *Oxidase I (COI)* gene for housekeeping purposes and the 40S ribosomal protein gene *S7*
273 (*RSP7*) as an additional normalization marker. Our PCR screening revealed that only the
274 *DIAP57/41* and *Keren* genes are actively expressed in the midgut, steering our subsequent
275 analysis toward these targets (Figure S1).

276 To quantify these two genes' expression dynamics, real-time quantitative PCR assays were
277 conducted on cDNA derived from RNA extracted from pooled samples of ten midguts, across
278 three replicate experiments for each mosquito group. Normalization of expression levels
279 against the *RSP7* reference gene did not unveil any significant differences between
280 mosquitoes harboring *Wolbachia* and those without, for both the *DIAP57/41* and *Keren* genes
281 (Figs. 4B and 4C).

282 This result suggests that the regulatory impact of *Wolbachia* on midgut homeostasis at
283 the studied time points does not mainly go through the modulation of these genes, diverging
284 from patterns observed in responses to pathogenic bacterial infections, particularly concerning
285 the *Keren* gene (30,31)

286 **Protective effects of *Wolbachia* on midgut homeostasis are unaffected by DENV infection**

287 After showing that *Wolbachia* is required to maintain midgut homeostasis in *Aedes albopictus*,
288 we next asked whether the different *Wolbachia* effects described above could be modulated
289 by DENV infection. We first observed that in the presence of *Wolbachia* (*W+/D+*), midgut nuclei
290 exhibit only minor disruptions compared to the stable structure observed without DENV
291 infection (*W+/D-*) (Fig.5A). Conversely, in *Wolbachia*-absent (*W-/D+*) conditions, significant

292 nuclear distortions were noted, potentially intensified by DENV, in agreement with the earlier
293 findings shown in figure 1B'.

294 When examining the ISC division at 1-day post-DENV infection, no substantial impact
295 was detected, regardless of *Wolbachia* presence. The division rates between DENV-infected
296 and uninfected groups remained comparable, in the presence or absence of *Wolbachia*.
297 Notably, in *Wolbachia*-free midguts post-DENV infection, ISC division increased, contrasting
298 with the more stable division rates in *Wolbachia*-positive scenarios. This pattern suggests that
299 DENV infection does not alter *Wolbachia*'s protective effects on ISC division.

300 In addition, apoptotic cell counts were unaffected in the context of DENV infection when
301 *Wolbachia* was present. Apoptosis rates stayed consistent in *Wolbachia*-positive conditions,
302 irrespective of DENV presence. However, a slight reduction in apoptosis was observed in
303 *Wolbachia*-negative scenarios following DENV infection, underlining *Wolbachia*'s significance
304 in maintaining cellular equilibrium even amidst viral challenges.

305 Finally, exploring the DENV impact on the intestinal muscular structure highlighted *Wolbachia*'s
306 protective role (Fig. 5D). In *Wolbachia*-positive mosquitoes with DENV (W+/D+), the muscular
307 structure exhibited minimal disruption, maintaining integrity that contrasts starkly with the
308 compromised structure seen in *Wolbachia*-negative and DENV-infected (W-/D+) mosquitoes,
309 where significant muscular distortion was evident.

310 Collectively, these findings underscore *Wolbachia*'s crucial function in safeguarding
311 midgut homeostasis, particularly in ISC division, apoptosis regulation, and muscular structure
312 preservation. *Wolbachia*'s protective mechanism seems unaffected by DENV at the observed
313 stage, highlighting its potential as a stabilizing force within mosquito midgut ecology,
314 regardless of viral infection dynamics. This elucidates the symbiont's broader role in host
315 protection and disease transmission, emphasizing the complexity of host-microbe-pathogen
316 interactions.

317 **DISCUSSION**

318 In this comprehensive study, we elucidate the temporal dynamics of *Wolbachia*'s influence on
319 the intestinal homeostasis of *Aedes albopictus*. Our findings provide compelling evidence that
320 *Wolbachia* plays a pivotal role in modulating cellular dynamics within the mosquito midgut,
321 including ISC division, apoptosis, and the structural integrity of the visceral muscle. These
322 effects collectively highlight the endosymbiont's significance far beyond its previously
323 recognized impacts on reproduction (5,32), pathogen defense (7–9), and host fitness (10,11),
324 positioning it as a key regulator of midgut physiology with potential implications for the
325 mosquito's vectorial capacity.

326 Our research delineates a clear temporal pattern in *Wolbachia* regulatory effects. At 1
327 DPB, the presence of *Wolbachia* significantly stabilizes mitotic activity within the midgut, a
328 critical period for initial regenerative responses to feeding. This stabilizing effect is absent by
329 5 DPB, suggesting a transient yet crucial role in immediate post-feeding homeostasis.
330 However, by 13 DPB, the resurgence of mitotic activity in *Wolbachia*-free mosquitoes
331 underscores the endosymbiont's long-term influence on cellular turnover and midgut
332 regeneration. These findings highlight specific periods where *Wolbachia* regulatory functions
333 are most pronounced, providing insights into its role in the mosquito lifecycle that were
334 previously unexplored. Examining potential mechanisms, we inferred a role of apoptosis in
335 *Wolbachia*-mediated intestinal cell turnover at 1 DPB. *Wolbachia* elimination led to a significant
336 increase in apoptotic cells, highlighting its role in apoptosis inhibition and the preservation of
337 intestinal epithelium homeostasis. The correlation between the pronounced increase in ISC
338 division and midgut cell apoptosis in *Wolbachia*-free mosquitoes immediately following a blood
339 meal emphasized the bacterium's role in moderating the regenerative response to feeding,
340 potentially leading to midgut epithelial stability and controlled responses to environmental
341 challenges. Additionally, the impact of *Wolbachia* on the muscular architecture of the midgut
342 underlines its broader influence on tissue integrity and function. The disorganized actin
343 filaments observed in *Wolbachia*-free mosquitoes point to a disruption of gut motility and

344 nutrient absorption efficiency, which could have significant implications for mosquito health,
345 oogenesis, and vector competence. Similar cellular events were described in the midgut of
346 *Drosophila melanogaster* following various damages (27,33,34,34–37), which induce cell
347 death and consequently ISC proliferation, highlighting the involvement of conserved regulatory
348 networks in maintaining gut integrity across Diptera.

349 Interestingly, the crucial involvement of *Wolbachia* in regulating cellular turnover and
350 tissue homeostasis has been previously underscored across different organisms. For example,
351 *Wolbachia* has been shown to stimulate stem cell division in filarial nematodes and *Drosophila*
352 *auritiana* ovaries (20,21). Additionally, its influence encompasses cellular apoptosis, as
353 evidenced in the *Asobara tabida* parasitic wasp, *Drosophila melanogaster*, and *Culex*
354 *quinquefasciatus* ovaries (22,23). Notably, *Wolbachia*'s presence in these species acts to
355 suppress/increases apoptosis in ovarian cells, facilitating normal oogenesis. Therefore, our
356 findings, within the context of *Aedes albopictus* midgut homeostasis, alongside the regulatory
357 roles observed in other hosts, illustrate the vital dependency that a diverse range of organisms
358 have developed on *Wolbachia* presence for maintaining tissue integrity and functions.

359 At the molecular scale, the absence of significant changes in the expression levels of
360 classical cell proliferation and apoptosis regulators, such as Keren and DIAP, suggests that
361 influence of *Wolbachia* extends through more complex or alternative pathways. Future
362 research could explore the involvement of other signaling pathways, such as JNK, JAK/STAT
363 and Wnt, known for their roles in apoptosis and cell proliferation (25,38,39). Such
364 investigations could reveal novel targets for manipulating *Wolbachia*-mosquito interactions to
365 control mosquito populations and disease transmission.

