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SURVEY AND ENTOMOLOGICAL CHARACTERIZATION OF MOSQUITOES AS POTENTIAL VECTORS OF ARBOVIRUSES, IN MOZAMBIQUE

ANA PAULA ABÍLIO, MPhil

MAPUTO, October 2021

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I declare that this thesis was never presented to obtain any degree or in any other field and it is the result of my individual work. This thesis is presented in partial compliance with the requirements of the Eduardo Mondlane University for Doctoral degree (PhD).

Signed: ..... (Candidate)

Date: 17/October/2021

DEDICATION

To my family the pillars of my life

Special to my greatest father, Abílio V. João

Who left this world at the onset of this Doctoral project

I know you are always there for me.

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ABSTRACT

Mosquito-borne diseases such as arboviruses represent expanding threats to sub-Saharan Africa, imposing a considerable burden on human and veterinary public health. Mozambique is located in a region suitable for arboviruses outbreaks. Increasingly available evidence suggests that the country is endemic to various debilitating and life-threatening arboviral diseases such as dengue (DEN), Rift Valley fever (RVF), chikungunya (CHIK) and others. Thus, the goal of this thesis is to describe the occurrence and distribution of mosquito arboviruses vectors in Mozambique and detect in them the presence of arbovirus. The thesis includes a total of three (I-III) studies that culminated with four (I-IV) manuscripts. **Study I** (Papers I and II) aimed at determining the occurrence and distribution of immature mosquitoes with the potential for transmitting arboviruses. Between March and April 2016, a cross-sectional study was conducted in 32 districts to determine the distribution and breeding sites of *Ae. aegypti and Ae. albopictus. Aedes aegypti* was found in every sampled district, while *Ae. albopictus* was only found in Moatize district (Tete Province). This study detected the occurrence of Ae. luteocephalus for the first time in the country, in the Lago district (Niassa Province). The highest Container Index (CI) of *Ae. aegypti* was found in used tires (35.3%), cement tanks (32.3%) and drums (22.1%). These results show that the risk of arboviruses transmission is likely to have been underestimated, highlighting the need to establish a solid national entomological surveillance program for *Aedes spp*. in Mozambique. **Study II** (Paper III) was mainly to determine the abundance, composition and main drivers with the influence of the dynamics of mosquitoes associated with the transmission of arboviruses in Mopeia (Zambézia Province) and Goba (Maputo Province) districts. Longitudinal surveys were conducted from 2014 to 2015. Mosquitoes were sampled overnight, once a month, using CDC light traps and Tent/Net traps, both bated with CO2. Sporadic collections were also performed in Maputo and Massingir districts. The mosquito population dynamics between sites and climate factors influencing it were investigated. A total of 33,621 mosquitoes were collected, in districts of Mopeia (86.6%) and Goba (12.2%), where a total of 37 and 31 mosquito species were found, respectively. The remaining 1.2% specimens were collected from complementary surveys carried out in Maputo and Massingir districts. The results indicated high diversity of vector species in Goba and Mopeia sites. There was significant variability of abundance and composition between sites season, and a significant association with rainfall and high average monthly air temperature. These findings underscore the need for further investigation on factors contributing to the establishment and abundance of mosquito vectors and arboviruses transmission in the studied sites. **Study III** (Paper IV) aimed at describing the presence of arbovirus groups in mosquitoes from Mozambique. Overall mosquito collection processes are described in study II. The viral screening was performed by targeting the detection of *Alphaviruses, Flaviviruses, and Bunyavirales*. The results revealed genetically distinct insect-specific flaviviruses detected in multiple species of mosquitoes from different genera, three lineages of putative members of the *Phenuiviridae* family, two of which correspond to the novel viral genetic lineages. Despite that pathogenic arboviruses have not been found in the collected mosquitoes, this work still represents an important contribution to inform the establishment of a vector control program for arbovirus in the country. The evidence presented in this thesis may guide the implementation of an integrated mosquito-borne diseases control program in Mozambique.

Keywords: Occurrence, Distribution, Seasonality, Mosquito, Vectors, Arbovirus, Flaviviruses, Bunyaviruses, Mozambique

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ABSTRACT IN PORTUGUESE

Doenças transmitidas por vectores envolvendo arbovírus representam ameaças em expansão na África Subsaariana, impondo um fardo considerável para saúde pública humana e veterinária. Moçambique está localizado numa região propícia a ocorrência de vários surtos de arboviroses. As evidências mostram que o país é endémico para várias doenças debilitantes e fatais, como a dengue (DEN), febre do Vale do Rift (RVF), chikungunya (CHIK) e outras doenças arbovirais. Esta tese teve como objectivo descrever a ocorrência e distribuição de mosquitos vectores de arbovírus em Moçambique e detectar neles a circulação de arbovírus. A mesma abrange um total de três (I-III) estudos que culminaram em quarto (I-IV) manuscritos. **O estudo I (Manuscritos I e II)** teve como objectivo determinar a ocorrência e distribuição de mosquitos com potencial na transmissão de arbovírus. Nesta pesquisa, foi realizado um estudo com intuito de determinar a distribuição e os principais criadouros de *Ae. aegypti e Ae. albopictus* em 32 distritos nos meses de Março e Abril de 2016. *Aedes aegypti* foi encontrado em todos os distritos estudados, enquanto que Ae. albopictus foi encontrado no distrito de Moatize (província de Tete). Este estudo detectou pela primeira vez a ocorrência de *Ae. luteocephalus*, uma nova espécie na fauna de Moçambique, no distrito do Lago (província de Niassa). O maior índice de recipiente (CI) de *Ae. aegypti* foi encontrado em pneus usados (35,3%), tanques de cimento (32,3%) e tambores (22,1%). Estes resultados mostram que o risco de transmissão de arbovírus está a ser subestimado, o que pode justificar a necessidade urgente de estabelecimento de um programa nacional de vigilância entomológica para *Aedes spp.* em Moçambique. **O estudo II (Manuscrito III)** teve como objectivo determinar a abundância, composição e os principais factores que influenciam a dinâmica dos mosquitos associados à transmissão de arbovírus nos distritos de Mopeia (província da Zambézia) e Goba (província de Maputo). Foi aplicado uma amostragem longitudinal de 2014 a 2015, fazendo capturas de mosquitos mensalmente, durante a noite com recurso a armadilhas luminosas de CDC e armadilhas de Rede/Tenda, ambas com CO2 como isco. As amostragens esporádicas também foram realizadas nas províncias de Maputo e Massingir. A dinâmica populacional entre os locais e os factores climáticos foram estudados. Num total de 33.621 mosquitos colectados, 86,6% eram de Mopeia e 12,2% de Goba, dos quais 37 e 31 espécies de mosquitos foram identificados em Mopeia e Goba respectivamente. O remanescente 1,2% pertencem a amostragens complementares de Maputo e Massingir. Estes resultados indicam uma alta diversidade de espécies de vectores de Mopeia e Goba. Verificou-se também uma variabilidade significativa para abundância e composição entre os locais estudados nas diferentes estações do ano. Igualmente, constatou-se uma significativa associação entre abundância de mosquitos, a precipitação e altas temperaturas. Este achado reforça a necessidade de se realizar mais pesquisas sobre factores que contribuem para o estabelecimento de espécies de vectores com importância para transmissão de arbovírus nos locais estudados. O estudo **III (Manuscrito IV)** descreve a presença de grupos de arbovírus em mosquitos de Moçambique. Todo o processo de amostragem de mosquitos foi descrito no estudo II. O rastreio de vírus foi realizado com objectivo de identificar a presença de vírus dos grupos *Alphavirus, Flavivirus e Bunyavirales.* Os resultados revelaram a presença de flavivírus específicos de insectos em várias espécies de mosquitos de diferentes géneros e três linhagens supostas de pertencerem a membros da família *Phenuiviridae*, das quais duas correspondiam a vírus de novas linhagens genéticas. Uma vez que este estudo não detectou vírus patogénicos em mosquitos analisados, a revelação de novos ISV ainda representa uma contribuição importante para a tomada de decisão informada sobre a necessidade de estabelecimento de um programa de controlo de vectores de arbovírus no país. As evidências apresentadas nesta tese podem orientar a implantação de um programa de controlo integrado de doenças transmitidas por mosquitos em Moçambique.

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LIST OF PAPERS

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Nº	Paper	Impact factor
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III.	**Abílio, A. P.**, Kampango A., das Neves, L., Parreira, R., Fafetine, M. F., Sidat, M., & de Almeida, A. P. G., Abundance and diversity of mosquito communities potentially associated with arbovirus transmission in two districts of Mozambique. *Submitted*.	Under review
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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CB-UEM	Centro de Biotecnologia-Universidade Eduardo Mondlane
CDC	Centre for Disease Control and Prevention
CI	Container Index
cISF	Classical Insect Specific Flavivirus
CFAV	Cell-Fusion Agent virus
CHIKV	Chikungunya virus
COI	Cytochrome oxidase gene subunit I
CPE	Cytopathic Effect
cRNA	Complementary Ribonucleic acid
DEN	Dengue
DENV	Dengue virus
DNA	Deoxyribonucleic acid
IHMT	Instituto de Higiene e Medicina Tropical
INS	Instituto Nacional de Saúde
IRS	Indoor Residual Spraying
ISV	Insect Specific virus
ITN	Insecticide Treated Net
manyGLM	Multivariate Abundance Linear Models
MCC	Maximum Clade Credibility
MCMC	Markov chain Monte-Carlo
MEB	Midgut Escape Barrier
MIB	Midgut Infection Barrier
mt*Cox*1	Mitochondrial cytochrome oxidase gene subunit I
mtDNA	Mitochondrial Deoxyribonucleic acid
mRNA	Messenger Ribonucleic acid
NACIDS	National Africa Centre for Infection Disease Surveillance
NICD	National Institute for Communicable Disease
nt	Nucleotide
PCR	Polymerase chain reaction
PNCM	Programa Nacional de Controlo da Malária
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase chain reaction
RVF	Rift Valley Fever
RVFV	Rift Valley Fever virus
SACIDS	Southern Africa Centre for Infection Disease Surveillance
UEM	Universidade Eduardo Mondlane
VBD	Vector-Born Disease
WHO	World Health Organization

GLOSSARY OF TERMS

Arboviruses	Are all viruses that are maintained in nature through biological transmission between susceptible vertebrate hosts and blood feeding arthropods such as mosquitoes, sand flies, ceratopogonids and ticks.
Context	Part of something considered together with the surrounding understandable words or idea.
Medical Entomology	Or public health entomology is the branch of entomology concerned to the studies of insects and other arthropod-related problems affecting humans and the public health in general.
One-Health	Known as a combined approach working at the local, regional, national, and global levels to improve public health outcomes recognizing the interconnection between people, animals, plants, and their shared environment.
Primers	Primer is a small sequence of nucleic acid with the role of starting the DNA synthesis. They can be found in living organism as short strands of RNA.

CHAPTER 1

CONTEXTUALIZATION

1. Introduction

Vector-borne diseases (VBDs) - involving arboviral agents including *Phlebovirus, Flavivirus* and *Alphavirus* - represent emerging and expanding threats within the Africa sub-Saharan region and account for 17% of all the burden of infectious disease worldwide (Campbell-Lendrum et al., 2015; Araujo et al., 2017). More than one billion people are infected, and more than one million die from VBDs, every year, despite increased funding for the control and eradication of those emerging diseases. It has been estimated that more than half of the world's population is at risk of VBDs transmission, particularly those from low-income countries (WHO 2014).

Rift Valley fever virus (RVFV) is one of *Phlebovirus* that cause severe zoonotic viral VBD that primarily affects ruminants, but it can also infect humans, causing life-threat disease to both animals and humans (Peters 1997; Faye et al., 2007; Bird et al., 2009; Campbell-Lendrum *et al.*, 2015). Other major groups of arbovirus, namely *Flavivirus* and *Alphavirus*, also comprise a group of rapidly expanding VBDs that affects preferentially humans (Campbell-Lendrum *et al.*, 2015). It has been estimated that between 390-500 million people around the world are infected by dengue virus (DENV) (one of the most common *Flavivirus*) each year (Bhatt et al., 2013; Araujo et al., 2017). On the other hand, the prevalence of chikungunya virus (CHIKV) (a virus of the genus *Alphavirus*) has experienced an alarming and unprecedented increase in the last decades, causing enormous and severe outbreaks in several countries, arguably due to the urbanization and poor sanitation, (Pierre *et al.*, 2006; Wahid *et al*., 2017). Indeed, long-term changes in globalization, urbanization and climate change are the main drivers for the rapid progression and frequency of recent and ongoing arbovirus outbreaks worldwide that occur on a scale (geographic, economic and human) and that is without precedent in human history (Gould *et al*., 2017; Mayer *et al.*, 2017; Powers & Waterman 2017).

Mozambique has also experienced dengue outbreaks, and existing laboratory evidence indicates that natural transmission of Rift Valley fever (RVF), dengue (DEN) and chikungunya (CHIK) may have also been occurring (Fafetine et al., 2013b; Gudo *et al*., 2015b; Gudo *et al*., 2016d; Mugabe *et al*., 2018). Additionally, South Africa, a neighbouring country of Mozambique, has also been disclosing several arbovirus such *Alphavirus*, namely Sindbis, Middelburg and Ndumu virus, *Orthobunyavirus*, namely Shuni virus, *Flavivirus* such as West Nile virus (WNV) and the *Phenuivirus* RVFV, the arbovirus with importance to human and animal health (Venter 2018; Guarido *et al*., 2021; Motlou & Venter 2021). The circulation of those numerous arboviruses in the southern African region poses a threat to a public health concern.

This project aims at updated and accurate information on the current knowledge of occurrence, distribution and dynamics of mosquito populations with potential to transmit arboviruses of public health relevance in Mozambique. The research also explores the presence of different groups of arboviruses and has potential to work as a bridge of translational transference of technology of virus detection and isolation among field and laboratory scientists based on public and private institutes dedicated to research in VBDs. This information provides site-based evidence to guide the implementation of programs for integrated and effective control and prevention of arbovirus transmission in a One-Health Approach in Mozambique.

2. Literature review

2.1. Arboviruses: taxonomic classification and transmission cycle

The concept that mosquitoes can transmit filariasis worms was the first discovery of an arthropod vector role in human pathogens (Manson 1878). In 1881, Carlos Finlay postulated that mosquitoes could transmit the yellow fever virus, which was confirmed in 1900 by Walter Reed (Manson-Bahr & Bell 1987). The term arbovirus firstly appeared in the 1940's, referring to an animal virus transmitted to a vertebrate host by a haematophagous insect. Later, the concept and term "arthropod-borne" virus transmission was first introduced in 1942 (Hammon & Reeves 1945). Consequently, coined by WHO to refer to any virus transmitted by arthropods (arthropods-borne viruses). Arthropod-borne viruses, i.e. arboviruses, "are viruses that are maintained in nature through biological transmission between susceptible vertebrate hosts and blood-feeding arthropods (mosquitoes, sandflies, ceratopogonid, and ticks)" (WHO 1967; Kuno & Chang 2005). From there on, the term arthropod-borne virus, or arbovirus, evolved (Huang *et al*., 2019).

The group of arboviruses, comprising those of medical and veterinary importance, are taxonomically highly diverse, most of them being implicated in causing severe diseases to humans, various domestic animals and wildlife (Tab. 1). In general, the major group of arboviruses belongs to at least eight viral families/orders. However, those of importance in terms of public health have been grouped into three viral families/orders, namely: *Bunyavirales*, *Flaviviridae* and *Togaviridae* (Pabbaraju *et al*., 2009; Campbell-Lendrum et al., 2015; Mayer *et al*., 2017) (Tab. 1).