366 Remarkably, the absence of pronounced *Wolbachia*-modulated changes in response
367 to DENV infection suggests that the bacterium protective effects on gut homeostasis operate
368 independently of external challenges. This autonomy features the potential of *Wolbachia* as a
369 stabilizing force within the mosquito intestinal environment, capable of safeguarding against

370 both endogenous and exogenous perturbations. It also highlights the complexity of interactions
371 between mosquito, symbiont, and pathogen, indicating that the benefits conferred by
372 *Wolbachia* may extend beyond direct pathogen blocking to include enhancement of the host's
373 physiological resilience.

374 Our findings have significant implications for mosquito management strategies,
375 particularly in the context of *Wolbachia*-based interventions aimed at controlling vector-borne
376 diseases (8,40–43). By elucidating the mechanisms by which *Wolbachia* contributes to midgut
377 homeostasis, our study opens new avenues for exploring how modulation of this symbiotic
378 relationship can influence mosquito fitness, pathogen transmission, and ultimately, disease
379 spread. For instance, enhancing *Wolbachia*'s beneficial effects on gut physiology could
380 potentially augment the vector's resistance to pathogens or reduce its lifespan, thereby
381 decreasing disease transmission risk. Moreover, the identification of molecular signatures
382 associated with *Wolbachia* regulatory effects offers potential targets for novel control
383 strategies. Understanding the specific genes and pathways involved in mediating these effects
384 could lead to the development of genetic or chemical interventions designed to mimic or
385 enhance *Wolbachia*'s protective role, offering a complementary strategy to existing *Wolbachia*
386 release programs (44,45).

387 In conclusion, our study sheds light on the essential role of *Wolbachia* in maintaining
388 midgut homeostasis in *Aedes albopictus*, revealing intricate interactions that contribute to the
389 mosquito health and its capacity to transmit diseases. Further research is needed to unravel
390 the complex regulatory networks involved and to explore the full potential of leveraging
391 *Wolbachia*-midgut relationships for public health benefits. Additionally, elucidating the long-
392 term effects of *Wolbachia* removal on mosquito fitness and disease transmission could offer
393 valuable insights into its role in the ecological and evolutionary dynamics of *Aedes albopictus*.
394 Consistently, the broad spectrum of *Wolbachia* influence across various hosts offers a
395 compelling insight into the evolutionary significance of this symbiotic relationship. It suggests
396 that *Wolbachia* role in biological processes is deeply embedded in the life history of numerous

397 species, indicating a co-evolutionary trajectory that has allowed these organisms to harness
398 the symbiont regulatory capabilities for their survival and reproductive success. This shared
399 evolutionary path underscores the potential of *Wolbachia* as a target for novel biological control
400 strategies, leveraging its universal influence on host biology.

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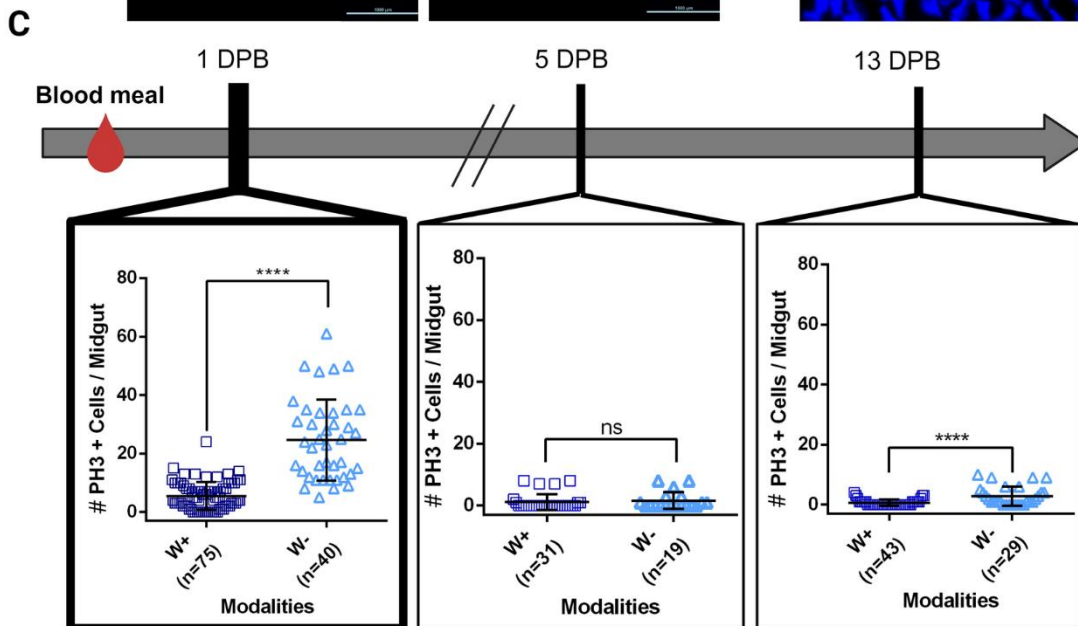
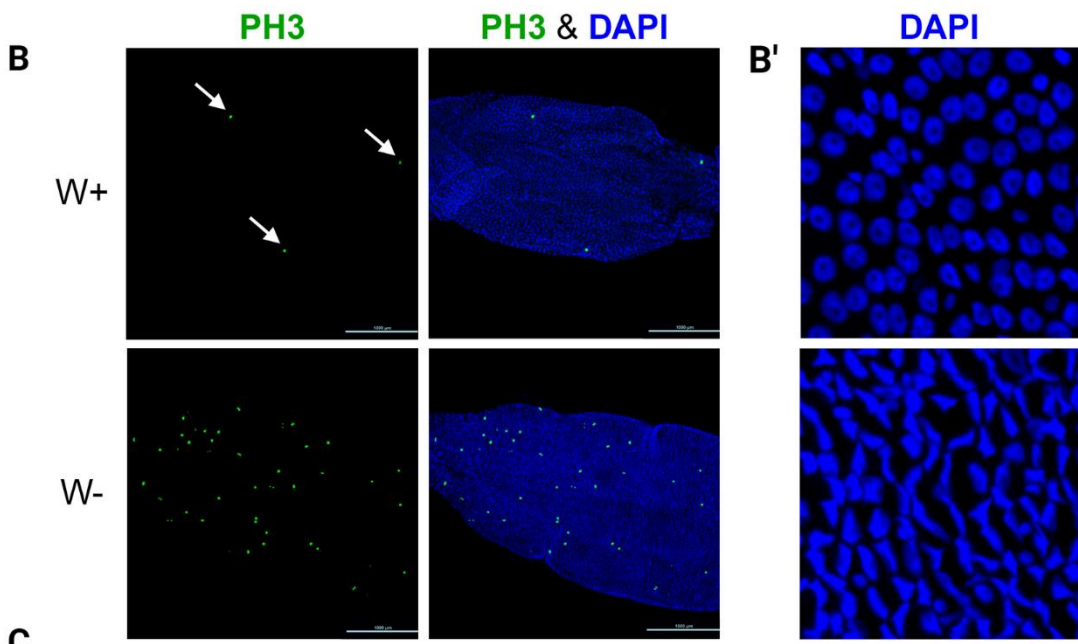
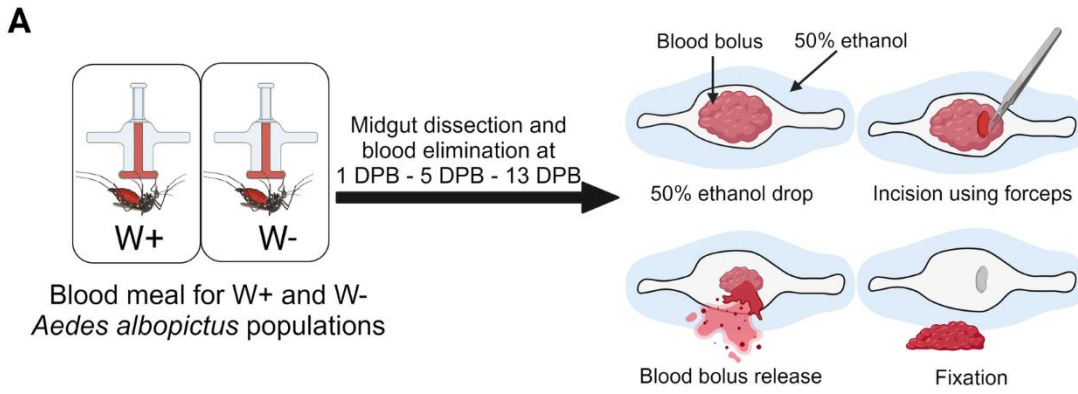
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514 **List of Figures**