Arboviruses are mainly transmitted via biological processes that can be divided as follows: vertical and horizontal transmission. However, for all types of biological transmission to occur, the virus must firstly replicate into an arthropod vector before being passed onto another susceptible animal or human host. Vertical (trans-ovarial or trans-stadial) transmission occurs when an actively replicating virus manages to trespass the ovary barrier of an infected female insect and infects the eggs during the maturation process or when replicating viruses pass from one immature life stage to the next life stage -immature or adult. However, horizontal transmission is considered the most common, and the one most responsible for the epidemiological pattern of transmission worldwide. This transmission type occurs mainly when an infected haematophagous insect acquires an infected blood meal, then the viruses disseminate within the arthropods, replicate in their tissues and reach the salivary glands. The virus is passed to a susceptible host or amplification host through injection of saliva-laden while the insect is having a blood meal (Goddard 2008). There have also been studies reporting an horizontal transmission via matting or sexual intercourse or via oral by co-feeding (Kuno & Chang 2005; Weaver & Reisen 2010).

Table 1. Example of important medical and veterinary arboviruses belonging to Order/families *Flaviviridae*, *Togaviridae* and *Bunyavirales*. Adapted from (Gubler 2002; Mayer *et al.* 2017)

Order/Family	Virus	Human disease	Vertebrate host	Arthropod vector	Geographic distribution
Flaviviridae/ Flavivirus	Dengue virus 1-4 (DENV)	Dengue hemorrhagic fever/shock syndrome	Primates, Human	Mosquitoes: *Aedes spp*	Africa, Americas, Asia, Europe, Oceania
	Yellow fever virus (YFV)	Yellow fever-hemorrhagic fever	Primates, Humans	Mosquitoes: *Aedes* and *Haemogogus spp*	Africa, Americas
	Japanese encephalitis virus (JEV)	Encephalitis	Birds, Pigs	Mosquitoes: *Culex spp*	Asia
	Saint Louis encephalitis virus (SLEV)	Encephalitis	Birds, Bats, Other Mammals	Mosquitoes: *Culex spp*	Americas
	West Nile virus (WNV)	FAR syndrome, encephalitis	Birds, Horses, Other Mammals	Mosquitoes: *Culex spp*	Africa, Asia, Europe, Oceania, Americas
	Murray Valley encephalitis virus (MVEV)	Encephalitis	Birds	Mosquitoes: *Culex spp*	Oceania
	Zika virus	Fever	Primates	Mosquitoes: *Aedes spp*	Africa, Asia, Europe, Oceania, Central and South America
	Tick-born encephalitis virus (TBEV)	Encephalitis	Rodents, Goats, Sheep, Cows, Other Mammals, Birds?	Ticks: *Ixodes spp*	Europe, Asia
Togaviridae/ Alphavirus	Chikungunya virus	FAR syndrome	Primates, Humans, Birds, Cattle, Rodents	Mosquitoes: *Aedes* and *Culex spp*	Africa, Asia, Europe
	Ross River virus	FAR syndrome	Marsupials, Other Mammals, Birds?	Mosquitoes: *Aedes* and *Culex spp*	Oceania and Asia
	Sindbis virus	Fever/Rash	Birds	Mosquitoes: *Aedes*, *Culex*, and *Culiseta spp*	Europe, Africa, Asia, Oceania, Asia
	O'Nyong nyong virus	Fever	Unknown	Mosquitoes: *Anopheles spp*	Africa
	Equine encephalitis virus (EEV,WEV)	Encephalitis	Birds, Horses, Other Mammals	Mosquitoes: *Culiseta*, *Aedes*, *Coquillettidia*, and *Culex spp*	Americas
Bunyavirales					
Peribunyaviridae Orthobunyavirus	La Cross virus	Encephalitis	Rodents	Mosquitoes: *Aedes spp*	North America
	Bunyamwera virus	Fever	Rodents	Mosquitoes	Global
	California encephalitis virus	Encephalitis	Mammals	Mosquitoes	North America
	Tanyna virus	Fever, respiratory disease, encephalitis	Mammals	Mosquitoes	Asia, Europe
	Shuni virus	Neurologic disease	Humans, Mammals	Mosquitoes: *Culex theileri*, and *Cullicoides midges*	Africa, Asia
Phenuiviridae Phlebovirus	Rift Valley fever virus	Fever/ hemorrhagic fever	Cows, Sheep, Camels, Goats, and Other Mammals	Mosquitoes: *Aedes*, *Ochlerotatus*, *Culex*, *Stegomyia*, *Anopheles*, *Neomelaniconion*, *Eretmapodites*, and *Others*	Africa, Asia
	Sand fly virus	Systemic febrile illness	Birds, Mammals	Sandflies: *Phlebotomous spp*	Europe, Africa, Asia
	FAR (Fever/arthralgia/rash)				

Not all infected arthropods are capable of transmitting these pathogens. Thus, for an active transmission cycle to occur, the vector must be competent and must be susceptible to infection by the pathogen permitting the replication and dissemination, and consequently becoming infective and able to transmit the pathogen through an infective bite during the next bloodmeal event (Kuno & Chang 2005; Goddard 2008). Henceforward, arboviruses transmission can merely happen when the three components are present: the virus, the vector and the vertebrate host (Fig. 1). In general, arbovirus transmission is seasonal, with it being limited by the distribution of the vector population, the density of animal reservoir and variation of microclimatic variables, which in turn influences vegetation patterns and other ecological parameters that determine the distributions of arthropod vectors and vertebrate hosts (Kraemer *et al.*, 2015).

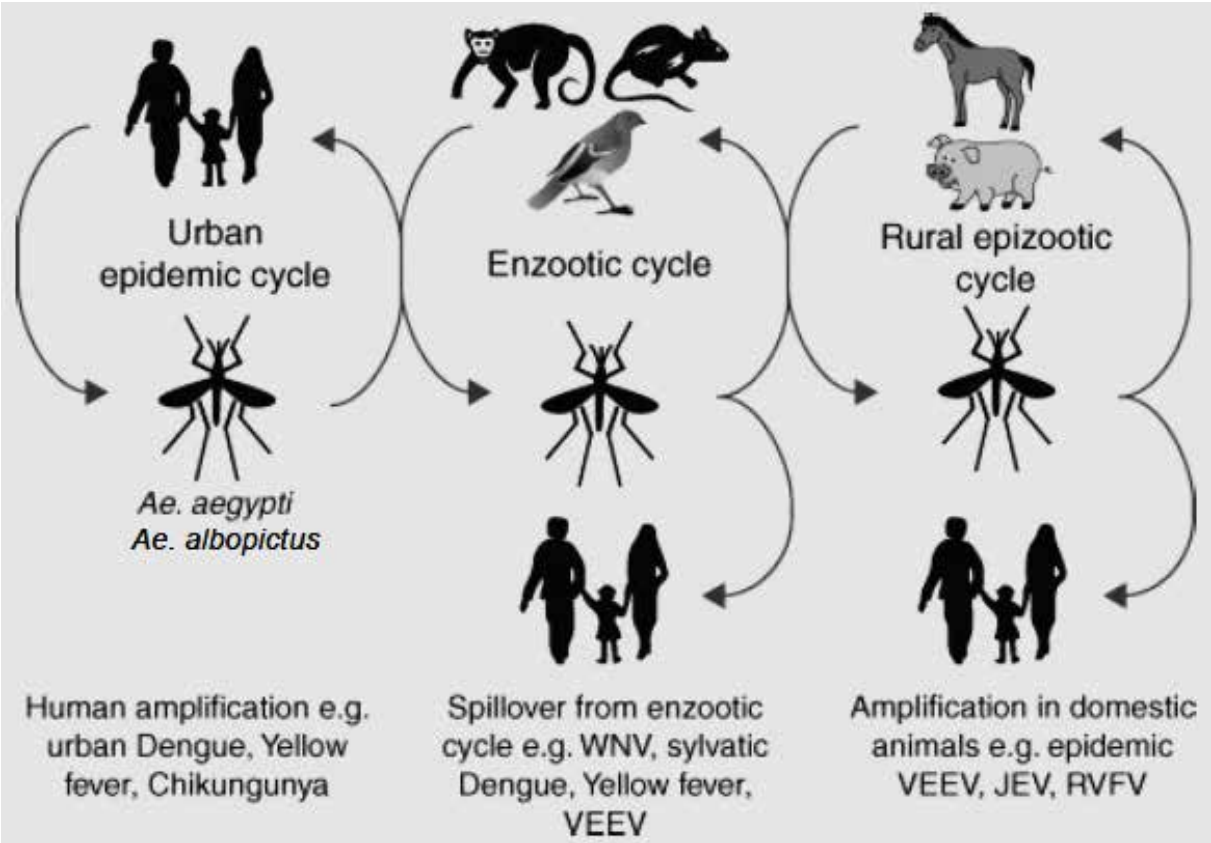

Figure 1. Maintenance cycles of mosquito-borne arboviruses in nature. DENV- dengue virus, YFV- yellow fever virus, CHIKV- chikungunya virus, WNV- West Nile virus, JEV - Japanese encephalitis virus. Adapted from (Hanley & Weaver 2008).

2.2. Medical and Veterinary Entomology

Entomology is a science dedicated to the study of insects and other arthropods, and all the aspects involved in their relationship with humans, animals, plants, the environment and other organisms (Idridge & Edman 2000; Mullen & Durden 2009). In analogy, Medical entomology, or public health entomology, is the branch of entomology concerned studying insects and other arthropod-related problems affecting humans and public health in general. Whilst, Veterinary entomology is a field of entomology dedicated to the study of insect and arthropod-related complications that affect domestic animals, mainly livestock and companion animals (such as dogs, cats, horses, caged birds, etc.). The scope of veterinary entomology also incorporates the studies of arthropod-borne diseases affecting captive animals in zoological parks and wildlife in general (Idridge & Edman 2000; Mullen & Durden 2009).

Medical and veterinary entomology also encompasses scientific research on arthropod disease vectors' behaviour, ecology and epidemiology. Currently, there has been widespread awareness that the control of any arthropod-borne disease, in the context of the One-Health approach, will only be possible if its epidemiology of entomological transmission has been deeply understood (Idridge & Edman 2000; Mullen & Durden 2009).

2.3. Mosquito Biology and life cycle

Mosquitoes are widely distributed throughout the world and are by far the most dominant vectors of arbovirus throughout their distribution, from tropical to temperate regions. More than 3,500 mosquito species are described worldwide (McGavin 2001).

Mosquitoes are holometabolous insects, which means they undergo complete metamorphosis during their life-cycle, passing through four distinct evolutive stages, viz: egg, larva, pupa and adult (Fig. 2). The entire cycle occurs in two different habitats, that is, aquatic the immature forms (egg, larva and pupa) and terrestrial/aerial habitats (the adults) (Eldridge 2005; Rutledge 2008; Becker et al., 2010). The mosquito eggs are mostly deposited in water surfaces or moist ground or surfaces, in groups or individually. The eggs' hatching can happen either within a day or when flooding happens. The larvae usually experience four moults, four larval stages, before becoming a pupa. Adult mosquito emergence frequently occurs 1-3 days after pupa formation (Eldridge 2005; Rutledge 2008). The entire life cycle can last for approximately 10 to 14 days in the tropics and temperate regions. Thus, food supply and temperature seem to be the two most influential factors that affect the speed of mosquito development during the aquatic stage (Eldridge 2005; Rutledge 2008) of the entire mosquito life cycles. The temperature rise within certain ranges of developmental threshold increases the rate of virtually most important processes governing mosquito life cycle, such as egg maturation and survival, larval growth, development and survivorship, larva to pupa development, pupation rates, adult growth, development and survivorship. Temperature above the threshold increases the growth, but, at the same time, the mortality rate is higher than at the ideal temperature (Hopkins 1952; Clements 1963; Clements 1992; Rutledge 2008; Rejmánková et al., 2013; Christiansen-Jucht *et al*., 2014).

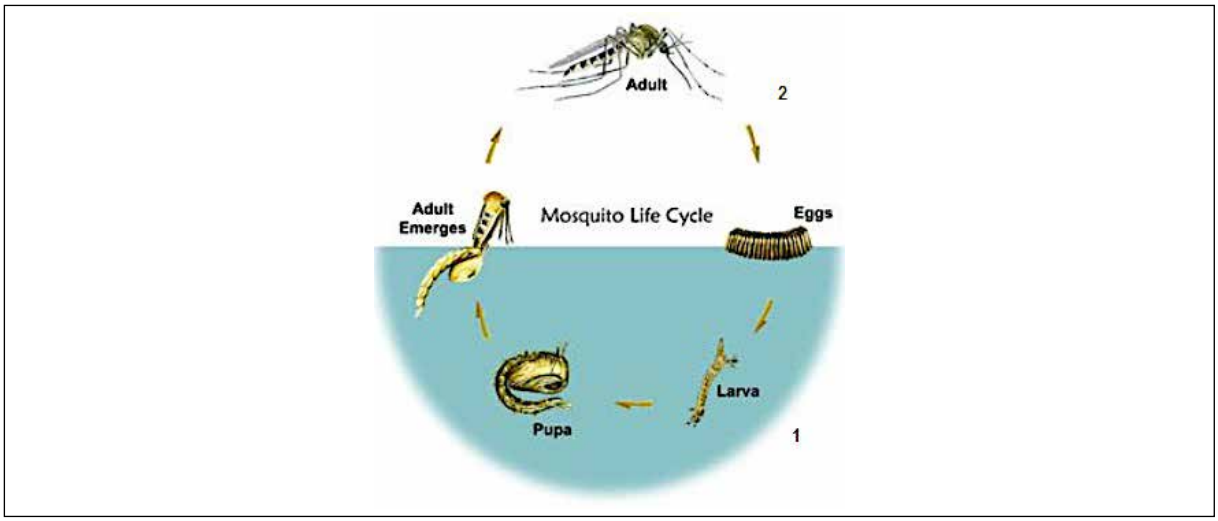

Figure 2. Infographic of mosquito (Diptera: Culicidae) life cycle showing both aquatic (1) and terrestrial stages (2). Adapted from (https://www.mosquito.org/page/lifecycle.

2.4. Taxonomy and classification of mosquito species vectors of arbovirus

Mosquitoes belong to the order Diptera, Sub-order *Nematocera* and the family *Culicidae* (Edwards 1941; Jupp 1996; Harbach 2015). The family *Culicidae* comprises vastly number of species, including

some of the most important haematophagous mosquito vectors of several pathogenic agents (Jupp 1996; Eiras 2004; Eldridge 2005). This family has been divided into three subfamilies: *Anophelinae, Culicinae and Toxorthynchinae*. The *subfamilies, Anophelinae and Culicinae* comprise over 3,500 mosquitoes species and subspecies, most of which are of medical and veterinary importance and capable of efficiently transmitting arboviruses, nematode worms and protozoa (Manson-Bahr & Bell 1987; Rutledge 2008).

2.4.1. Subfamily *Anophelinae*

The subfamily *Anophelinae* formally comprises 485 species, currently divided into three distinct genera: namely, Chagasia (restricted to Neotropical region), *Bironella* (restricted to Australasian region), and *Anopheles* (cosmopolitan) (Mattingly 1969; Harbach & Kitching 1998). The genus *Anopheles* has, in turn, been divided into seven subgenera, viz: *Anopheles* (cosmopolitan, 182 species), Baimaia (Oriental, only one species described), *Cellia* (Afrotropical, 220 species), *Kerteszia* (Neotropical, 12 species), *Lophopodomyia* (Neotropical, six species), *Nyssorhynchus* (Neotropical, 39 species) and *Stethomyia* (Neotropical, five species) (Harbach 2015). Four of the subgenera (*Anopheles, Cellia, Kerteszia and Nyssorhynchus*) comprise both species and complexes of sibling species that transmit human and simian malarial parasites, as well as the bancroftian filariasis and arbovirus (Krzywinski & Besansky 2003; Harbach 2013b). However, the genus Anopheles is by far the greatest important pathogen-carrying mosquito in the public health context due to its exclusive involvement in transmitting human malaria parasites (WHO 2012). As also a vector of causative agents of filariasis and some arboviruses (e.g. On`ongy ongy virus), it has been argued that the Anopheles genus affects the lives of humans more than any other insects (WHO 2012; Harbach 2013).

2.4.1.1. Genus *Anopheles* (Meigen, 1818)

There are 472 formally recognised *Anopheles* (An.) species (Knight & Stone 1977; Harbach 2015), 70 of these have been considered vectors of public health importance (De Meillon 1947, 1951; Hay et al., 2010), whilst 40 from this were identified as vector of primary of secondary importance in the Southern Africa region (Gillies & De Meillon 1968). Some *Anopheles* species have also been incriminated as vectors of arbovirus. The vector of RVFV comprises *An. (Ano) coustani, An. (Ce) squamous, An. (Ce) gambiae, An. (Ce) arabiensis and An (Ce) pharoensis* (Linthicum et al., 1985; Seufi & Galal 2010; Ratovonjato *et al*., 2011). O´nyong´nyong virus and Ilesha virus can be transmitted by *An. gambiae and An. funestus* (Williams *et al*., 1965; Service 1990). Each vector species or complex has its own geographical and ecological characteristics that determine the local diversity and patterns of pathogen agent transmission (Mouchet 1999).

In Mozambique, several entomological studies on *Anopheles* species have been undertaken. Currently, there have been 46 species recorded in the country. However, *An (Ce) gambiae, An (Ce) arabiensis, An (Ce) merus and An (Ce) funestus and An (Ce) pharoensis* have been the most frequently found (De Meillon 1941; Petrarca et al., 1984; Mendis *et al*., 2000; Casimiro *et al*., 2006; Coleman et al., 2008; Cuamba & Mendis 2009; Abilio et al., 2011; Charlwood *et al*., 2013; Abilio *et al*., 2015; Kampango 2016). The role of these vectors species on the transmission of arbovirus in Mozambique is yet to be determined.

2.4.2. Subfamily *Culicinae*

Culicinae is the largest and the most diverse group of mosquitoes in the whole world, with 3546 formally recognized species (Harbach 2007; Becker et al., 2010). The subfamily has been subdivided into 11 tribes namely *Culicini, Aedeomyiini, Aedini, Mansoniini, Culisetini, Ficalbiini, Orthopodomyiini Sabethini and Uranothaeniini* and others (Jupp 1996; Harbach 2007). In the tropical and subtropical regions, the main portion of mosquito belongs to the tribes of *Culicini, Aedini and Mansoniini*, which includes numerous genus of public health importance, such as *Aedes, Culex, Mansonia, and Coquillettidia* (Becker et al., 2010). A brief account of these four genera is described below.

2.4.2.1. Genus *Aedes* (Meigen, 1818)

This is the largest described and cosmopolitan genus of Culicines mosquito, it includes great diversity of species, usually difficult to tell apart from the whole group by morphological identification (Edwards 1941; Hopkins 1952). This genus comprises great variety of species, which in recent years has undergone major systematic changes, the last of which is that of 2015 (Wilkerson *et al.* 2015), which includes the major and cosmopolitan vectors of DEN, Zika (ZIK) and CHIKV, *Aedes (Stegomyia) aegypti* and *Ae. (Stg.) albopictus*. In southern Africa at least 5 floodwater *Aedes* species were incriminated as efficient vectors of other arbovirus, with particular emphasis to the transmission of RVFV. These vectors include *Ae. (Neo.) mcintoshi*, *Ae. (Adm.) vexans, Ae. (Neo.) circunluteolus*, considered as reservoir and maintenance vectors and, *Ae. (Ocherotatus) caballus* and *Ae. (Och.) juppi* considered as potential reservoir vectors (Gear *et al.,* 1955; Jupp, 1996).

In Mozambique, there is no information related to arbovirus seroprevalence in Mosquitoes. Tentative of screening of individuals belonging to aforementioned mosquito *Aedes* species suspected the presence of arbovirus (Worth & De Meillon 1960; Kokernot *et al.* 1962). However, due the lack of advanced technology applied the results are far away to be reproductive. This situation, indicates that the actual role of this genus of mosquitoes in the maintenance of arbovirus group of global public health importance, such as DEN, ZIK and CHIK and other arbovirus remain to be resolved.

2.4.2.2. Genus *Culex* (Linnaeus, 1758)

This genus, *Culex*, is the largest Culicini tribe in Southern Africa. There are currently 46 species, and six subspecies among six subgenera described namely, *Afroculex, Culex, Culiciomyia, Eumelanomyia, Lutzia, Mallotia* (Jupp 1996). Many *Culex* species are nearly difficult to identify morphologically. The final diagnosis usually depends on the differences in male genitalia or some cases, on the morphological features found in the larval stages (Edwards 1941; Hopkins 1952). Similarly, this genus also comprises the major vectors of arbovirus of medical and veterinary importance, such as vectors of WNV, Japanese encephalitis virus, Sindbis, CHIKV, RVFV and others (van den Hurk *et al*., 2009; Diaz-Badillo et al., 2011). The species *Cx. (Culex) antennatus, Cx. (Cux.) pipiens s.l., Cx. (Cux.) poicilipes, Cx. (Cux.) theileri and Cx. (Cux.) zombaensis* have been found infected by RVFV and implicated in the epidemics of RVF in many countries of the sub-Saharan region (Swanepoel & Coetzer 1994). For instance, *Cx. pipiens and Cx. poicilipes* were found infected in Sudan (Seufi & Galal 2010).

Mosquitoes of the genus of *Culex* have been frequently collected in the entomological surveillance of malaria vectors. However, despite earlier serological surveys have indicated that the *Culex* fauna of Mozambique may also harbour an important vector of arbovirus (Worth & De Meillon 1960), their actual role as arbovirus vectors in the country has yet to be clarified.

2.4.2.3. Genus *Mansonia* (Blanchard, 1901)

This genus includes 25 species worldwide distributed, most of them are tropical species, and several varieties are found in the colder part of the world. The genus is divided into two subgenera: *Mansonia* comprised of 15 species occurring in New World and *Mansonioides,* with ten species occurring in Old World (Harbach 2008). Adults of the genus *Mansonia* are vectors of RVFV, CHIKV, Sindbis virus, Ilesha virus and others (Jupp *et al*., 1981; Service 1990; Jupp 1996; Lam *et al*., 2001; Sang *et al*., 2010).

In Mozambique, two species of the genus *Mansonia (Ma. (Mnd.) africana and Ma. (Mnd.) uniformis*) occur, with a tendency for *Ma. africana* to predominate (De Meillon & Worth 1960; Worth & Paterson 1961). More recently, *Mansonia spp*. mosquitoes from Zambezi Valley were found infected with a new flavivirus, tentatively named Cuacua virus (Cholleti *et al*., 2016).

2.4.2.4. Genus *Coquillettidia* (Dyar, 1905)

Mosquitoes from the genus *Coquillettidia* are usually large, distinguished in the adult stage with visible yellow colours similar to the specie of *Mansonia* and resemble certain species of *Culex* and *Aedini*. The genus includes 58 species divided into three subgenera, namely *Austromansonia* encompassing only one species, *Coquillettidia* with 44 species and *Rhynchotaenia* comprising 13 species (Harbach 2008). Several adult mosquitoes of subgenera *Coquillettidia and Rhynchotaenia* are native arboviruses vectors such as eastern equine encephalitis and RVF (Weaver & Reisen 2010).

Species of *Coquillettidia* have been systematically found since the initial entomological surveys carried out so far in Mozambique (Worth & De Meillon 1960; Worth & Paterson 1961; Jupp 1996).

2.5. The transmission and epidemiology of arbovirus: an overview

Arbovirus that affects humans and animals can be transmitted by a myriad of blood-feeding arthropods, including ticks, biting midges, flies and mosquitoes. Depending on the vector species, the transmission cycle can be either simple or complex, usually with the participation of at least two or more amplifying hosts or reservoirs. The description of the arbovirus transmission cycle involving other vectors species, different from mosquitoes, is out of the scope of this work, so only mosquito-borne arbovirus will be addressed.

In mosquito vectors, after ingesting an infective blood meal, there is an extrinsic incubation period of approximately one to two weeks, depending on environmental temperature, before virus transmission can occur. In this period, the arbovirus replicates in the midgut cells, escaping to the haemocoel where it is disseminated via the haemolymph to the salivary glands and other organs where replication occurs. However, in some proportions of infected mosquitoes, the infection remains confined to the midgut, implying that there is a mesenterial barrier to the spreading of the infection. In contrast, in a reduced number of mosquitoes, the virus is quickly disseminated via the ring of cells at the junction of the foregut and midgut (Hardy *et al*., 1983; Faran *et al*., 1988; Lerdthusnee *et al*., 1995). In general, following a mosquito oral acquisition of a viremic bloodmeal, from an infected vertebrate host, the arbovirus infection cycle in the mosquito follows different steps until it becomes a productive infection in the cellular environment of the arthropod vector. These steps can be divided into six categories, namely; (I) the initiation of virus infection in the midgut; (II) the spread of infection within the midgut epithelium; (III) dissemination of the virus infection from the midgut to secondary tissues; (IV) occurrence of secondary amplification of the virus in various tissues beyond midgut; (V) infection of salivary glands (and occasionally reproductive tissues for vertical transmission to offspring); and (VI) release of the virus into salivary ducts for horizontal transmission to uninfected vertebrate host (Fig. 3). However, in order to systemically infect the vector, the virus must manage to escape the innate immune responses, and the virion might overcome several tissue barriers associated with the midgut and the salivary glands. In this case, persistent arbovirus infection of a mosquito vector requires a successful crossing of specific tissue barriers, namely, the midgut infection barrier (MIB), midgut escape barrier (MEB) (Fig. 3), salivary gland infection barrier, and salivary gland escape barrier (Hardy *et al*., 1983).

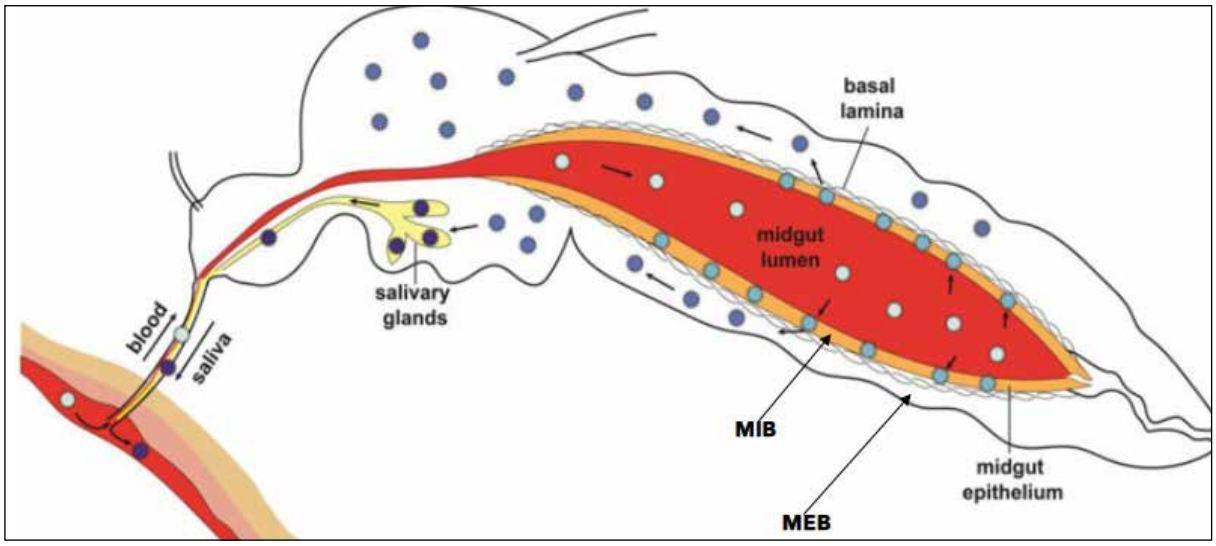

Figure 3. Example of arbovirus cycle in response of virion ingestion by the mosquito vector. Virions (light blue circles), midgut infection barrier (MIB) and midgut escape barrier (MEB). Adapted from (Rückert & Ebel 2018).

Additionally, there are other essential mechanisms preventing virus replication in the mosquito that is considered antiviral immune response where RNA interference (RNAi) is part of them (Blair & Olson 2015). The RNAi is known as the specific and potent mosquito antiviral defence. In the process of mosquito cell infection, the viral intermediates are recognized by endonuclease Dircer-2 that are split into 21nt virus-derived small RNA. That smaller RNA is incorporated into an RNA-induced silencing complex (RISC) that can target viral RNA for degradation (O'Neal *et al.*, 2014). Thus, mosquito barriers coupled with antiviral responses constitute the important phenotype for the vector to be competent for arbovirus transmission.

The key requirement for the virus to replicate in both the vector and vertebrate host is the process of biological transmission that creates numerous chances for interaction among the vertebrate host, vectors, and viruses. These interactions can happen in numerous stages that can eventually influence transmission patterns and disease pathogenesis. In nature, the transmission cycle of arboviruses can be influenced by the specific vector or vertebrate host involved in the specific transmission cycles (Kuno & Chang 2005). The Enzootic cycles are usually silent and involve mostly horizontal virus transmission between vector and vertebrate host (mammals and wild birds), while the Epizootic or epidemic cycle is the transmission that occurs to an unusual vertebrate host or humans respectively, which can cause high morbidity and mortality to the population involved (Weaver & Barrett 2004).

There are different types of hosts in arbovirus transmission (Kuno & Chang 2005). One of them is the so-called primary hosts that normally play an essentials role in the virus perpetuation in nature (e.g. birds, rodents and primates). In this situation, the manifestation of the disease doesn't occur, as there is a symbiosis interaction between the virus and the host that creates antibodies to the host. Another type is said secondary, accidental, connecting or amplifying hosts (e.g. small mammals, wild birds). This group of hosts increases the occasional transient virus reservoir and weight of the infection to which the human is exposed, in some cases acting as a dead-end host (Weaver & Barrett 2004). In fact, depending on the arbovirus strain, humans and other vertebrates can be either reservoir, amplifying or dead-end hosts, as observed, for instance with, DENV, CHIKV, ZIKV, WNV, RVFV infection (where domestic mammals, feral birds and primates can be both amplifier or reservoir host). Humans and cattle are usually dead-end hosts of the Japanese encephalitis virus, whilst domestic pigs are virus amplifiers (Scherer *et al.*, 1959). Diverse mosquitos' species differ in their blood-feeding behaviour and host preference. Thus, the mode of transmission between maintenance hosts may differ from that responsible for the infection as they may have susceptibility to different vectors species (Rückert & Ebel 2018; Huang *et al.*, 2019).

2.6. The *Bunyavirales* group

Bunyaviruses used to be a family, offering a considerable taxonomic challenge with confusing nomenclature comprising a substantial number not yet been assigned to a genus or serogroup. This controversy led to the establishment in 2017 of the order *Bunyavirales* by the International Committee on Taxonomy of Virus (ICTV) Executive Committee (EC) to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes (Abudurexiti *et al.*, 2019). As a result, the order actually comprises twelve families, of which the family Phenuiviridae is part, where RVFV from the Phlebovirus genus belongs (Maes et al., 2018; Abudurexiti *et al.*, 2019; Maes et al., 2019). In this thesis, the attention is turned to RVF due to its importance as VBD for animals and humans in Africa. However, others, such as the Shuni virus, have been recently detected in neighbouring South Africa (Motlou & Venter 2021).

2.6.1. Global epidemiology of Rift Valley fever virus

2.6.1.1. Geographic Distribution and Antecedents

Evidence from serological studies and epizootic reports indicates that RVFV is widely distributed in Africa. There are reports of RVF in several countries throughout the continent, including Kenya, Tanzania, Somalia, South Africa, Madagascar, Egypt, Sudan, Mauritania (Faye *et al.*, 2007), Niger (Tambo *et al.*, 2016) and recently in Turkey, Tunisia and Libya (Nielsen *et al.*, 2020). Transmission seems to be absent in the arid regions of the Sahara desert and Northwestern Africa (Fig. 4). The virus continues to disperse further afield, where in September 2000, RVF cases were confirmed in Saudi Arabia and Yemen, outside Africa (Jupp *et al.*, 2002; Miller *et al.*, 2002; Bird *et al.*, 2009; Nanyingi *et al.*, 2015).