515

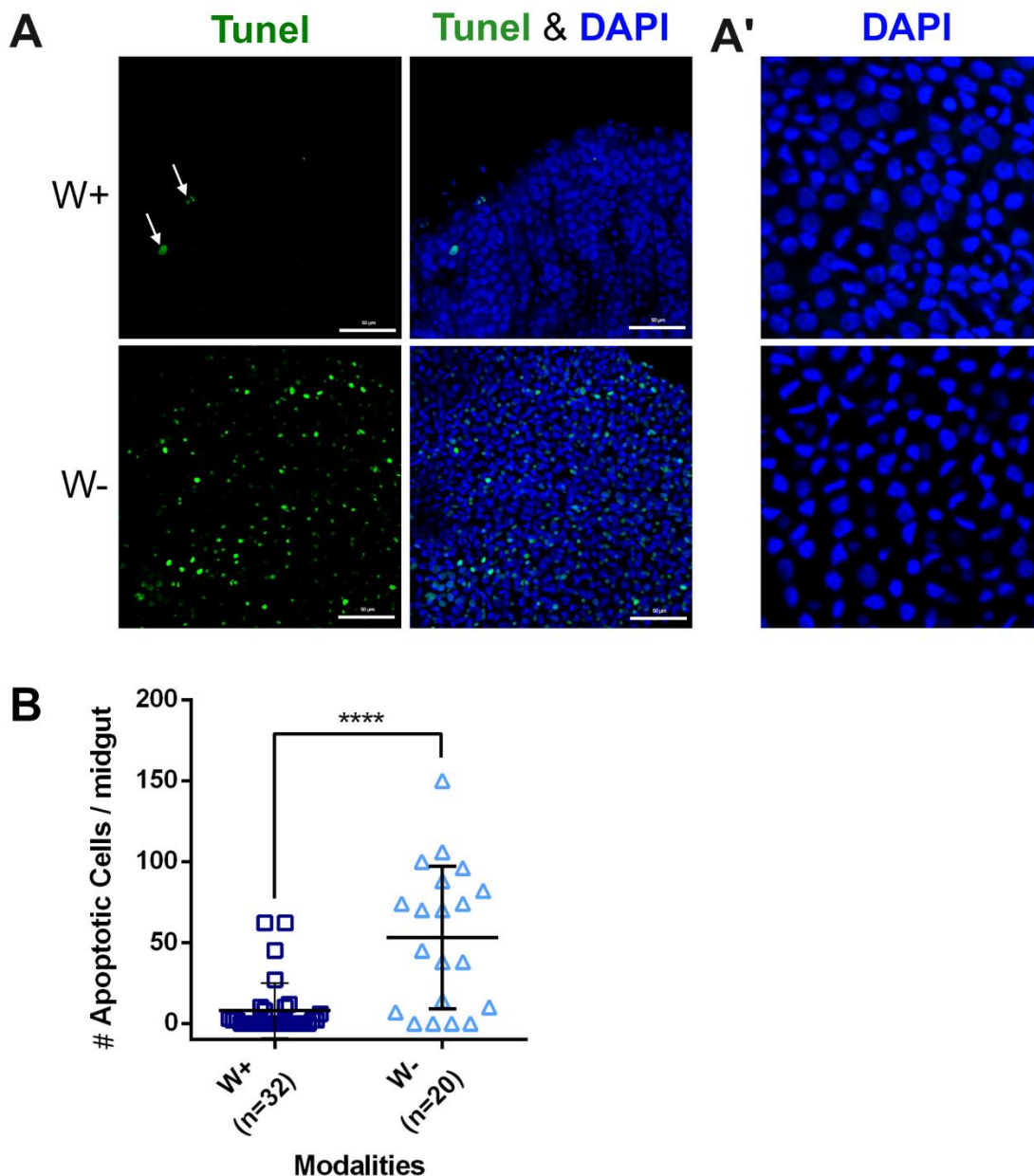
516

517 **Figure 1: *Wolbachia* controls ISC proliferation status in *Aedes albopictus* midgut.**

518 (A) Overview of the experimental design, where *Aedes albopictus* mosquitoes, both
519 *Wolbachia*-infected (W+) and uninfected (W-), were subjected to blood feeding and
520 subsequently dissected at 1, 5, and 13 days post-blood meal (1DPB, 5DPB, and 13DPB) to
521 analyze ISC proliferation.

522 (B) Representative fluorescence microscopy images show mitotic cells (highlighted with green
523 fluorescent anti-PH3 antibodies), cell nuclei are stained with DAPI in blue. Green arrows point
524 to the mitotic cells. Close-up images in (B') detail the nuclear structure of epithelial cells for
525 both W+ and W- mosquito lines, offering insights into proliferation differences.

526 (C) Quantitative analysis of mitotic cell counts in the midguts across different time points
527 (1DPB, 5DPB, and 13DPB). Counts were obtained from anti-PH3 antibody staining and
528 analyzed using a t-test to compare W+ and W- groups. Experiments were conducted in
529 triplicates, and statistical significance is indicated with asterisks: **** $P < 0.0001$, * $P < 0.05$,
530 indicating significant differences, and ns ($P > 0.05$) indicating non-significance.



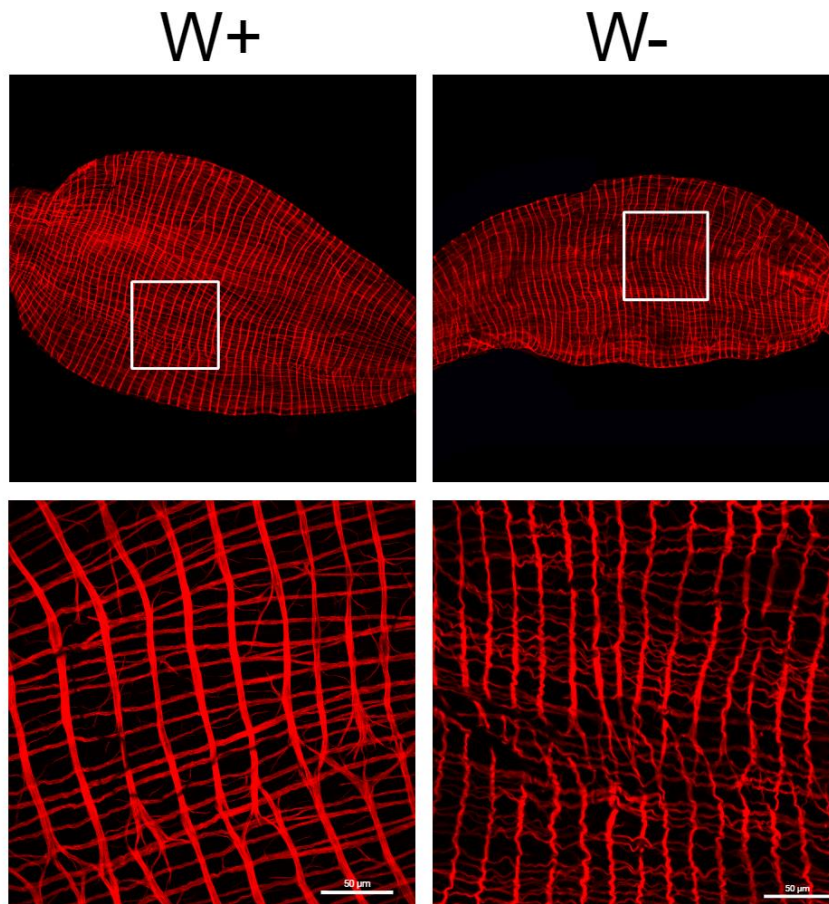
531

532 **Figure 2: *Wolbachia* reduces apoptosis in *Aedes albopictus* midgut at 1DPB**