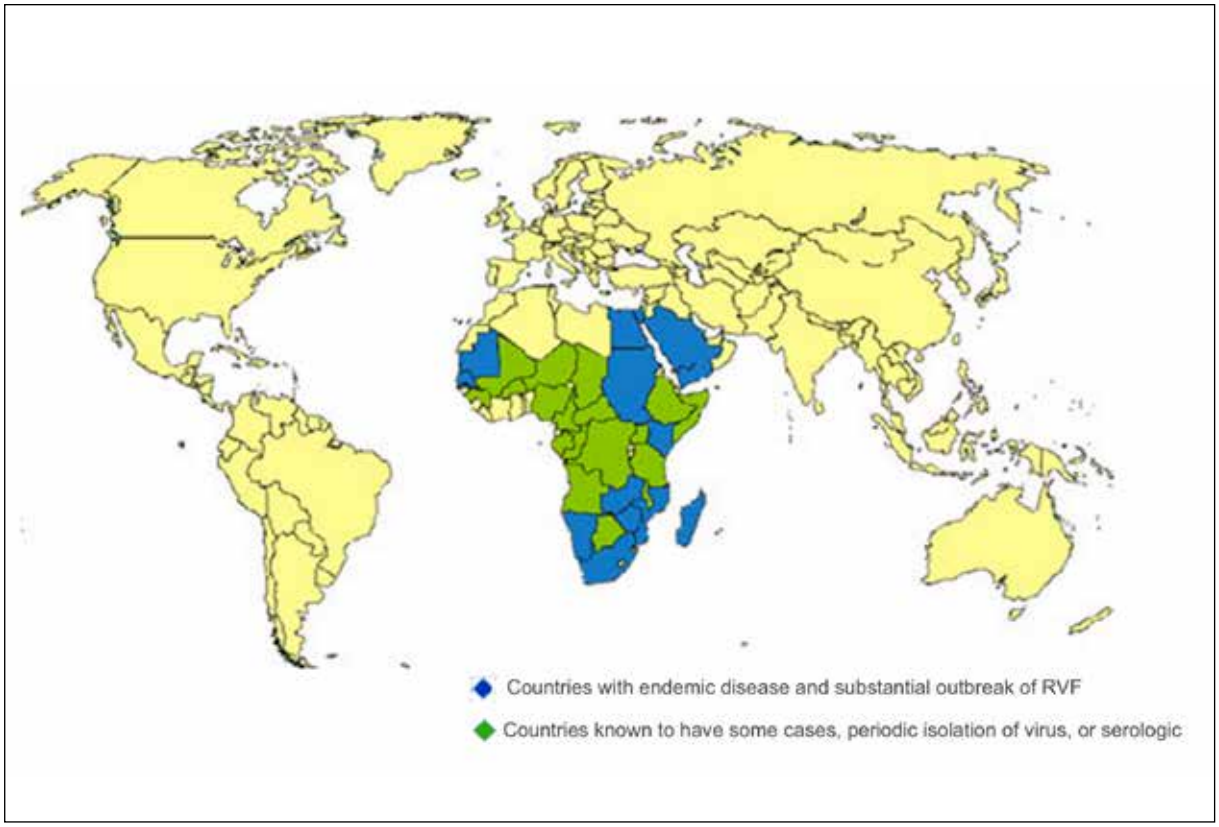

Figure 4. Global distribution of Rift Valley Fever (CDC 2011).

The virus was named after the Rift Valley region in East Africa, where the etiologic virus was firstly isolated in 1930 by Daubney and Hudson (1932) following an outbreak of "enzootic hepatitis" in new born lambs and isolated from humans near Naivasha in the part of Rift Valley region in Kenya (Daubney *et al*., 1931). It was possible to retrospectively identify epizootic outbreaks as far back as 1912 (Eddy & Peters 1980; Davies 2010). In 1951 the disease was recorded for the first time in South Africa when humans became ill after having assisted in a necropsy on a bull near Johannesburg. At that time, the virus caused 500 000 abortions and 100 000 estimated deaths of sheep (Alexander 1951; Mundel & Gear 1951). Comparably, large livestock losses were reported in Zambia and Zimbabwe (Meagan 1981), whereas Namibia, Mozambique, Sudan and East Africa reported further epizootics outbreaks of the virus (Meagan 1981). A severe outbreak occurred in Egypt in 1977/1978, causing approximately 200 000 human infections and 18 000 cases of illness, of which at least 598 resulted in death. At the same time, an outbreak occurred in Zimbabwe with associated human fatalities (Swanopoel *et al*., 1979). In Mauritania, in 1987, there was a human outbreak with approximately 200 human deaths (Swanepoel & Coetzer 2004; Swanepoel & Paweska 2011). Another occurred in East Africa and Madagascar in 1991 with 89 000 human infections that resulted in more than 500 deaths, while in 1998, a further outbreak resulted in 98 000 human infections and 250 deaths (Swanepoel & Coetzer 2004; Swanepoel & Paweska 2011). The virus outbreak spread outside its endemic range in September 2000, and cases were confirmed, in Saudi Arabia and Yemen, affecting 882 humans, of which 124 resulted in deaths. This represented the first reported outbreak of haemorrhagic cases of RVFV infections occurring outside Africa and Madagascar, raising concerns about the risk of expansion to Asia or Europe. Approximately 66% of those 882 infected individuals referred to have contact with animals, and 99% have received repeated mosquito bytes (Jupp *et al*., 2002; Miller *et al*., 2002; Balkhy & Memish 2003; Bird et al., 2009; Paweska 2015). In 2007, a large epizootic/epidemic RVF outbreaks also occurred in Kenya, Tanzania and Somalia, were 392, 309, and 114 correspondingly human cases resulted in 90, 144 and 51 deaths, respectively (WHO 2007; Mohamed et al., 2010; Nguku et al., 2010). In the same period, in Sudan occurred a large outbreak that caused 747 human cases, of which 230 resulted in deaths (Hassan et al., 2011). In May 2008, it was detected for the first time in the Archipelago of Comores, located between Mozambique and Madagascar, on the French island of Mayotte (Sissoko *et al*., 2009). In 2010, the government of South Africa reported 237 laboratory-confirmed cases of RVF in humans, including 26 deaths in Free State Province, Cape Province and North West Province outbreaks. In the same outbreak a large number of animal cases were diagnosed, mainly in sheep, goats cattle, and wild bovines (mostly Cape buffalo) and camelids (Paweska *et al*., 2010; WHO 2010). In May 2016, RVF epizootics occurred in Niger caused 90 human cases that resulted in 28 deaths with substantial livestock and cattle loss (Tambo *et al*., 2016). More recently, seropositivity was detected in Turkey, Tunisia and Libya with no death but raised the attention of the European region (EU) for a possible spread to the neighbouring countries (Nielsen *et al*., 2020).

In Mozambique, few studies have been undertaken to understand the epidemiology and entomology of RVF, despite recurrent outbreaks reported in the surrounding countries. The first documented report of possible circulation of RVFV in humans dated from 1981-1983, when the anti-RVFV antibodies were found in 2% of the sera obtained from 1163 people tested (Niklasson *et al*., 1987). Regarding animal infection, a recent serological survey involving domestic animals detected a high level of circulant IgG anti-RVFV among ruminants from Maputo and Gaza, Southern Mozambique and in five rural districts of Zambézia Province (Maganja da Costa, Mocuba, Morrumbala, Mopeia and Nicoadala) in which 35.75% of serum collected from sheep and 21.15% from goat were positive for neutralizing RVFV (Fafetine *et al*., 2013; Lagerqvist *et al*., 2013; Fafetine *et al*., 2016). Furthermore, seroconversion of IgG anti-RVFV was observed in 5% (10/200) of humans suffering from acute febrile illness, whereas specific IgM anti-RVFV antigen was detected in the serum of one (1/200) acute febrile patient (Gudo *et al*., 2016d).

2.6.2. The structure and composition of RVFV genome

Like all the Bunyavirales, the RVFV genome has a three-segmented, single-stranded, negative-sense RNA genome, e.g., the large (L), medium (M) and small segments (Fig 5). Each of the three RNA segments is inserted in a separated ribonucleocapsid or ribonucleoprotein (RNP) within the virion (Raymond *et al*., 2010). The L and medium M segments are of negative polarity, coding respectively for the L protein, which is the viral RNA-dependent RNA polymerase (Muller *et al*., 1994), and for the precursor to the glycoproteins (Collett et al., 1985), while the third and the small (S) segment is ambisense RNA, i.e. has bi-directional coding (Giorgi *et al*., 1991). The L segment encodes the RNA polymerase used for replication and mRNA transcription. The M encodes for the precursor of the envelope glycoprotein Gn and Gc of 78 kilodaltons (kDa) minor structural glycoprotein and a non-structural 14 kDa protein named NSm. The S segment is expressed with the open reading frame (ORF) of the nucleocapsid (N) protein in the negative sense and a non-structural protein (NSs) in the positive sense (Fig. 5) (Giorgi *et al*., 1991).

The matured particles of RVFV are usually spherical and enveloped with a diameter of 90-110 nanometre (Ellis *et al*., 1979). The envelope comprises a lipidic bilayer composed of Gn and Gc glycoproteins within the surface sub-units of 5-8 nm in length that are arranged frequently on its surface (Besselaar and Blackbum 1992), comparable to those observed with related Uukuniemi *phlebovirus* (Pettersson & von Bonsdorff 1975). Several copies of the nucleoprotein N and RNA dependent RNA polymerase L, connected with the viral ribonucleoproteins (RNP) resulting in each of the three genomic segments, are packaged into the virion. Some studies by cryo-electron microscopy on RVFV and Uukuniemi virus showed that some *phlebovirus* has a modified pleomorphic structure (Bishop *et al*., 1980). The evidence also showed that designated types of virions are liable to contain icosahedral symmetry: a surface shell of 120-122 glycoprotein capsomers organised in an icosahedral lactic with T=12 close to the highly ordered structure. The capsomers look like hollow cylinders located at five and six-coordinated positions, revealed by a three-dimensional reconstruction at 22 or 27 Ao resolution. In the inner envelope, a coating of RNP is situated proximal to the inside booklet of the membrane, robustly evoking an interaction between the cytosolic tail of the glycoproteins and the RNP that would balance in the absence of matrix protein in the viruses from this family (Freiberg *et al*., 2008; Over by *et al*., 2008; Huiskonen *et al*., 2009; Sherman *et al*., 2009). The complementarity between terminal sequences 3′ and 5′ of the nucleic acid chain in the virus genome corroborates its circular nature (Bishop *et al*., 1980).

The two nucleoproteins (N) proteins and a non-structural protein, namely (NSs), are encoded within S segments using ambisense and L, M employing negative-sense arrangement (Giorgi *et al*., 1991). The general view that highlights that a single viral genome is incorporated into the mature particle was reconsidered as a small but important fraction of the antigenomes, i.e. replicative intermediates are being detected in purified RVFV particles (Ikegami *et al.*, 2005). This finding corroborates with historical studies on Uukuniemi virus, which showed that it is possible to detect the S genomic segment with purified virions and determine its antigenomic polarities (Simons *et al*., 1990). The common RVFV transcription and replication characteristics closely resemble those of other negative-stranded RNA viruses of the *Phenuiviridae* family (Bishop *et al*., 1980; Tercero *et al*., 2019). During the replication cycle, each segment contains an un-translated region (UTRs) that contains the promoters for transcription. The replication is transcribed into mRNA and is replicated through a process that involves the synthesis of the exact copy of the genome called complementary RNA (cRNA) or antigenome. All the replication steps occur in the cytoplasm of infected cells, and virions mature by budding in the Golgi compartment (Giorgi *et al*., 1991).

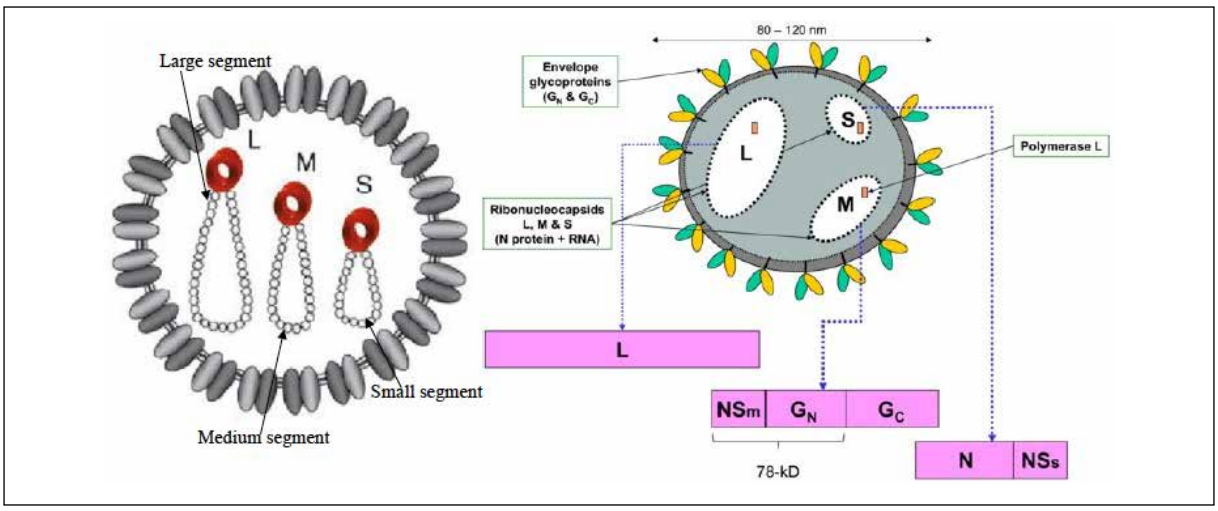

Figure 5. Organizational structure of RFV genome showing the three single-stranded segments of the virus RNA genome and the open reading frame that encode for viral proteins of the envelope and capside. Electron microgram adapted from Linda Stannard (Hanley & Weaver 2008), and (A colour version of this figure is available online at www.vetres.org) and https://www.researchgate.net/figure/Rift-Valley-fever-virus-virion-and-transcription-strategy-a-Rift-Valley-fever-RVF_fig2_283088046.

2.6.3. Transmission cycle of RVFV

Rift Valley fever virus transmission between animals and humans has been associated almost exclusively with mosquitoes (Diptera: Culicidae), particularly those belonging to the genera *Aedes, Culex, Anopheles and Mansonia* (Daubney *et al.*, 1931; Easterday *et al.*, 1962; Woods *et al.*, 2002; King et al., 2010; Ba et al., 2012). Unlike the majority of arboviruses, which tend to be adapted to a narrow range of vectors, RVFV can also be transmitted by ticks and a variety of flies (Davies & Highton 1980; Linthicum *et al.*, 1985; Turell & Perkins 1990; Fontenille *et al.*, 1998; Mellor & Hamblin 2004; Diallo *et al.*, 2005). The transmission is mostly horizontal, but a vertical mode was described in some *Aedes* species (Linthicum *et al.*, 1985).

Rift Valley fever virus was isolated from eggs of *Aedes* mosquitoes which breed in isolated depressions called dambos found in the vast grassland areas. These depressions also serve as good habitats for *Culex and Anopheles* mosquitoes (Mondet *et al.*, 2005). Animals were infected when the virus was transmitted from an infected mosquito during blood-feeding. A couple of days after infection, viral amplification occurs in these vertebrate hosts, resulting in the propagation of the virus infecting several cells throughout the mosquito body. Transmission to humans usually occurs through infected mosquito bites, but there are also reports on transmission to humans via contact with either blood or other blood-related fluids (Fig. 6). The latter type of transmission can happen through aerosols created during slaughtering or through contact with raw meat (Daubney *et al.*, 1931; Easterday *et al.*, 1962). Sheep are favourable highly susceptible animals to RVFV infection, and the rate of mortality in infected lambs and adults can reach 90-100% and 20-30%, respectively, whilst more than 80% of pregnant ewes abort after being infected (Daubney *et al.*, 1931; Easterday *et al.*, 1962).