533 (A) This panel features representative images of apoptotic cells stained in green fluorescent
 534 following TUNEL staining, along with DAPI nuclear staining. Arrows indicate apoptotic cells
 535 identified by TUNEL. Arrows point to the apoptotic cells distinguished by TUNEL staining. In
 536 the zoomed-in view (A'), the structure of nucleus cells is detailed for both W+ and W- lines.

537 (B) Apoptotic cell quantification: the number of apoptotic cells per midgut was quantified for
 538 both W+ and W- lines at 1DPB. Statistical analysis was performed using t-test to compare the

539 differences in apoptotic cell numbers between W+ and W- mosquitoes. The study was
540 performed in triplicate, ensuring reliability. Statistical significance is denoted by ****P<0.0001,
541 **P<0.01, *P<0.05, and ns (P>0.05) for non-significant differences.

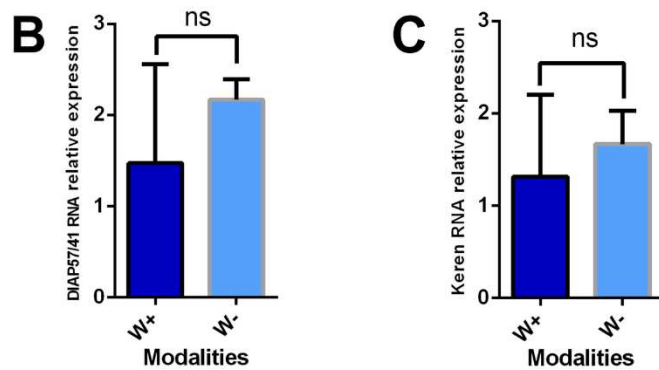
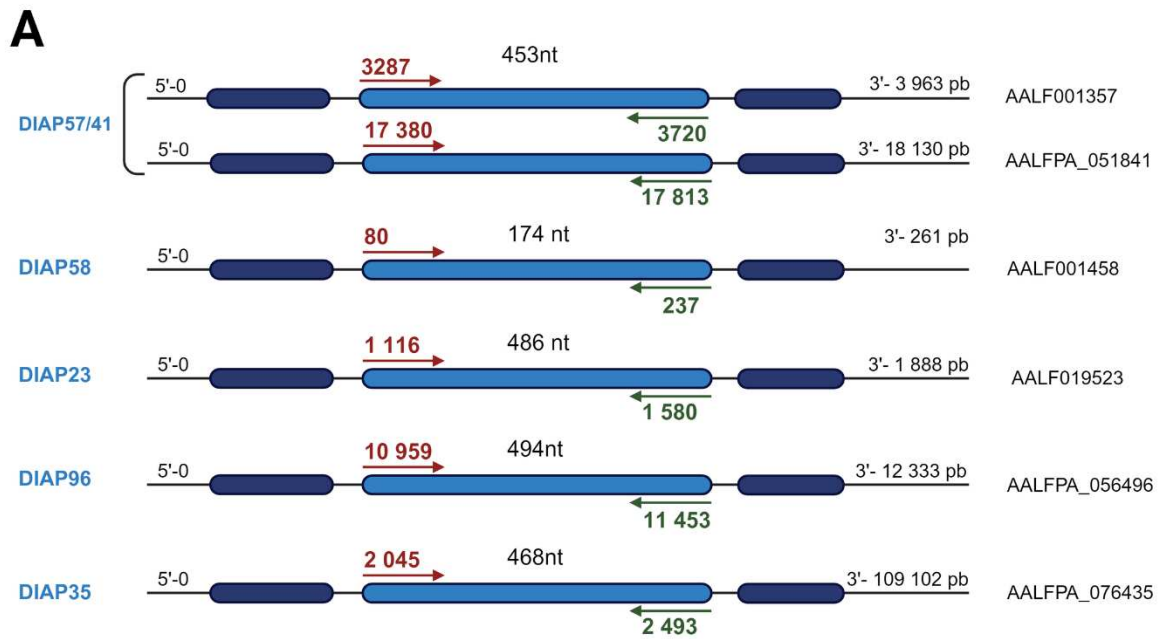


542

543 **Figure 3: The absence of *Wolbachia* affects midgut muscular structure in mosquitoes.**

544 This illustration presents the muscular architecture of the midgut in mosquito lines with (W+) and without (W-) *Wolbachia* infection. Muscular structures were visualized using Phalloidin staining, a specific marker for actin filaments, to detail the organization within the midgut. The upper images display the overall structure of the midgut musculature, while the lower images offer detailed views, emphasizing the arrangement and distribution of actin filaments.

549



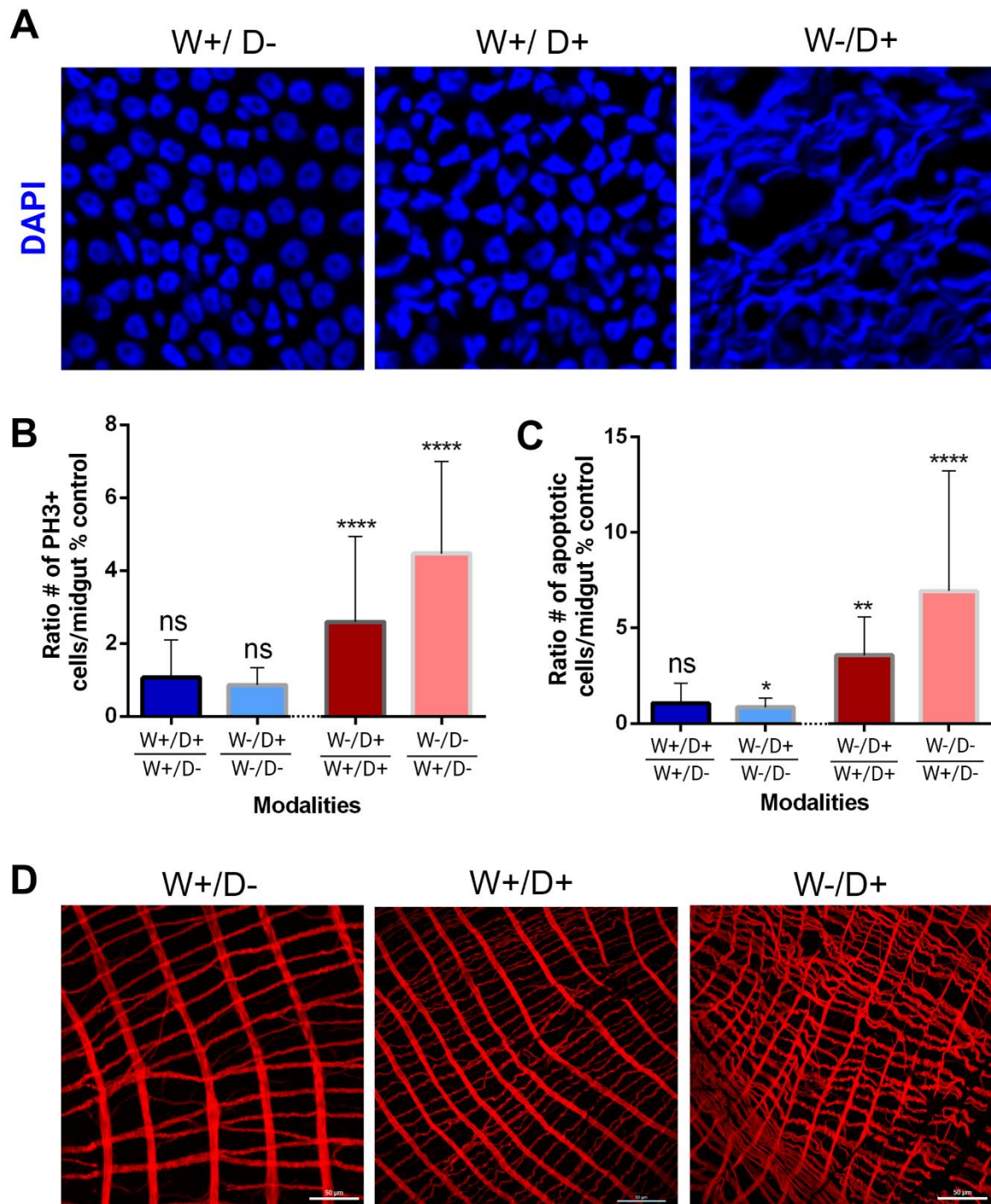
550

551 **Figure 4: DIAP and Keren gene expression unaffected by *Wolbachia* presence in *Aedes***
 552 ***albopictus* midgut**

553 (A) Schematic detailing the annealing sites for DIAP gene-specific primers. Primers
 554 DIAP57/41, DIAP96, and DIAP35 target the DIAP gene isoforms
 555 AALF001357/AALFPA_051841, AALFPA_056496, and AALFPA_076435, respectively.
 556 Forward primers are marked with red arrows, and reverse primers with green arrows.

557 (B) The relative expression levels of DIAP57/41 RNA were normalized to the housekeeping
 558 gene RSP7: Analysis shows no significant difference in DIAP mRNA expression between W+
 559 and W- midguts.

560 (C) The relative expression levels of Keren RNA were normalized to the housekeeping gene
561 RSP7: Keren mRNA expression levels at 1DPB do not significantly differ between W+ and W-
562 midguts.
563 Statistical analysis: Mean differences are categorized as follows: ***, mean difference >1.4; **,
564 mean difference >1.3; *, mean difference >1; ns, mean difference <0.01. Significance levels
565 are denoted by * $P < 0.05$, with ns indicating a lack of significant difference ($P > 0.05$).



566

567 **Figure 5: Limited effects of Dengue virus on midgut homeostasis**

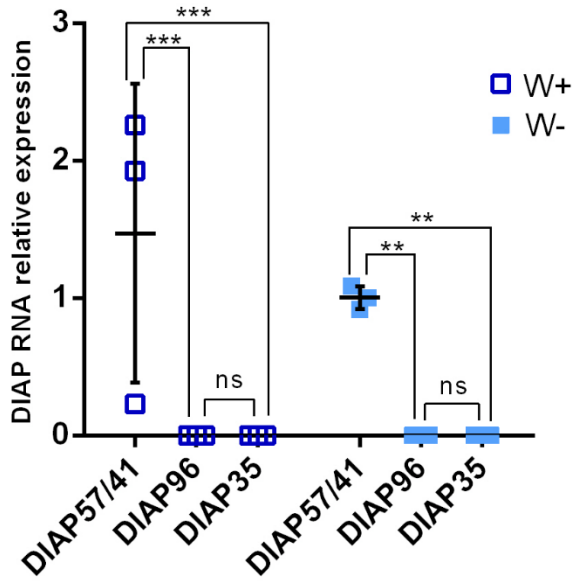
568 (A) Nuclear structure in midgut cells is depicted for W+ and W- strains following DENV
 569 infection, alongside a control group DENV-uninfected W+ strain.

570 (B) Quantification of mitotic cells post-DENV infection showcases the cell division ratios in
 571 W+/D+ compared to W+/D- controls, and in W-/D+ relative to W-/D- controls. Additionally, the

572 impact of *Wolbachia* in the context of DENV is evaluated by comparing cell division ratios in
573 W-/D+ against W+/D+ controls and W+/D- against W-/D- controls.

574 (C) Apoptotic cell ratios in W+/D+ are contrasted with W+/D- controls, and in W-/D+ versus W-
575 /D- controls. Furthermore, the influence of *Wolbachia* amidst DENV infection is analyzed by
576 comparing apoptotic cell ratios in W-/D+ to W+/D+ controls. Statistical significance is
577 determined using T-tests, denoted as follows: ****P<0.0001, *P<0.05, indicating significance,
578 and ns (P>0.05) indicating non-significance. (D) Magnified views show changes in the
579 intestinal muscular structure of the midgut following DENV infection in both W+ and W- strains.
580 The left image represents the muscular structure in the control W+/D- strain for reference,
581 while the middle and right images display observed alterations post-DENV infection in W+ and
582 W- strains, respectively.

583 **SUPPORTING INFORMATION:**



584

585 **S1 Figure. Transcriptional profiling of DIAP Isoforms in *Aedes albopictus* midgut.**

586 Assessment of the transcriptional levels of DIAP isoforms, utilizing DIAP57/41, DIAP58, and
 587 DIAP35 primers, at 1DPB in both W+ and W- mosquito strains. A two-way ANOVA multiple
 588 comparison statistical test revealed that the relative expression of the DIAP57/41 isoform was
 589 approximately 1.5 times higher compared to DIAP58 and DIAP35 in both mosquito strains.

Gene	Primer Forward				Primer Reverse				Amplicon Length	Locus	Location
	Primer	Length	Tm	GC[%]	Primer	Length	Tm	GC[%]			
DIAP_AALF001357	CAAGGACTGGGAAGCTGAAG	20	59.982	55.000	ACATTGGTGAAAGGTTTCCG	20	59.830	45.000	453	JXUM01S001116	195,570..199,532(-)
DIAP_AALFPA_051841										SWKY01000002	59,574,634..59,652,855(+)
DIAP_AALF001458	AGGCAAGTCCACCGATTCC	19	62.380	57.895	TAGCGGTAATCACCTTTGGC	20	60.096	50.000	174	JXUM01S008787	27,615..34,357(-)
DIAP_AALF019523	AGATGAAGAAGCTCGAGCCC	20	59.950	50.000	ACCGGACAATTCGTTACTGC	20	60.000	50.000	486	JXUM01S004611	10,180..12,309(+)
DIAP_AALFPA_056496	TAGGTGCTTCCCAAATGTC	20	59.933	50.000	AGCCGACGACTACTTCTCCA	20	60.012	55.000	494	SWKY01000007	66,299,401..66,311,733(-)
DIAP_AALFPA_076435	TTCTCTGAGCACCATTGACG	20	59.984	50.000	GACAGGGTTCGACGATACGTT	20	59.997	55.000	468	SWKY01000009	43,700,706..43,809,807(-)
Keren_AALFPA_047463	GACCTCGTCGGCAAAGATTA	20	60.214	50.000	TGATTGAAACAATGCAGGA	20	60.049	40.000	493	SWKY01000050	999,788..1,055,274(+)
Keren_AALFPA_061379										SWKY01000050	2,931,433..2,953,633(+)
Keren_AALFPA_070949	SWKY01000050	1,796,869..1,867,442(+)									
Keren_AALFPA_071273	TCCCTACGTTGCAATTTTCC	20	59.938	45.000	GAGATCCAATGGTCGCTTC	20	59.635	50.000	500	SWKY01000038	2,617,079..2,835,848(-)
Keren_AALFPA_075378	TGTTGGAAACGACATTGAC	20	59.547	45.000	CCGAACCAATGCAACAATG	20	59.964	45.000	498	SWKY01000038	184,870..387,959(+)
Keren_AALFPA_054420	TCCGACGGCTAGACCAAATA	20	60.597	50.000	GTGAGATCCAATGGTCGCT	20	60.081	50.000	409	SWKY01000664	238,054..245,625(+)
Keren (Janež et al., 2017)	TGATGATCCATTTGCAAGA				CTTATCCGTCCTCGCTGA						