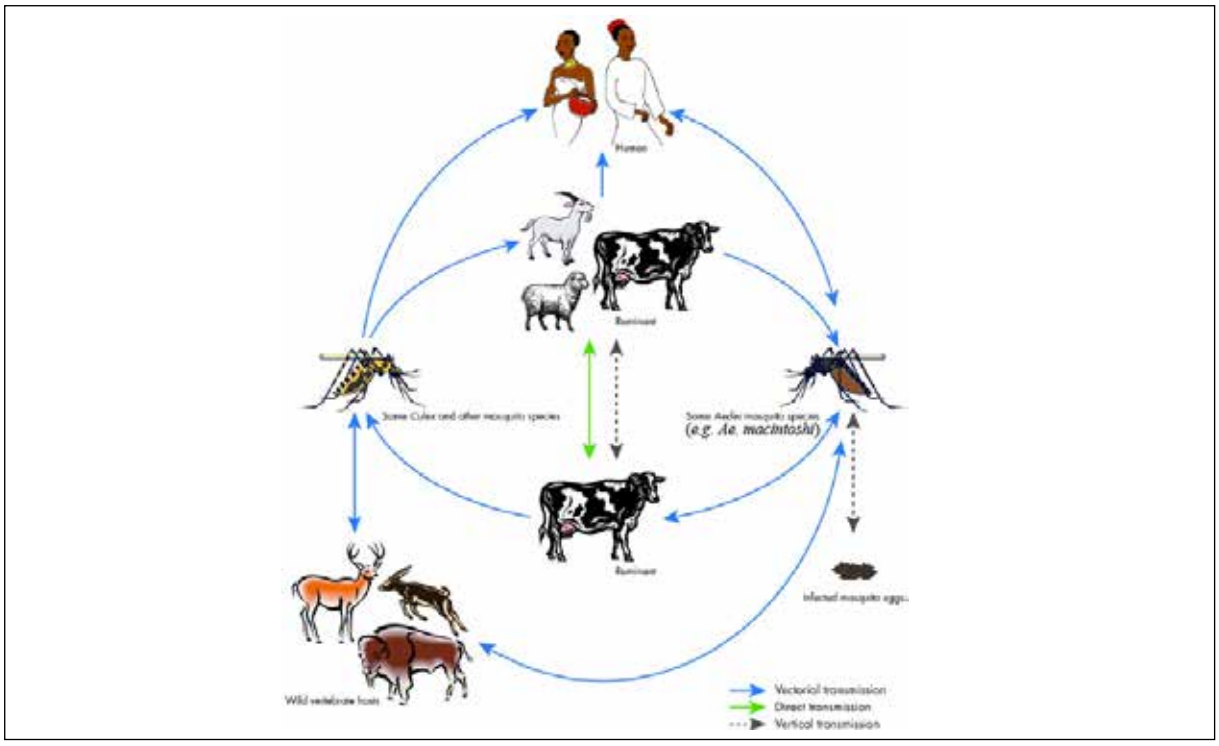

Figure 6. Schematic representation of transmission cycles of RVF, showing the type o transmission and component involved. Adapted from (Balenghien et al. 2013).

2.6.4. The Rift Valley fever virus in mosquitoes

The replication of RVF in an infected mosquito did not differ much from the replication of many other arboviruses. After ingesting an infective blood meal by a mosquito vector, an extrinsic incubation period of approximately one to two weeks can occur before transmission, depending on environmental temperature. RVFV replicates inside the midgut cells, in this period, escaping to the haemocoel, which it is disseminated via the haemolymph to the salivary glands and other organs. However, in some proportions of infected mosquitoes, the virus infection has been confined to the midgut, implying that there is a mesenteronal barrier to the spread of the infection (Faran *et al*., 1988). In a reduced number of mosquitoes, there is quick dissemination of RVFV via a ring of cells at the junction of the foregut and midgut (Lerdthusnee *et al*., 1995). If the mesenterial barrier is broken by intra-thoracic inoculation of the RVFV, the rates of transmission increase to 100 percent in some species of mosquitoes (Faran *et al*., 1988). Nevertheless, there are other species where some or all individuals remain refractory to transmission. This situation demonstrates that there may be a barrier to infection by parasites at the salivary gland level (Franz *et al*., 2015). Laboratory evidence has suggested that co-infection with *Plasmodium* increases the changes in penetration and transmission of RVFV (Vaughan & Turell 1996).

2.7. Other arbovirus groups of Public Health relevance

2.7.1.The *Flavivirus* group

The Flaviviruses are a widely distributed group of arbovirus belonging to the genus *Flavivirus*, family *Flaviviridae*. The name was derived from Latin "flavus", which means yellow, as the first *Flavivirus* isolated was the virus causing Yellow Fever.

The Flaviviridae is an extensively spread and genetically diverse RNA virus that infects both humans and animals (Lindenbach et al., 2007; Cook et al., 2009; Huang et al., 2014). The family comprises four genera: genus *Pestivirus, Hepacivirus, Flavivirus and Pegivirus* (Lindenbach *et al*., 2007; Cook *et al*., 2009; Huang *et al*., 2014). Seventy species of the genus *Flavivirus* are recognized, and probably

many other species to be described (Pybus *et al.*, 2002; Cook & Holmes 2006; Junglen *et al.*, 2009; Evangelista *et al.*, 2013). Many *Flaviviruses* are the main cause of outbreaks and epidemics of life-threatening infectious diseases affecting humans.

They are among the most virulent and deadly groups of arboviruses and mosquitoes of the species, *Ae. aegypti, Cx. tritaeniorhynchus, Cx. pipiens spp* are, respectively, the most important vectors (Petersen & Marfin 2005). Flaviviruses can also be transmitted by tick bites (Junglen *et al.*, 2009; Huang *et al.*, 2014). Transgenerational vertical transmission maintaining the virus circulation in the mosquito population has also been reported (Khin & Than 1983; Rosen 1988), contributing to the virus's circulation between mammals and birds.

Avian hosts are the main reservoirs for some *Flaviviruses*, making eradication almost inconceivable and unfeasible, hence an effective control depends on the integrated application of approaches involving vaccination and vector control measures programs (Blitvich 2008; Go *et al.*, 2014; Huang *et al.*, 2014). More than half of the global human population is at risk of flaviviruses infection emphasising dengue DENV serotypes DENV-1, -DENV2, -DENV3 and DENV4 (Durbin *et al.*, 2013), Yellow fever (YF) virus, Japanese encephalitis virus and WNV. These all together cause thousands of deaths each year worldwide (Mukhopadhyay *et al.*, 2005; Petersen & Marfin 2005).

2.7.1.1. *Flavivirus* genome structure

The genome of flaviviruses is a single-stranded positive polarity RNA molecule containing approximately 11 kilobases in length. The genome encodes three structural proteins (capsid [C], membrane [M] and envelope [E]) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Fig. 7). The polyprotein was flanked by 5' and 3' non-coding regions (Sánchez-Seco et al., 2005; Harris 2006; Hoshino et al., 2009).

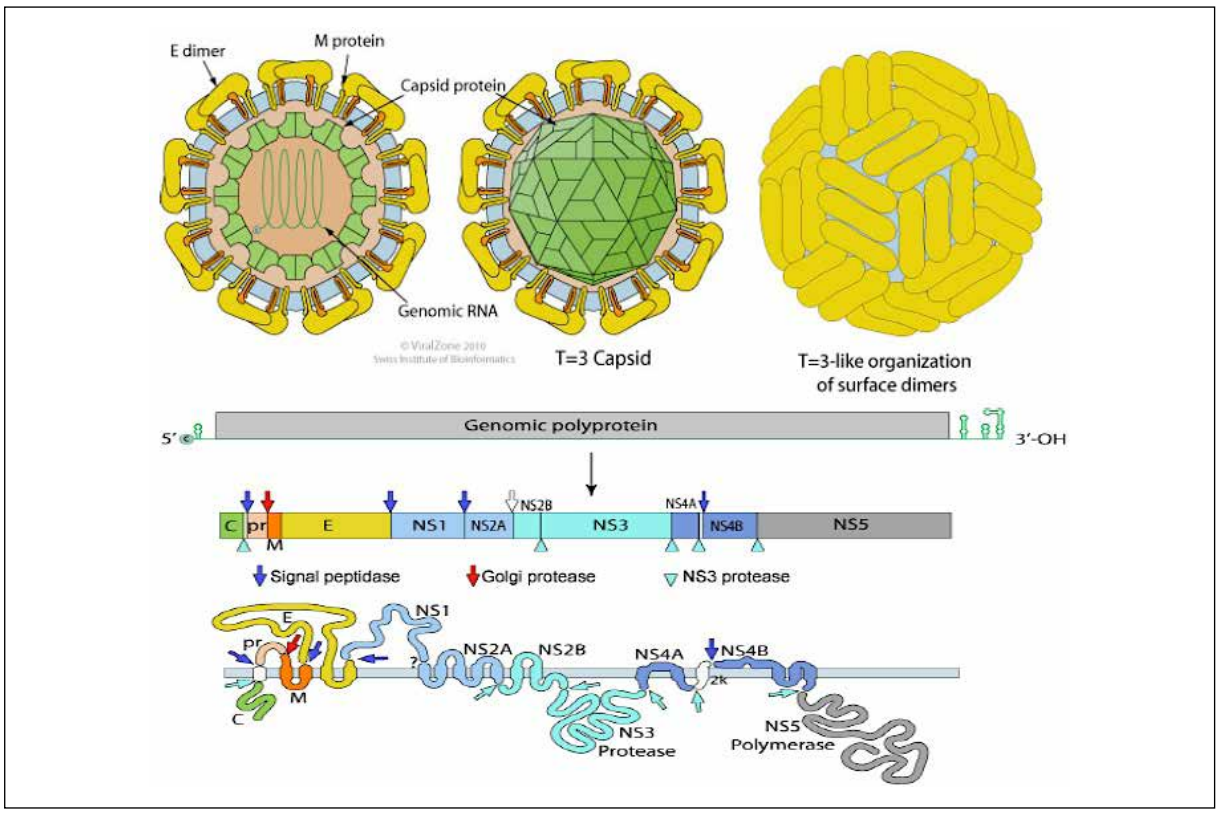

Figure 7. Schematic and cross-sectional representation of the structure of Flavivirus virion (Above) and physical map of Flaviviruses genome showing the arrangement of the open reading frames that encode for structural and non-structural proteins (Below). Adapted from: http://viralzone.expasy.org/24?outline=all_by_species.

2.7.1.2. Flavivirus *transmission in Mozambique*

The first suspect of a possible ongoing arbovirus transmission to humans in Mozambique came about with the first confirmed case of dengue (DENV-3) reported in the North of the country in 1984. The two recent dengue (DENV-2) virus outbreaks that occurred in 2014 and 2015 in the same region have confirmed previous findings and, overall, the country has reported at least two DENV outbreaks since 1984, which recently occurred in 2014 and 2015, all in northern of the country (Gubler *et al*., 1986; Massangaie *et al*., 2016). However, positive samples for IgG of DENV and WNV were detected in the same patients from the southern region of the country, probably as previous independent infection, co-infection or cross-reactivity (Gudo *et al*., 2016b). More recently, IgM anti-DENV antibodies were detected in 1 (0.9%) of 104 tested patients from Quelimane district in the country's central region (Mugabe *et al*., 2018), representing a wide circulation of the flaviviruses in Mozambique. Apart from that, one study discovered mosquitoes specific flaviviruses detected in *Monsonia spp*. (Cholleti *et al*., 2016).

2.7.2. The *Alphavirus* group

Alphaviruses are viruses belonging to the genus *Alphavirus*, family *Togaviridae*. According to the VIIIth edition of the International Committee of the Taxonomy of Virus (ICTV) (Weaver *et al*., 2005), the genus Alphavirus currently comprises 29 different species, including some of the Rubivirus genus (Weaver *et al*., 2005). The *alphaviruses* are arthropod-borne viruses (arbovirus), while rubiviruses are wind-borne viruses that affect the respiratory system. All alphaviruses are antigenically similar, identified manly by cross-reactivity tests (Clarke & Casals 1958; Chanas *et al*., 1976). *Alphaviruses* are divided into eight antigenic complexes identified, viz. Eastern, Western, and Venezuelan equine encephalitis, Trocara (complex assigned based only on genetic divergence), Middelburg, Ndumu, Semliki Forest and Barmah Forest (Clarke & Casals 1958; Chanas *et al*., 1976).

Alphaviruses are widely spread disease-causing agents affecting humans, domesticated and wild terrestrial vertebrates and fishes (Weaver et al., 2005). In humans, Alphaviruses are usually characterized by joint pain, rash and arthritis, occasionally with a severe impact on human health and productivity (Gould & Higgs 2009). In the last decades, the world has experienced epidemics of emerging and re-emerging alphaviruses of public health concern, such as CHIKV, O'nyong nyong virus, Ross River virus, and Sindbis virus, which have resulted in high morbidity in humans (Rulli *et al*., 2007; Gould *et al*., 2010). The transmission dynamics of Alphaviruses remain poorly understood. However, it is well established that these viruses circulate among mammals, birds and several arthropod vectors (Weaver *et al*., 1992; Martina & Osterhaus 2007; Power & Logue 2007).

2.7.2.1. *Alphavirus* genome structure

The genome of *Alphavirus* virions is spherical single-stranded positive-sense RNAs with approximately 70 nm in diameter. Usually, the genome length has between 11.000 to 12,000 nucleotides (Kuhn 2007). It includes a 5' cover and 3' poly-A tail that encodes two open reading frames (ORF) for the non-structural (nsP) and structural polyproteins, respectively (Aguilar *et al*., 2007; Garmashova *et al*., 2007). The non-structural ORF encodes proteins for transcription and replication of viral RNA, polyprotein of cleavage, and RNA capping, while the structural ORF encodes the capsid protein, envelope glycoproteins E2 and E1 (Aguilar *et al*., 2007; Garmashova *et al*., 2007). The expression of those proteins and replication of the viral genome altogether occur in the cytoplasm from the host cells, even though the nsP2 and/or capsid proteins of certain alphaviruses insert the nucleus where they get in the way with host cell gene transcription (Fig. 8) (Aguilar *et al*., 2007; Garmashova *et al*., 2007).

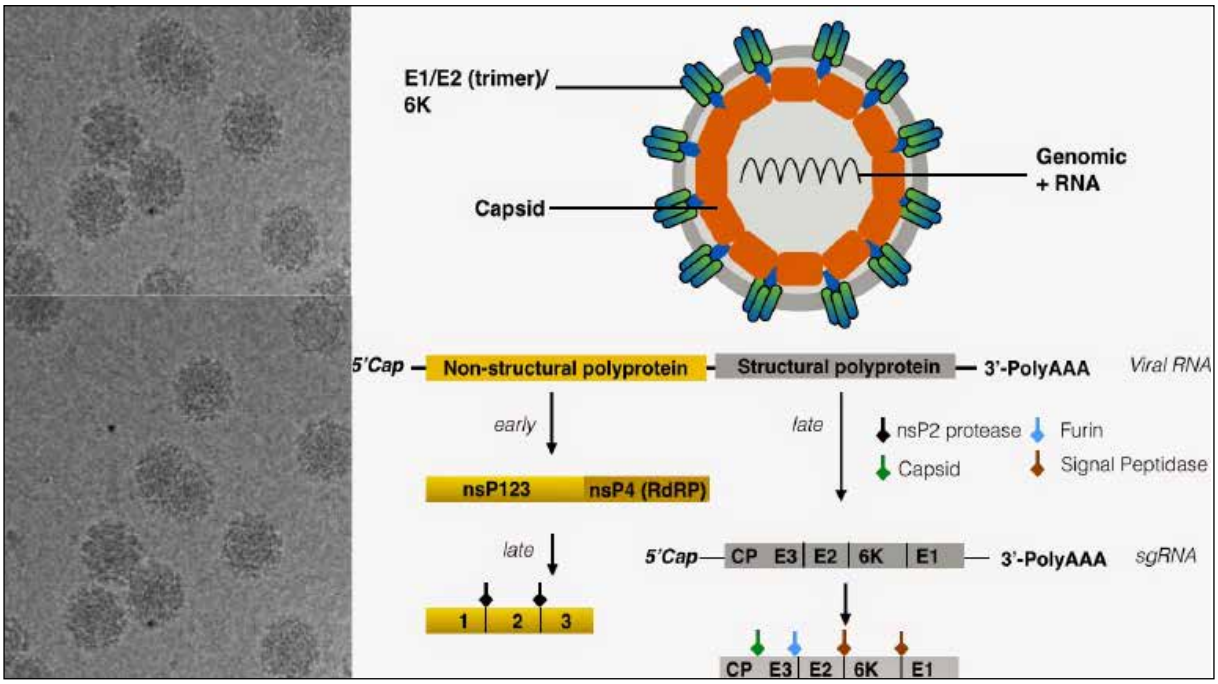

Figure 8. Schematic representation of genome of a typical Alphavirus showing the physic map of o ORF responsible for encoding of structural and non-structural proteins. Adapted from chikungunya virus and NDP52: a deadly association? http://virologytidbits.blogspot.com/2014/08) and https://phys.org/news/2015-12-effective-mechanisms-block-chikungunya-virus.html.

The epidemiology of Alphaviruses is by far not clear and incomprehensive due to the sporadic nature of the epidemics and outbreaks caused by these viruses (Suhrbier *et al*., 2012). The virus has varied geographical distribution across all the continents (Toivanen 2008). In general, most Alphaviruses outbreaks are known to be enzootic and usually disseminate to other regions due to the high capacity of vectors to adapt and colonize ecological niches (Weaver & Reisen 2010). The dissemination can also occur via air travel, seaborne trade, and virus evolution, among other factors (Lwande *et al*., 2015). In general, the transmission of Alphaviruses incorporates diverse vectors and reservoirs with different biology (Tsetsarkin *et al*., 2007; Lwande *et al*., 2015).

The transmission of Alphaviruses is multi-vectorial and somehow vector-arbovirus species-specific. Numerous mosquito species have been associated with Alphaviruses that affect human health (Weaver *et al*., 2005). For example, the natural transmission of CHIKV, one of the most common Alphavirus species infecting humans and animals, has been maintained by several Aedes spp species of mosquitoes. The virus transmission is maintained in two cycles, an urban, maintained by *Ae. aegypti and Ae. albopictus* and humans, the latter serving as host and reservoirs of the virus. The second type of transmission cycle (the rural cycle) is usually maintained by *Ae. furcifer, Ae africanus,* as well as wild primates, which are the main host and reservoir (Volk *et al*., 2010). Humans have been the main reservoirs during the epidemic transmission cycle of CHIKV, whilst primates and other animals, such as monkeys, rodents and sometimes birds, have been important reservoirs of the virus during inter-epidemics periods (Caglioti *et al*., 2013).

Several *Anopheles* mosquitoes have also efficiently transmitted Alphaviruses. One particular case is ONN virus, which is transmitted by the most efficient malaria vectors in the sub-Saharan region (*An. funestus s.l and An. gambiae s.l*). Strains of ONN virus have also been isolated from *Ma. uniformis* mosquitoes (Lutwama et al., 1999; Vanlandingham et al., 2005). The actual reservoir host of ONN virus has not been identified yet, but humans can function as amplification hosts during epidemics (Seymour et al., 2013). Differently, *Culex spp., Aedes spp., and Culiseta spp*. mosquitoes frequently transmit the Sindbis virus, another important *Alphavirus* species.

The transmission cycle involves birds as reservoirs (Lundstro¨m *et al*., 1990; Lundstrom & Pfeffer 2010), whereas the Ross River virus is transmitted by a large range of mosquito species that fall in the genera *Culex spp, Aedes (Och.) spp., Anopheles spp., Coquillettidia spp., and Mansonia spp*. (Russell 2002).

2.7.2.2. Alphavirus transmission in Mozambique

The initial discovery of some alphaviruses resembling CHIKV and ONN virus, geographically linked to Mozambique, was reported in 1959 and subsequently in 1960 (Lumsden 1955; Kokernot *et al*., 1960; Vanlandingham et al., 2005; Bessaud *et al*., 2006; Atkins 2013).

In 2015, 26.4% (55/208) of febrile patients seeking medical assistance in the Central Hospital of Maputo were positive for anti-CHIKV IgG antibodies detected during the period of convalescence. Seroconversion of the four-fold rising of anti-CHIKV IgG titres was confirmed in 9 (4.3%) of the patients (Gudo et al., 2015a). Recently, in Quelimane district, central Mozambique, out of 163 patients, IgM and IgG anti-CHIKV antibodies were identified from 17(10%) and 103 (63.2%) samples, respectively (Mugabe *et al*., 2018).

The circulation of *Ae. aegypti*, the most important vector of CHIKV has been documented in the country (Worth & De Meillon 1960; Jupp *et al*., 1981). In fact, *Ae. aegypti* specie is originated from Africa. For instance, with the exception of an exploratory study conducted recently in four districts during a dengue outbreak in 2014 (Higa *et al*., 2015), there has been no systematic study concerning the distribution of *Aedes spp*. populations in Mozambique. Nevertheless, its counterpart *Ae. albopictus* seems to occur in restricted ecological settings in the south of the country (Kampango & Abilio 2016). Despite the confirmed occurrence of major and secondary CHIKV vectors in Mozambique, the actual contribution of these vector populations in the transmission of the arbovirus remains to be studied. This is a barrier to implementing preventive and control interventions for arbovirus and other VBDs.

2.8. Insect Specific virus

The knowledge that arthropods could transmit viruses is centuries old, and the discovery that arthropods-specific viruses exist (often found in insects) is much more recent. Hereafter, the first insect-specific virus (ISV) was discovered 40 years ago when Stollar and Thomas isolated the virus originated from a culture of *Ae. aegypti* cells (Aag2 cell line), which caused a large number of syncytia in *Ae. albopictus* cells (cell line C6/36). Then, the cells came to be called cell-fusion agent virus (CFAV). Notable, once injected into another vertebrate cell line, no cytopathic effect (CPE) could be detected and the virus could not isolated, indicating that the virus has restricted replication in insect cells (Stollar & Thomas 1975).

The second evidence of insect-specific *flavivirus* (ISFs) was described 25 years later, which came to be named as Kamiti River virus, isolated for the first time from *Ae. macintoshi* from Kenya (Crabtree *et al*., 2003). Similar to CFAV, the virus also causes cytopathic effects in C6/36 cells, but not in the vertebrate cells. Since then, the study of viruses in general, and ISFs in particular, has grown considerably in the last few decades due to their high frequency in nature. Subsequently to the CFAV detection in the last two decades, ISVs have been discovered in a large number with growth regularity that includes also several ISVs belonging to the family *phenuiviridae* (Marklewitz *et al*., 2011; Carapeta *et al*., 2015; Li *et al*., 2015). This interest is deeply coupled with the advancement of viral intensified mosquito surveillance, metagenomics and sequencing methods (Bolling *et al.*, 2015) and the fact that at least in some cases their position in phylogenetic trees, suggests that they also represent a useful model for evolutionary steps as ancestral viruses from which those pathogenic humans (e.g. RVFV, DENV, YFV, WNV, and Sandfly virus) may have originated (Cook & Holmes 2006).

The recent investigation of these viruses offers valuable evidence on the evolution and genetic discrepancies of distinct viral species, and molecular bases of transmissibility and pathogenesis. Additionally, there are indications that insistent infection with ISVs may interfere with the ability of mosquitoes to transmit viral pathogens of vertebrates (Kent *et al*., 2010; Newman et al., 2011; Bolling *et al*., 2012; Schultz *et al*., 2018; Öhlund *et al*., 2019). This analyse could be important in light of current evidence displaying that some ISF namely Palm Creek and Nhumirim viruses, somewhat suppress the replication of co-infection West Nile and Murray Valley encephalitis viruses (Hobson-Peters *et al*., 2013), or West Nile and Saint Louis encephalitis viruses (Kenney *et al*., 2014), respectively, notwithstanding the elevate level of genetic discrepancies between them. On the contrary, there is evidence showing that to preserve cells against oxidative pressure, following a blood-feeding, the antioxidant competence of the midgut is increased. This event occurs when the infection by viruses like DENV in the midgut epithelial cell is accelerated, influenced by discharged catalase. Concurrently, in the blood-feeding taken by mosquitoes such as dengue, existing pathogens also require overcoming the similar oxidative challenges, and the antioxidant platform stimulated by the arthropod is likely to affect the infection significance of the mosquito including its vectorial capacity (Goic *et al*., 2016; Oliveira *et al*., 2017; Shrinet *et al*., 2018). However, other experiments show that some ISVs such as CxFV had no effect on the replication and their vector competence for arboviruses as WNV and RVFV (Kent *et al*., 2010; Talavera *et al*., 2018). These contradictions recall for further investigation in order to better understand the most accurate role of ISVs in the transmission of pathogenic viruses.

CHAPTER 2

RATIONAL AND OBJECTIVES

1. Motivation

Mozambique is located in a tropical region, the climatic and environmental conditions of which are optimal for the occurrence and transmission of several arboviruses, which can cause several health problems to humans and animals. The circulation of these arboviruses is maintained by a wide range of vector populations occurring in different eco-geographical regions.

Recently, Mozambique was affected by two dengue virus outbreaks. Also, outbreaks of RVF and CHIK have been previously reported in the country. However, the transmission dynamics of these arboviruses remain unknown and, at the same time, have received less attention. Therefore, the main goal of this work was to (i) determine the occurrence and the composition of arboviruses in mosquito populations, occurring in selected areas of Mozambique and (ii) understand the dynamics of the transmission of RVFV and other arboviruses of medical and veterinary importance in a One Health approach.

Control methods targeting vector populations have long proved to be the most efficient and cost-effective means to tackle vector-borne disease transmission worldwide. However, the success and sustainability of any vector control approach and vector population surveillance systems, which will be implemented in Mozambique, would certainly depend on the existence of in-depth knowledge and understanding of both vector bionomics and disease transmission dynamics. Consequently, it is crucial to have accurate and higher resolution information on the bionomics and epidemiology of arboviruses transmission in Mozambique.

This work will gather field and laboratory-based evidence that will be used to support an efficient control of vector populations and arboviruses transmission in Mozambique and as a baseline for the establishment of the nation-wide mosquito-borne arboviruses surveillance program.

2. Rational and Problem

Mozambique is located in a region suitable for arbovirus outbreaks, and in very recent times, the country was affected by a dengue virus outbreak, which occurred in the northern regions (Bhatt *et al*., 2013; WHO 2019). Increasing evidence also suggests that the country may be endemic to other debilitating and life-threatening arboviral diseases such as Rift Valley Fever (RVF) (Fafetine et al., 2007; Fafetine et al., 2013a; Gudo et al., 2016d), dengue (DEN) (Gubler et al., 1986; Bhatt *et al*., 2013; Higa *et al*., 2015) and chikungunya (CHIK) (Gudo *et al*., 2015a). Furthermore, historical and global risk projection has suggested that the country may also be suitable for the establishment of ZIK (Bogoch *et al*., 2016; Gudo *et al*., 2016a; Samy *et al*., 2016), a virus recently linked to cases of microcephaly in newly born children reported during the most recent epidemic in some Latin American countries, with particular emphasis to Brazil (Cugola *et al*., 2016). The circulation of these arboviruses is maintained by a wide range of vector populations occurring in different types of eco-geographical regions (Weaver & Reisen 2010).

Despite increasing evidence indicating the circulation of public health-relevant arboviruses in Mozambique, the actual burden of the diseases they cause remains unknown. Besides, more than a hundred potential arbovirus vectors have been identified in Mozambique, including vectors from taxa of global concern such as *Aedes spp., Culex spp., Mansonia spp. and Anopheles spp.* (Worth & De Meillon 1960; Gillies & De Meillon 1968; Jupp 1996; Charlwood *et al*., 2013; Kampango & Abilio 2016), the role of which, arboviruses transmission are maintained in Mozambique is yet to be elucidated.

It has been widely accepted that, in the absence of an efficient and affordable vaccine, vector control remains the most effective approach for the prevention and control of arbovirus transmission (Wilson

et al., 2020). However, the design and implementation of state-of-the-art control measures will greatly depend on a thorough understanding of the local mosquito community's ecology, spatiotemporal dynamics of vector species assemblage, and modulating factors.

This information has proven to be crucial for designing and implementing high sensitive prediction systems for the detection of climate-sensitive mosquito-borne disease outbreaks, such as dengue, across both temporal and geographical ranges (Bhatt *et al.*, 2013). The dynamics of the mosquito population of public health relevance occurring in Mozambique is poorly known. Therefore, vector control has been usually implemented based upon the extrapolation of the information reported from neighbouring countries or published accounts on global vectors' habitat suitability. Hence, high-resolution field-based data is fundamental to designing a successful vector control program at the regional and national level. As such, the main goal of this research was to produce updated information on the occurrence and seasonal dynamics of mosquito populations associated with the transmission of arboviral diseases in selected settings of Mozambique.

3. Contribution

This project has been planned as a contribution to:

- Updated and accurate information on the current knowledge of the occurrence, distribution and dynamics of mosquito populations with the potential to be vectors of RVF and other arboviruses of public health relevance in a One Health Approach in a selected setting of Mozambique.
- Input of these mosquito species on the transmission of arboviruses and/or insect specific virus in Mozambique, aiming to approximate characterization of the circulation, or co-circulation, of these among different vector populations.
- Acquisition of essential field and laboratory training needed to establish the country-wide surveillance program to monitor the dynamics of vector-borne diseases transmission in endemic settings of Mozambique.
- Obtainment of tools to strengthen national laboratory capacity for surveillance and early detection of vector-borne arboviruses of public health importance.
- Provision of site-based evidence to guide the implementation of programs for integrated and effective prevention and control of arbovirus transmission of public health relevance in Mozambique.
- Obtainment of the potential to work as a bridge of translational transference of virus detection technology and isolation from the field and laboratory scientists based on public and private institutes dedicated to research in vector-borne diseases.
- Strengthening collaboration between national and international professionals interested in insect-borne diseases, whose legal mandate includes performing the entomological characterization of endemic and emerging vector-borne diseases.

4. Objectives

4.1. Aim and Objectives

The overall aim of this thesis was based on describing the occurrence and distribution of mosquito arbovirus vectors in Mozambique and detecting in these mosquitoes the circulation of the virus of the groups *Bunyavirales, Flavivirus and Alphavirus.*

4.2. Specific objectives

Study I (Papers I and II): To map the occurrence and distribution of immature mosquito species with the potential for transmitting RVFV, DENV and other arboviruses of human and veterinary relevance in Mozambique.

Study II (Paper III): To determine the abundance and composition of host-seeking adult mosquito populations associated with the transmission of arboviruses in the districts of Mopeia (Zambézia Province) and Goba (Maputo Province); and to determine the spatiotemporal dynamics of mosquito community abundance, composition and the main drivers.

Study III (Paper IV): To investigate the presence of arboviruses of *Flavivirus, Alphavirus and Bunyavirus* groups in mosquito populations from different regions of Mozambique.