590

591 **S1 Table. DIAP and Keren genes' isoforms.** Table presenting i) the Length [nt], Tm [°C], and

592 %GC information for "Primers" forward and reversed synthesized sequences on www.dkfz.de,

593 ii) the locus, location, of Keren and DIAP genes' isoforms found on VectorBase for Keren in

594 *Aedes albopictus* and DIAP in *Aedes albopictus* and *Aedes aegypti*

III.3.D.3. Perspectives

Our study unveils a critical mutualistic relationship between the endosymbiont *Wolbachia* and the Asian tiger mosquito, *Aedes albopictus*. *Wolbachia* appears to be essential for mosquito midgut health, regulating cellular processes like division and apoptosis while maintaining the structure of midgut cells and muscles. This highlights the dependency *Aedes albopictus* has developed on *Wolbachia*. Interestingly, DENV infection caused no significant changes in midgut physiology, suggesting *Wolbachia*'s protective role outweighs viral damage and ensures midgut homeostasis.

To gain a deeper understanding of the molecular mechanisms involved, RNA sequencing can be employed. This technique allows us to analyze the complete set of RNA molecules (transcriptome) within the midgut. Employing RNAseq can offer a deeper understanding of the molecular mechanisms implicated in midgut physiology, potentially manipulated by *Wolbachia*. Thus, we might be able to identify genes that might be responsible for the observed physiological effects and represent potential targets for manipulation.

Further research directions can also include the investigation of this *Wolbachia*-midgut relationship in other arbovirus vectors, both naturally infected and those amenable to transinfection by *Wolbachia*. Moreover, delving deeper into the *Wolbachia*-DENV interaction within the midgut to determine if the lack of cellular phenotype changes is due to *Wolbachia*'s protection, delayed DENV effects, or specific intestinal responses. Similar studies could be conducted with other arboviruses like Zika, Yellow Fever, and West Nile.

Our findings offer a strong foundation for developing a vital biological control strategy against arboviruses. By targeting mosquito fitness, longevity, and resistance to viral infection, we can explore novel approaches to curb the spread of DENV by the *Aedes albopictus* vector.

IV. General discussion and perspectives

Part IV. General discussion and perspectives

Given the emergence of arbovirus epidemics in human populations, investigating the factors influencing interactions between arboviruses and mosquito vectors is a significant challenge. Our study aims to explore the interaction between circulating dengue strains and the local *Aedes albopictus* vector.

After genetically characterizing the DENV-1 and tracing the origin of its emergence during the last epidemic, which caused a significant burden of incidence (objective 1, article 1), we pursued the second objective of this thesis consisting of characterizing, at the local interpopulation level of *Aedes albopictus*, the impact of extrinsic factors on the overall vector competence toward DENV-1 (objective 2, article 2). Finally, the third objective focused on exploring the effect of an intrinsic biological factor, the *Wolbachia* bacterium, on the maintenance of intestinal homeostasis. This interaction was studied at the intrapopulation level of a Reunionese *Aedes albopictus* laboratory population, under basal conditions and following dengue virus ingestion (article 3).

Our research has unveiled numerous novel discoveries. We have demonstrated that the recent outbreaks of DENV-1 in La Réunion Island and the Seychelles were initiated by the introduction of distinct DENV-1 genotypes originating from Asia. Additionally, the complete genome sequencing of DENV-1 identified fifteen non-synonymous mutations, warranting further investigation into their biological and functional significance.

In the subsequent phase, we revealed two key findings. First, we demonstrated that extrinsic parameters, including mosquito generations, days post-infection, and the geographical areas of mosquito location, significantly influence vector competence towards DENV-1. Secondly, we elucidated the significant impact of the intrinsic biological parameter, *Wolbachia*, on maintaining the intestinal homeostasis of *Aedes albopictus* following a blood meal. Moreover, we observed that this protective effect persists after DENV-1 infection, as in the absence of *Wolbachia*, the virus-induced modulation of the apoptotic rate of midgut cell epithelium. This modulation may contribute to the maintenance of viral replication in the *Aedes albopictus* midgut.

In the following section, we will delve into the three major concerns of the thesis, discussing further investigations and exploring new perspectives based on the results we have obtained.

IV.A. DENV-1 epidemic strain harbors genomic characteristics that may contribute to its virulence and transmission potential

In 2016, a significant epidemic was recorded in the Seychelles, then spreading to neighboring islands, including Madagascar and Mauritius, and locally impacting La Réunion (78–82). The emergence of this epidemic began in La Réunion at the end of 2017 when DENV-2 circulated, followed by DENV-1 in early 2019, contributing to an epidemic crisis that persisted until the end of 2022 (77,171). The link between emergences in both Réunion and Seychelles was suggested, as the DENV-2 circulating strains were clustered together in the Cosmopolitan sub-lineage 1, which contains sequences from India and China (172). Both DENV-2 genomes share 99.8% identity and showed 93% identity with the genomic sequence

of the DENV-2 strain from Thailand, suggesting an Asiatic origin in the emergence of DENV-2 in the Seychelles and La Réunion (79).

Considering the shared origin of the emerged DENV-2 strains in both islands, along with their close geographical proximity, and the frequent exchanges and interactions between residents and travelers, there also appears to be a likely connection between the recent DENV-1 outbreaks in both countries. Surprisingly, our initial study revealed a contrast: DENV-1 strains that emerged on each island in 2016 and earlier in 2019 belong to different genotypes. La Réunion strain belongs to genotype I, whereas the Seychelles strain belongs to genotype V, and both probably originated from different countries in Asia. This mirrors the spread of DENV-1 in 2003 and 2004 in La Réunion and Seychelles, and in the eastern of Madagascar two years later, when no regional links were revealed for those emergences (75,76).

Interestingly, the observation of fifteen non-synonymous amino acid mutations in both structural and non-structural proteins across all La Réunion DENV-1 full genome sequences suggests discrete micro-evolutionary processes that may either be neutral or contribute to positive or negative selection. In all cases, their prevalence will be influenced by natural selection pressures within the environment, leading to either their disappearance or their persistence in a minority (337,338). These findings offer insights into the heightened transmission of DENV-1 by the vector and its infectious virulence among Reunionese patients during the last epidemics, particularly as some mutations occur within proteins associated with DENV virulence. The fifteen non-synonymous amino acid mutations occurred in structural and non-structural proteins (NS1, NS2B, NS3, NS4B, and NS5). The NS1 protein serves as a marker for rapid DENV diagnosis, plays a pivotal role in efficient virus production, is responsible for DENV-1 virulence, and is linked to disease severity. It modulates host immunity and causes hemorrhage and vascular leakage. NS1 mutations impact dengue clinical detection and are associated with higher viral production, secretion, and greater disease severity (341–343). Mutations were also detected in NS2B and NS5, both of which play a role in NS3 protease activity, a therapeutic target against *Flaviviruses*. Moreover, NS3 and NS5 proteins play important roles in viral replication through interactions with viral or host proteins to regulate important pathways and enzymatic activities (344,345). It was demonstrated that mutations in NS2B can enhance ZIKA transmission potential and provide evasion from pre-existing DENV immunity (346). In addition, the link between NS5 mutations and DENV fatality was established, as they contributed to higher viral replication, and thermal stability (347). However, affecting NS3 protein activity through NS2B and NS5 mutations can negatively impact DENV selection. Studies have shown that affecting NS3 functionality by substitution mutations of alanine at ultra-conserved residues abolishes NS3 protease activity, resulting in reduced viral protein production and RNA replication (345,348).

These observations indicate that these mutations would affect the virus virulence and replication of the DENV-1 in the vector, the host, or both simultaneously. This possibly explains the increased virulence observed in patients infected during the last epidemic, as well as the high rate of transmission by the mosquitoes (147). Thus, it is crucial to further investigate the functionalities of these genomic mutations to have a clearer idea if these genetic variations can affect negatively or positively viral virulence. This could be achieved by utilizing viral infectious clones of the DENV-1 strains from La Réunion and the Seychelles, into which the

non-synonymous mutations can be engineered. The infectivity of the mutated strain could be assessed in several ways: i) *in vitro* using appropriate *Aedes albopictus* cell lines such as the Aag4 lineage, ii) *in vivo* within the vectors by studying the infectivity of the new viral strains in the mosquito midgut, and iii) transmission within the mosquito's body and dissemination through the salivary glands.

IV.B. Extrinsic factors of different regions In La Réunion Island as potential contributors in shaping the vector competence of *Aedes albopictus*.

We conducted a study to explore the Vector Competence (VC) of different populations of *Aedes albopictus* towards the DENV-1 serotype together with the DENV-2 serotype. VC is a key factor in determining the capacity of mosquitoes to transmit DENV and it is affected by numerous extrinsic factors. Here, we focused on three majors of them: geography zone of collection, mosquito generation, and days post-exposure (dpe) after the infected blood meal. Furthermore, given that the viral titers of the two DENV-2 strains were low and *Aedes aegypti* has been included mainly as a control, in the following paragraphs we restricted our discussion on VC towards the DENV-1 strain, which presented viral titer suitable for such study and was, fortunately, also used in all the work done in the thesis.

We found that *Aedes albopictus* species exhibit a higher transmission efficiency for DENV-1, as expected. Moreover, our study, conducted from 2020 to 2021, overlapped with the peak of DENV-1 circulation and the decline in DENV-2 cases (350). These observations align with what was observed previously in different countries where DENV-1 circulated in areas dominated by *Aedes albopictus* (351,353,354).

When investigating the influence of selected extrinsic factors, differences were observed. These factors may provide insights into the evolutionary processes shaping vector permissiveness to viral infection and virulence.

The impact of dpe on VC parameters is significant, with higher values observed as more dpe passes and as mosquitoes age. This correlation is attributed to the dynamics of viral infection within the mosquito's body and the duration of the EIP. Furthermore, the aging process of mosquitoes can influence their immune response to viral infections (355), potentially enhancing vector competence and susceptibility.

Consistently, few studies show that at both small and large scales, *Aedes albopictus* exhibits different levels of VC and is responsible for transmitting specific arboviruses within particular regions (356,357), also we showed that the geographic areas within La Réunion island have an impact on the VC. Despite the presence of *Aedes aegypti* in La Réunion in certain isolated areas, *Aedes albopictus* is considered the primary vector, responsible for the epidemic of DENV as well as other arboviruses such as CHIKV (77). These observations highlight the influence of regional factors such as climate, temperature, humidity, host blood source, etc. on the species VC for specific arboviruses. A notable impact of the collection area was observed on TE of *Aedes albopictus* mosquitoes, where the number of infected individuals per generation or DPE varied depending on the collection area. Previous studies show that this effect was consistent across *Aedes albopictus* mosquito populations from various regions within a territory such as China and temperate regions in Europe (352,358), as well as *Aedes*

aegypti populations tested from specific regions (197). The demographic history of *Aedes albopictus* populations reflects rapid and intricate patterns of historical lineage diversification and divergence, which have an impact on the populations' genetics and evolution (359). The genetic differences between populations affect their physiology and consequently, their competence (191,196,197). Thus, this competence may vary depending on species migration and colonization in regions with specific environmental conditions.

Furthermore, a significant difference was detected between F0, F1, and F2 generations (F2 generations were more adapted to laboratory conditions) for IR, DE, and TE. F2 *Aedes albopictus* mosquitoes that are more adapted to laboratory conditions have higher vector competence. This suggests the probable implication of other ecological and natural factors in modulating the VC. Numerous distinctions between laboratory and field populations, such as genetic variability, midgut microbiota, and host blood source, could account for the discrepancies observed in VC outcomes (208,360,361). Moreover, in laboratory settings, modifications in traits due to adaptation can occur as a result of inbreeding and genetic drift. Laboratory environments often lack diversity and complexity, which may lead to reduced selective pressures associated with fluctuating conditions, thereby diminishing the selection for stress tolerance, fitness, and competence in the natural environment (362).

Given the wide range of methodologies utilized in laboratories for VC analysis, it is important to consider the preparation of the viral titer utilized for experimental bloodmeal, and the recognition of the importance of research on natural populations compared to laboratory populations. Thus, for future investigations, it would be interesting to compare the replicative fitness of DENV-1 and DENV-2 strains from La Réunion in *Aedes albopictus* field populations using similar viral titers for both DENV serotypes. Additionally, it is essential to note that findings derived from studies conducted on laboratory or field populations cannot be universally applied or validated across other populations. This limitation arises from the potential divergence observed within the same species reared in different laboratories, as well as the high selection, genetic, and physiological alterations occurring over successive generations in natural populations (360).

In addition, the adaptation of the virus and vector within a region, and the adaptation of mosquitoes to laboratory conditions, would affect the genetics of both the vector and the pathogen. Thus, it is crucial to conduct a comprehensive genetic analysis of mosquito determinants of VC to ascertain the independence of these populations and explore the actual molecular mechanisms controlling VC development. Several factors can be explored and explained by the genetic analysis. This includes variations in genetic diversity between laboratory and natural populations, the influence of specific environmental factors like climate change and temperature (210,363), and the role of vertebrate hosts and blood sources within specific geographic regions (208). These factors collectively influence the molecular regulation of several genes involved in the VC of the host and the infectivity of the virus (364).

Besides, it is suggested that the MIB and MEB in mosquitoes are quantitative traits influenced by multiple genes that likely regulate VC and determine infection and transmission rates within populations. Strong MIBs and MEBs reduce the potential for transmission (115,365). Thus, it would be interesting to genetically analyze the effect of extrinsic factors on

the MIB and MEB efficiencies in limiting viral infection and dissemination in vectors. Also, the difference in midgut microbiota diversity between laboratory and field populations can be analyzed through metagenomics, to explore on the molecular level, its implication in the VC modulating within specific environments. This approach will open up several perspectives regarding the interactions between extrinsic and intrinsic factors and their effect on VC towards DENV or other arboviruses.

We discovered that at the inter-population level of *Aedes albopictus* populations, *Wolbachia* density varies among populations within specific regions (article 2). Despite the studies demonstrating the role of *Wolbachia* in manipulating the VC (203,210,352), in Reunionese populations, *Wolbachia* does not seem to affect viral infectivity or any of the VC parameters. Consistent with our findings, *Wolbachia* does not significantly impact DENV replication nor vector susceptibility in the *Aedes albopictus* population in Malaysia (354). The variability in these observations, emphasizes the importance of dual transcriptomics and metagenomics in analyzing on the molecular level how environmental conditions across different regions affect the genetic expression of *Wolbachia* and its potential effect on VC.