CHAPTER 3

MATERIAL AND METHODS

1. STUDY SETTINGS

Mozambique is located on the southeast coast of Africa, with 2,515 km of coastline (Fig. 9), and a projected population of 27 million inhabitants (INE 2007). According to the Köppen-Geiger classification (Köppen & Geiger 2021), the Mozambican climate is classified as a tropical climate, also known as savannah climate (Aw) with two distinct seasons, namely, a rain (wet) season from November-April and a dry season from May-October. The dry season is extended during the winter and rainfall is less than 1000mm occurring mainly in the summertime during the wet season. The average humidity ranges between 70-80%, with the highest values being reported in the Central and North regions. The average annual air temperature varies between 20ºC in the South to 26ºC in the Northern regions.

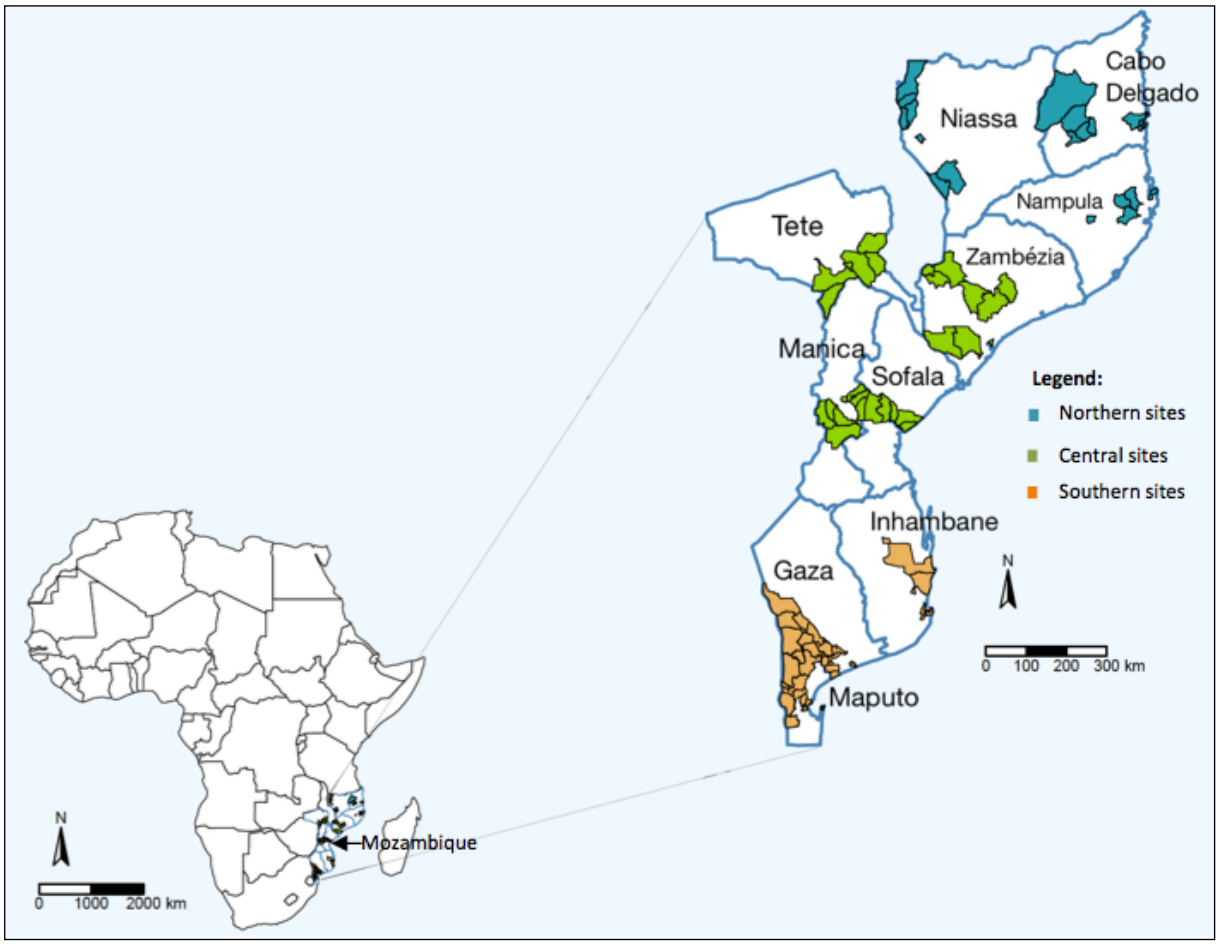

Figure 9. Map showing the entire project studied sites in the three main regions of Mozambique, namely, Northern (Blue), Central (Green) and Southern (Blue), incorporating the whole Provinces of Mozambique.

2. STUDY DESIGN, DATA COLLECTION AND ANALYSES

For this thesis, a quantitative study design was applied. The topics and questions of each study are summarized below (Tab. 2) and are all based on primary data gathered in each study sett characterized.

Table 2. Summary of the three studies related to this thesis.

Topic	Research questions	Study design and data collection	Participants	Analyses	Study
Occurrence and distribution of main arbovirus vectors in Mozambique	Are the information on occurrence and distribution of mosquito species with potential for transmitting arboviruses updated in Mozambique?	Entomological field and Laboratory based-Cross sectional mosquito larval survey conducted in all Provinces of Mozambique.	Human household and one forest, from Lago district in Niassa Province. n = 2807 container in all households - **Paper I** and, n = 92 specimens - **Paper II**.	Descriptive and analytical statistics "Stata 13"	I
Main drivers that influence for abundance, composition and seasonality of mosquito arbovirus vector	What is the abundance and composition of host-seeking mosquito population associated with the transmission of arboviruses and their main drivers that contribute for spatiotemporal dynamics of mosquito community?	Entomological field and laboratory based-Longitudinal study carried out in Gaza, Maputo and Zambézia Provinces.	Human household and animal premises n = 34,539, specimens - **Paper III**	Descriptive and analytical statistics, "R"	II
Characterize the mosquito species involved in *Alphavirus, Flavivirus* and *Phlebovirus* transmission	Is the information related to the susceptibility of mosquito population to arbovirus sufficient for better mitigation of any arbovirus emergence or outbreak in different regions of Mozambique?	Virology laboratory based-Cross sectional, RNA virus screen in adult mosquito collected from study II.	n = 14,519 specimens - **Paper IV**	Descriptive, incidence and phylogenetic analyses	III

2.1 STUDY I (PAPERS I AND II): OCCURRENCE AND DISTRIBUTION OF MOSQUITO SPECIES WITH POTENTIAL FOR TRANSMITTING RVFV, DENV, AND OTHER ARBOVIRUSES.

2.1.1 Study Design and Procedures

Entomological field surveys for this study were conducted from 19 March to 30 April 2016 in 32 districts covering all Provinces from the three administrative regions (northern, central and southern) to have an updated and approximated picture of the occurrence of mosquito species potential vectors of arbovirus in the different ecological settings throughout the country and to investigate the risk of the dengue outbreak occurred in 2014 and 2015 in Mozambique.

This study aimed at determining the distribution of urban mosquito species with the potential for transmitting RVFV, DENV and other arboviruses of public health relevance in the region.

2.1.2. <u>Paper I</u>. A cross-sectional study was conducted between March 19 and April 30, 2016, during the rainy season, in a total of 32 districts. The sampling approaches applied to select the household were stratified in three stages. The first stage involved the selection of all the eleven provinces of Mozambique to ensure that every province was represented in this survey. In each Province, three districts were selected for the second stage and in each district one village or neighbourhood was selected to complete the third stage, based on the following criteria: i) occurrence of confirmed dengue cases in the preceding months or years, and ii) climatic and socio-demographical factors (human population density and degree of urbanization) considered suitable for the occurrence and establishment of dengue vectors. The most populated and urbanized village or neighbourhood was preferentially chosen.

A spatial sampling procedure oriented to clusters of households was adopted to select households. A cluster was considered a geographical area comprising between 10-20 households located within a radius of 50-100 metres. The selection of a household cluster was carried out following the procedure described by Troyo *et al*., (Troyo *et al*., 2008). According to this procedure, an administrative map of each village/neighbourhood was obtained using Google Earth Pro v. 7.3.0 (Google Inc., USA). Then, grid cells of 10km2 of the area were drawn on the map. The number of grid cells varied according to the size of the region. Grids were numbered starting from the cell on the upper left corner of the map. Then, a random sample of three 10km2 area grids was selected for the household cluster survey. In each of these grids, three clusters comprising 10-20 households were selected, based on the accessibility of the location. The clusters were at least 400 metres apart, considered to be the maximum distance of *Ae. aegypti* flight (Reiter *et al*., 1995), to reduce the likelihood of pseudoreplication. A household was defined as a single unit of accommodation (individual household or an apartment) including the surrounding enclosure/compounds.

2.2.1. Entomological survey

In every household, intra and peridomestic breeding sites were inspected for the presence of immature stage (larva and pupa) of *Ae. Aegypti and Ae. albopictus* (Fig. 10). All selected households were assessed indoors and outdoors. We considered as outdoors any place outside the rooms, but inside the enclosure/compound, including the rooftop, while any place inside the household was classified as indoors. The immature stages were sampled in all water-holding containers following standard operating procedures for *Ae. aegypti* (WHO 2011). Containers were classified according to the presence of larvae (positive/negative). For small containers, the total number of larvae and pupae (as well as pupa carcasses) were collected using pipettes, whereas for containers ≥ 25 litres in volume or wells, the funnel and sweeping-net technique and dipper (500 µm of mesh diameter) were used (WHO 1975, 2011) and ten dips and sweeps were performed per container.

Figure 10. Investigator inspecting the presence of Aedes spp in a cement tank container.

Larvae were transported to the insectary and reared to adults under controlled environmental conditions of temperature (27ºC ± 2ºC). Adults were morphologically identified using the taxonomical key of Huang (Huang 1990). Two-experienced entomologists double-checked the identification of specimens. The field team in each province comprised four entomologists, two from the central level and two from the provincial level.

2.2.2. Mosquito collection, transportation, preservation and morphological identification

Water-holding containers were categorised according to the type of container. All information related to each container, including the presence of *Aedes spp*., and whether immature stages were sampled as larvae or pupae, was recorded in a field form. Immature forms were collected using a pipette or dipper net (5 x 7 cm, 500 µm mesh) depending on container type and its location in the household (Gratz 2004). All larvae and pupae were stored in a labelled specimen bottle and transported to local insectaries for growth until the adult stage according to the standard procedures for rearing mosquitoes (WHO 1975). Mosquitoes were sacrificed and preserved on a 1.5 ml tube containing silica gel upon adult emergence. . All preserved samples were transported to the Medical Entomology Laboratory at the National Institute of Health (INS) in Maputo for morphological identification of the *Aedes* species under a stereomicroscope using a taxonomic key (Huang 2004).

2.2.3. Data analysis

Data were entered into a database developed using Microsoft Excel 2013 imported into Stata 13 for descriptive data analysis to determine the frequencies and distribution of Ae. aegypti and Ae. albopictus. The container index (CI) was determined using the following formula: CI=Total nº of positive container / Total nº of water-holding containers ×100% (Focks 2003). The spatial variation of CI estimates for each region was visualised in maps operating ArcGIS 10.2 Software (ESRI Inc, Redlands, CA), used to produce occurrence maps.

2.3. <u>Paper II</u>: Entomological field surveys were conducted in April of 2016 in Lago District, a neighbourhood of Maniamba, Niassa Province, in Northern Mozambique. All potential types of natural and artificial mosquito breeding sites were surveyed for the presence of immature mosquito stages. Following standard operating procedures, mosquito larvae and pupae were sampled (WHO 2011). Additionally, used car tires filled with water were placed approximately 500 metres apart in a transect along the main road crossing Chapama forest and Luaui river to collect as many samples as possible at different sites in the vicinity to better sample the area. The tires were left in the field for eight days, after which they were surveyed for immature mosquitoes. Each breeding place was surveyed using a Pasteur pipette. Collected specimens were sorted, placed in the 500 ml plastic bottles, filled with up to 75% of the water from a specific breeding place and labelled accordingly. All samples collected were then transported to local insectaries for rearing in adults (WHO 1975, 2011). Preliminary morphological identification was conducted on adult stages that emerged, using taxonomic keys (Ribeiro 1967; Gillies & De Meillon 1968; Huang 1990; Jupp 1996; Mixao *et al*., 2016). For further morphological and molecular analysis, each adult specimen was preserved in single 1.5 ml Eppendorf tubes at -80 °C.. Whole male and female mosquitoes of *Aedes (Stg) luteocephalus* were re-observed, male terminalia was separated from the abdomen, and the genitalia was adsorbed in Marc André solution (Ribeiro 1967). Genitalia was dissected under a stereomicroscope and mounted in formic acid-polyvinyl alcohol (PVA) solution between slide and coverslip and photographed (Ribeiro 1967; Mixao *et al*., 2016) under Olympus stereo microscope SZ51, Olympus microscope (BX51) and an Olympus SC30 digital camera respectively.

2.3.1. Molecular analyses of adult mosquito specimens

Genomic DNA was extracted from remaining the abdomen and legs of 4 males, as described elsewhere (Mixao *et al*., 2016). Molecular analysis was targeted at the barcoding section between positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome oxidase gene subunit I (cox1 mtDNA). Amplification of cox1 mtDNA was performed using LCO1490 and HCO2198 specific primers, and PCR conditions as described by Folmer *et al*., (Folmer *et al*., 1994). The nucleotide (nt) sequences obtained were deposited in the GenBank database https//www.ddbj.nig.ac.jp/data-

release-policy-e.html (Clark *et al*., 2016) under accession numbers (LC536733; LC536734; LC536735 and LC536736).

The degree of correspondence between the barcode cox1 mtDNA gene sequences obtained in this study was compared against those at the GenBank database using BLASTn, (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Barcode of Life Data Sytems-v4 (available at http://www.boldsystems.org/) (Ratnasingham & Hebert 2007).

Phylogenetic reconstructions using cox1 molecular data were carried out from multiple alignments of nt sequences obtained using the iterative G-INS-I method implemented in MAFFT vs 7 (Katoh & Standley 2013). Subsequently, attained sequences were edited using both GBlocks (Castresana 2000) and visual inspection using BioEdit 7.0.5 (Hall 1999) to ensure the correct alignment of homologous codons. Phylogenetic analysis was carried out using the Maximum Likelihood (ML) optimization criterium and GTR+Γ+I (GTR-General Time Reversal, Γ-Gamma distribution, I-proportion of invariant sites) as the dataset best-fitting evolutionary model, as suggested by JModeltest2 (Darriba et al., 2012). The ML phylogenetic tree was constructed with W-IQ-tree (Trifinopoulos et al., 2016), using the bootstrap test (with 1000 random data resampling) to assess the tree's topological stability. The tree was edited for presentation with Figtree1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Due to the lack of a number of sequences and specimens, we were unable run haplotypes networking analysis for robust inference of the origin of *Ae. luteocephalus* collected in this study.

3. STUDY II (PAPER III): ABUNDANCE, DIVERSITY, AND SEASONAL ASSEMBLAGES OF MOSQUITO SPECIES POTENTIALLY ASSOCIATED WITH ARBOVIRUS TRANSMISSION IN MOZAMBIQUE.

3.1. Study Design and Procedures

Studies were conducted in two provinces, Maputo (southern region) and Zambézia (central region) Provinces. In Maputo Province, surveys were performed in the Goba locality (Namaacha district), whereas in Zambézia, surveys were undertaken in the Mopeia district, located along the Zambezi River. The two locations are highly endemic to malaria, and there has also been an indication of arbovirus transmission in the region (Fafetine *et al*., 2016). The study settings were selected based on the recent reports of confirmed and suspected cases of mosquito-transmitted arboviral diseases, particularly, the Rift Valley fever virus in Goba and Mopeia districts (Fafetine *et al*., 2013a; Fafetine *et al*., 2016).

Other preliminary sporadic collections were also performed on the logistics convenience in Maputo City and Massingir district (Limpopo National Park) in Gaza Province.