Our second study contributed to a more comprehensive analysis of the impact of various local extrinsic ecological factors and an intrinsic factor simultaneously on the VC of *Aedes albopictus* in Réunion Island. Through the differentiation of VC in different regions, we aim to develop more efficient methods for anticipating future outbreaks. This will enhance our ability to prevent the circulation of new epidemics in regions conducive to vector population growth.

IV.C. *Wolbachia* influences the *Aedes albopictus* intestinal homeostasis

Although the impact of *Wolbachia* on *Aedes albopictus* VC was not significant in our second study, this bacterium is known to manipulate the defense system of the vector against pathogens through several biological mechanisms (202,210,324–326,332). However, there is a lack of understanding regarding its role in the vector midgut, which serves as the major entry point of pathogens. Therefore, in our third study, we investigated the potential of *Wolbachia* to sustain the intestinal homeostasis of *Aedes albopictus* under basal conditions, – during normal blood digestion –, and following DENV ingestion via blood feeding.

Based on our findings, after a standard blood meal, removing *Wolbachia* from *Aedes albopictus* disrupts intestinal muscular structure and intestinal homeostasis by altering cells' nuclear phenotypes, leading to epithelial cell apoptosis. To compensate, intestinal stem cell division is triggered. We sought to investigate whether this effect occurs during normal blood digestion or also after DENV ingestion via blood meal. In the presence of *Wolbachia*, DENV did not modulate epithelial apoptosis; instead, apoptotic and dividing cell rates remained stable. This was demonstrated on the molecular level in *Aedes malayensis* when DENV affected the regulation of only one apoptotic gene at 1 dpi (254). Conversely, without *Wolbachia*, DENV slightly decreased cellular apoptosis, possibly to support viral replication in intestinal cells, as seen in previous studies (268,273). Refractory mosquito strains with higher apoptosis rates limit DENV proliferation (271,272). Our findings highlight the effect of *Wolbachia* on intestinal homeostasis through ISC division, cellular apoptosis, and changes in nuclear and muscular structure. Moreover, the fundamental reliance and evolution organisms have developed on

the presence of *Wolbachia* and the important role that this bacterium plays in the biology and physiology of the *Aedes albopictus* was observed.

To enhance understanding, *Aedes aegypti* used to be infected by *Wolbachia* (366), yet throughout its evolution, the presence of this bacterium is no longer naturally observed in this species. Therefore, investigating intestinal homeostasis regulation and epithelial integrity in this species is crucial. Such analysis allows for comparison to determine whether this effect is specifically and solely governed by *Wolbachia* in *Aedes albopictus*.

Despite the importance of understanding gene expression dynamics and their regulation in the mosquitoes' midgut under normal conditions or following viral infections, only a few studies have delved into the analysis of these processes at the molecular level. Previous studies have investigated the maintenance of *Aedes aegypti* midgut integrity post-blood meal by analyzing the overall transcriptome and proteome. After a normal blood meal in *Aedes aegypti*, they identified genes related to nutrient uptake, metabolism, cellular stress responses, innate immunity, and peritrophic matrix formation (367). Additionally, single-nucleus RNA sequencing studies explored individual midgut and function, revealing changes in ISC/EB proportion and ISC differentiation into EB and ECs (224). Other studies focused on midgut transcriptomic responses to DENV infection in *Aedes* mosquitoes, with upregulated or downregulated genes primarily associated with vector immune response, expressed at various time points post-blood feeding in each study (252–254). Therefore, our study raises the question remains whether *Wolbachia* also affects the overall midgut transcriptome in *Aedes albopictus* following a normal and DENV-infected blood meal.

The transcriptomic analysis of *Wolbachia* and the host elucidated the involvement of various molecular genes and mechanisms manipulated by *Wolbachia*. These may include epithelium regeneration processes directly affecting intestinal homeostasis, as well as immune responses and metabolic and digestion mechanisms that might indirectly impact intestinal homeostasis. Using RNAi or CRISPR/Cas9 (373, 374), we can validate the implication of these mechanisms by inhibiting the expression of candidate genes, thereby visualizing their direct or indirect implication in epithelium integrity and maintenance.

Another important question to explore is: how does *Wolbachia* influence the cellular composition of the intestinal epithelium during blood digestion and after DENV ingestion? This analysis can be carried out by labeling specific intestinal epithelial cells (such as ECs, EEs, ISCs, and EBs) with fluorescent markers and observing changes in the overall cellular composition of the intestine after both normal and DENV-infected blood meals, with and without *Wolbachia*. In *Drosophila*, bacterial stress alters ISC division and differentiation, resulting in a shift in epithelial composition marked by an increase number of EEs and ECs (236,255). Given the similarities between *Aedes* and *Drosophila* in morphology, physiology and midgut cell types (224), insights from *Drosophila* observations may shed light on potential changes in *Aedes albopictus* midgut cellular composition during homeostasis perturbations. Such investigations would reveal *Wolbachia's* ability, at the cellular and molecular levels, to modulate midgut epithelial cell fate during regeneration under normal conditions or following stress induced by DENV infection. This understanding is crucial for effectively maintaining intestinal integrity and homeostasis.

As demonstrated in previous studies, *Wolbachia* can alter the bacterial composition of the midgut microbiota in both *Aedes aegypti* and *Drosophila* (306,331). Hence, is the observed effect of *Wolbachia* on the midgut linked to changes in overall microbiota composition? To respond to this, two research approaches could be undertaken. Firstly, analyzing the bacterial metagenome of both *Wolbachia*-free and *Wolbachia*-infected populations to compare the impact of *Wolbachia* elimination on intestinal microbiota composition would clarify whether *Wolbachia* directly triggers the observed effect or indirectly affects the microbiota composition. Additionally, exploring intestinal homeostasis after reintroducing *Wolbachia* into *Wolbachia*-free *Aedes albopictus* females and stabilizing the strain for several generations would allow a comparison to determine if the presence of *Wolbachia* directly influences intestinal homeostasis.

To generalize observations from our study, several experiments could be conducted. Firstly, previous studies have shown that chemical and bacterial stresses induce similar ISC division responses in *Aedes albopictus* midguts (219,232). Hence, inducing such stress in populations with and without *Wolbachia* could reveal if the response is specific to viral stress or a common reaction to various damages. Second, given the global transmission of the four DENV serotypes by *Aedes albopictus* (189,351,352,354,368), studying the effect of *Wolbachia* on intestinal homeostasis following infection with each serotype would elucidate different interactions between *Wolbachia* and serotype, even why not the genotypes. Third, considering that rearing mosquitoes in laboratory settings for multiple generations affects microbiota variability (369,370), studying natural mosquito populations (F0) from various regions would provide deeper insights into arbovirus dynamics in natural conditions across multiple mosquito vector populations (185,289,300,371,372). This emphasizes the importance of investigating mosquito microbiota, particularly midgut bacteria, in modulating vector competence.

Our study lays the groundwork for future inquiries into the maintenance of intestinal homeostasis, epithelial integrity, and stem cell regeneration within the midgut of the *Aedes* mosquitoes. Furthermore, our research helps fill knowledge gaps regarding the endosymbiotic relationship between *Wolbachia* and one of the most significant arbovirus vectors, *Aedes albopictus*. Additionally, our findings will assist in developing innovative biological control methods to limit arbovirus invasion of the vector's midgut and subsequent transmission to humans.

Part V. References

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