3.2. Mosquito sampling strategy

Series of overnight (6:00 PM-6:00 AM) host-seeking adult mosquito collections were performed outdoors in each study site using conventional CO_2 – baited (dry ice) CDC light traps and Tent/ Net traps equally baited with CO_2 as dry ice (Fig. 11 A and C). Therefore, temporary mosquito sampling sentinel sites were set up close to previously identified homesteads, and mosquito traps were deployed near animal shelters (cows, goats, sheep herds) in an occupied room (Fig. 11 B). Traps were rotated between sites to reduce the influence of sites on mosquito catches sizes and composition. Collections in Goba and Mopeia were performed all year round, from November 2014

to December 2015, once monthly in each site, to estimate mosquito community fluctuation trends. However, due to logistic limitations, collections were not possible all year round in Goba.

Collections in Maputo city were performed in November and December in 2014; June in 2015, and June and July in 2017, while in Massingir were carried out in November 2014 and June 2015. Nevertheless, due to the lack of logistics, it was not possible to continue with the collection in those two locations.

Figure 11. Host-seeking adult mosquito collections using light trap (A.1. and A.2.), animal shelter (B.), and Tent/Net trap (C). Both "A" and "C" were baited with attractant CO_2.

3.3. Sample processing and identification

Mosquitoes were transported to the laboratory to sort and identify species in dry ice. Morphological identifications were performed using the keys proposed by Edwards (Edwards 1941), Gillies and De Meillon (Gillies & De Meillon 1968), Service (Service 1990), Jupp (Jupp 1996), and Ribeiro and Ramos (Ribeiro *et al.*, 1980). Immature mosquitoes were reared to adults in holding cages at insectary standard conditions of temperature (27 ± 2 ºC) and humidity (87 ± 10%). Newly emerged adults were removed from the cage, killed by freezing in the camping freezer for morphological

identification. Female mosquitoes were pooled according to species, locality and time of collection for arboviral screening. Male mosquitoes and specimens of either sex that, for some reason (e.g., loss of body parts, rubbed scales) were not possible to be identified morphologically, were submitted to identification by genitalia dissection and mounting for later and broad systematic study along with male genitalia dissection and mounting (ongoing study). They herein referred to as "genera sp". Pools submitted for virus screening, that were morphologically identified only to the general level were confirmed by molecular approaches, which consisted of sequencing of the barcoding section between positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome oxidase gene subunit I (cox1 mtDNA) in order to identify to the species level, as mentioned in (Abilio *et al*., 2020).

3.4. Statistical analysis

Overall mosquito community abundance was estimated as the average number of specimens sampled/ Trap/night. Observed mosquito community richness was determined as the total number of species observed by location/month. The diversity of mosquito community in each location was estimated as mosquito species richness (SR), representing the total number of all mosquito species expected to occur in the studied and the effective number of species (ENS), expressed by transforming the Shannon-Weaver and Simpson diversity indices into Hill's numbers (qD), where q is the Hill´s number order which controls the weights of common and rare species; q = 0 for species richness, q=1 for a number of equally common species, obtained by the exponential of the Shannon–Wiener Index [exp(H')], and q = 2 for highly abundant species resulting from the transformation of Gini-Simpson (D) (Jost 2007; Chao *et al*., 2014). The true SR was estimated by the Chao1 bias-corrected (Chao1 bc) species estimator (Chao *et al*., 2014). The completeness of overall mosquito samples for estimation of global community ecology metrics was assessed by the mean of species-accumulation curves (Hsieh et al., 2016). The degree of variability in mosquito abundance and composition between sites, season, and the association with climate variables, namely temperature, humidity, and rainfalls, was determined using multivariate abundance generalized linear models (manyGLM) proposed by Wang and colleagues (Wang et al., 2012). The manyGLM model assumed negative binomial distribution of mosquito abundance with a log-link function to predictors. The possible non-linear effect of continuous predictors (temperature, humidity, and precipitation) was modelled using Penalized spline function (Eilers & Marx 1996). Data processing tasks and statistical analyses were undertaken using R software version 4.0.2 (R Core Team 2020).

4. STUDY III (PAPER IV): PRESENCE OF FLAVIVIRUS, ALPHAVIRUS AND BUNYAVIRUS GROUPS IN MOSQUITO POPULATIONS FROM DIFFERENT REGIONS OF MOZAMBIQUE

4.1. Study Design and Procedures

A total of 14,519 mosquitoes were collected in rural settings in Mozambique (located in west southern Africa) between November 2014 and December 2015 as part of the work of study II paper III at Massingir (in the province of Gaza), Namaacha (in the province of Maputo), and Mopeia (in the province of Zambézia). The general biotypes for Goba were savanna with medium grassland located around 10 to 500 m from a water stream. Collection sites in Massingir and Mopeia corresponded to forest environments located close to the Limpopo and Zambezi rivers, respectively.

The adult mosquitoes were collected using a combination of sampling methods that included indoor resting, tent collections and those carried out using CO_2-baited miniature CDC-light traps.

These mosquitoes were stored in dry ice and then transported to the laboratory for sorting and taxonomic identification using keys proposed by Gillies and Coetzee (Gillies & Coetzee 1987) and Jupp (Jupp 1996), the systematic classification of which is followed in this thesis. The manipulations of specimens for identification were carried out at temperatures close to or approximate to 0 °C under a stereomicroscope equipped with an ice block. Male and blood-fed specimens were excluded from this study. All samples were then stored at -80 °C until viral screening was carried out.

4.2. Preparation of mosquito homogenates, and nucleic acid extraction

The preparation of mosquito homogenates was based on a preliminary grouping of the collected and identified specimens in pools according to their species, sex, geographic origin, and blood-fed status. These mosquitoes were mechanically disrupted in 15 ml Falcon tubes by vortexing using glass beads and aluminium oxide in 1 ml of phosphate buffer saline (PBS) buffer. After three pulses of 1 min (with 30 sec breaks on ice), the mosquito macerates were clarified by centrifugation, as previously described (Carapeta et al., 2015). RNA, and DNA, were extracted from 200 ml of clarified mosquito homogenate using NZYol® (NZYTech, Portugal), as indicated by the supplier. The extracted RNA was dissolved in 30 ml nuclease-free water, while the obtained DNA sediments were dissolved in 40-100 ml using a 1:1 mixture of 8 mM NaOH and TE buffer (Tris 100 mM, EDTA 1mM, pH=7).

4.3. Viral genome detection

The extracts of total RNA served as a template for the synthesis of cDNA, which was carried out with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) using random hexamers and a thermal profile including 10 min at 25 °C, 45 min at 52 °C and 10 min at 80 °C (for enzyme inactivation), followed by treatment with RNaseH (20 min at 37 °C).

Detection of flavivirus NS5 sequences (encoding the viral RNA-dependent RNA polymerase, or RdRp) was carried out using previously described primers and reaction conditions (Vazquez *et al*., 2012). A generic PCR method using degenerate primers targeting the nsP4 gene (also encoding the viral RdRp) was used to detect the presence of the genomes of alphaviruses (Sanchez-Seco *et al*., 2001), while RVFV genomic NSs coding sequences were tentatively detected as previously described (Sall *et al*., 2001). Finally, the presence of phleboviruses and orthobunyaviruses L sequences (also encoding an RdRp) was investigated using the ppL1/ppL2 sets of primers/reaction conditions previously described by Matsuno and others (Matsuno *et al*., 2015) and the technical modifications suggested by Pereira and others (Pereira *et al*., 2017), or as defined elsewhere (Silva *et al*., 2019). Nelorpivirus detection was carried out as previously defined (Carapeta *et al*., 2015). PCR amplifications were carried out using NZYTaq 2X Green Master Mix (NZYTech, Portugal). The obtained amplicons were purified and directly sequenced or cloned in either pGEMT-easy® (Promega, USA) or pNZY28-A using the NZY-A PCR cloning kit (NZYTech, Portugal), followed by DNA sequencing of individually purified plasmid recombinant-DNA molecules.

4.4. Cell culture and virus isolation

Aedes (Ste.) albopictus C6/36 cell line was used for virus isolation. Cells were maintained at 28 °C (in the absence of CO2) in L-15 Leibovitz Medium (Lonza, USA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (Lonza, USA), two mM L-glutamine (Gibco BRL, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, USA) and 1×tryptose phosphate broth (AppliChem GmbH, Germany). Approximately 500 ml of filter-sterilized mosquito homogenates were diluted in the same volume of phosphate-buffered saline (PBS) and inoculated onto semi-confluent layers of C6/36 cells grown in T25 culture flasks (Nunc, Denmark). After one h at room temperature (for viral adsorption), the viral inoculum was removed, 5 ml of L-15 Leibovitz Medium (2 % FBS) was added to each flask, and the cell cultures were incubated at 28 °C for a week. Culture supernatants

collected after a single blind-passage was used as viral stocks and stored at −80 °C. Cytopathic effect (CPE) was determined by microscopic observation of the inoculated cell cultures.

4.5. DNA sequencing and genetic analyses

Multiple alignments of nucleotide (nt) or amino acid (aa) sequences were performed using the iterative G-INS-I and E-INS-I methods as implemented in MAFFT vs 7 (Katoh & Standley 2013), followed by editing using both GBlocks (Castresana 2000) and visual inspection. The multiple sequence alignments of nucleotide sequences were systematically verified to ensure the correct alignment of homologous codons using BioEdit 7.0.5 (Hall 1999).

Phylogenetic trees were constructed using both Maximum Likelihood (ML) and Bayesian approaches. The best-fitting evolutionary models used were those suggested by JModeltest2 (Darriba et al., 2012) and W-IQ-tree (Trifinopoulos *et al.*, 2016) for the analysis of nt (GTR+Γ+I: GTR-General Time Reversal, Γ-Gamma distribution, I-proportion of invariant sites) or aa alignments (LG+G: Le-Gascuel, Γ-Gamma distribution). Phylogenetic analyses based on the ML optimization criterion were carried out using the Mega 6.0 software (Tamura *et al.*, 2013), and the stability of the obtained tree topologies was assessed by bootstrapping with different re-samplings of the original aligned positions (1000 for nt alignments, 100 for aa sequence data). Phylogenetic reconstructions following a Bayesian approach were carried out by running two independent Markov chain Monte-Carlo (MCMC) analyses using BEASTv1.7.5 (Drummond *et al.*, 2012), assuming a relaxed uncorrelated lognormal molecular clock model (Ho *et al.*, 2005) as suggested by the ML Clock Test implemented in Mega 6.0. The MCMC chains were run until 100,000,000 states were sampled using both logistic population growth and Gaussian Markov random field/GMRF skygrid demographic priors. The Tracer software (http://beast.bio.ed.ac.uk/tracer) was used to diagnose stationarity and adequate (>300) effective sample size (ESS). The trees were logged on every 5,000th MCMC step, and the tree sample was summarized using TreeAnnotator v1.8.3 as maximum clade credibility (MCC) trees, with median heights used as the node heights in the tree, after discarding 10% of them as burn-in. The FigTree v1.4.2 software was used to visualize the phylogenetic trees (http://tree.bio.ed.ac.uk/software/figtree/).

The molecular confirmation of the morphological identifications of mosquitoes was carried out based on the analysis of the barcoding section (from positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome oxidase subunit I-COI) essentially using Bold Systems-v4 (available at http://www.boldsystems.org/).

The nt sequences obtained in this study were deposited in the GenBank/ENA/DDBJ databases under accession numbers LC461994-LC462019, LC-462246-LC462257, and LC517270-LC517293. The reference sequences used for analyses presented in this manuscript were directly downloaded from the public sequence databases. Whenever necessary, nt sequence similarity searches were carried out using BLASTn, and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

5. LIMITATIONS OF THE PROJECT

This thesis is descriptive and relies on the characterization of potential vectors and the screening of arbovirus in mosquitoes from the selected settings of Mozambique, which could increase the probability of procedures bias. For the occurrence and distribution of *Ae. aegypti and Ae. albopictus* species, known as the major arbovirus vectors, it was possible only to collect samples from 32 out of the 152 districts of Mozambique. Expanding larvae and pupae sampling over more cities would produce a more detailed risk map for arboviruses transmission in Mozambique. The lack of sufficient logistics affected negatively the time needed for performing all necessary data collections. A reasonably longer sampling period would allow collecting sufficient data to estimate not only Container Index (CI) but also other entomological indicators of arboviral diseases epidemic risks,

notably House Index (HI) and Breteau Index (BI). Additionally, it was impossible to perform collections from February to June 2015 in the Goba district due to the logistics issue. That temporal gap in the data prevented an accurate characterization of the temporal dynamics of mosquito-borne infections exposure risks from being made. The very limited number of sampling days (one day/site/month) may have also contributed to the failure to detect all mosquito species occurring in the Mopeia district and the presence of human pathogens associated with a mosquito-borne arbovirus. Several other factors may affect the likelihood of mosquito detection, such as the differential response of mosquitoes to CO_2 baited light traps and tent traps used. Combining several trapping methods would help increase human-associated virus detection probability. Usually, detection probability is highly facilitated in the presence of outbreaks or visible clinical symptoms in vertebrates during the mosquito collection periods. Additionally, there is a technical limitation associated with the use in this study of a less technologically advanced virus detection approach based on conventional RT-PCR as opposed to more sensitive virus screening approaches such as metagenomic combined with the use of next-generation sequencing (NGS) methods. In general, the budget limitation was the main bottleneck for achieving this study's main goals.

6. ETHICAL CONSIDERATIONS

All households with homesteads where animals (cows, goats, sheep or buffalos) are housed were informed about the purpose and nature of the study. No human volunteers were used as mosquito bait during the study. Animal baits that might be used were obtained in the local communities and were handled by an experienced-trained veterinary technician from the staff certified to handle animals. The main sampling unit of the study was mosquitoes, so no human or animal fluids were collected or kept during the study.

Ethical clearance to undertake the study was obtained by the Faculty of Medicine of Eduardo Mondlane University (Ref #: CIBS FM&HCM/15/2018).

CHAPTER 4

RESULTS

This section presents the main findings based on the methodologies applied to gather all research information from the three studies' objectives as follows:

1. STUDY I (Paper I)

A total of 2,807 water-holding containers were inspected, of which 628 (22.4%) were positive for *Ae. aegypti*. *Aedes albopictus* was only found in a single breeding site located in the Moatize district (Central region), which was also positive for *Ae. aegypti* (Fig. 12).

The Container index (CI) of *Aedes spp*. was higher in the Central region (43.6%; 260/596), followed by the North (36.9%; 228/617), whilst the lowest CI was found in the South region (8.7%; 140/1594).

In the Northern region, the highest *Ae. aegypti* CI at the Province level was reported in Nampula (49.4%; 158/320), followed by Cabo Delgado (24.3%; 28/115) and Niassa (23.1%; 42/182) (Table 1). The districts of Nacala Porto (CI = 68.1%; 47/69) and Nampula city (CI = 46.7%; 78/167) in Nampula Province, and Pemba Metuge (CI = 42.8%; 9/21), in Cabo Delgado Province, exhibited the highest infestation levels of *Ae. aegypti.*

Regarding the Central region, the highest *Ae. aegypti* CI was registered in Manica (53.5%; 107/200), followed by Tete (46.2%; 24/52) and Sofala (38.4%; 53/138) Provinces. The lowest CI was found in Zambézia Province (35.0%; 75/214). The highest *Ae. aegypti* infestation levels were found in Milange district (CI = 62.3%; 33/53) in Zambézia Province, Changara district (CI = 61.1%; 11/18) in Tete Province and Sussundenga district (CI = 60.3%; 35/58) in Manica Province.

In Southern Mozambique, the highest CI was reported in Maputo city (37.5%; 15/40), followed by Maputo (16.8%; 48/285) and Gaza (13.1%; 52/396) Provinces. The lowest CI was reported in Inhambane Province (2.9%; 25/863). The districts with the highest *Ae. aegypti* CI in the South were Kamachaquene (50.0%; 2/4) and Kanfumo (36.1%; 13/36) in Maputo city and Matola district (30.2%; 29/96) in Maputo Province.

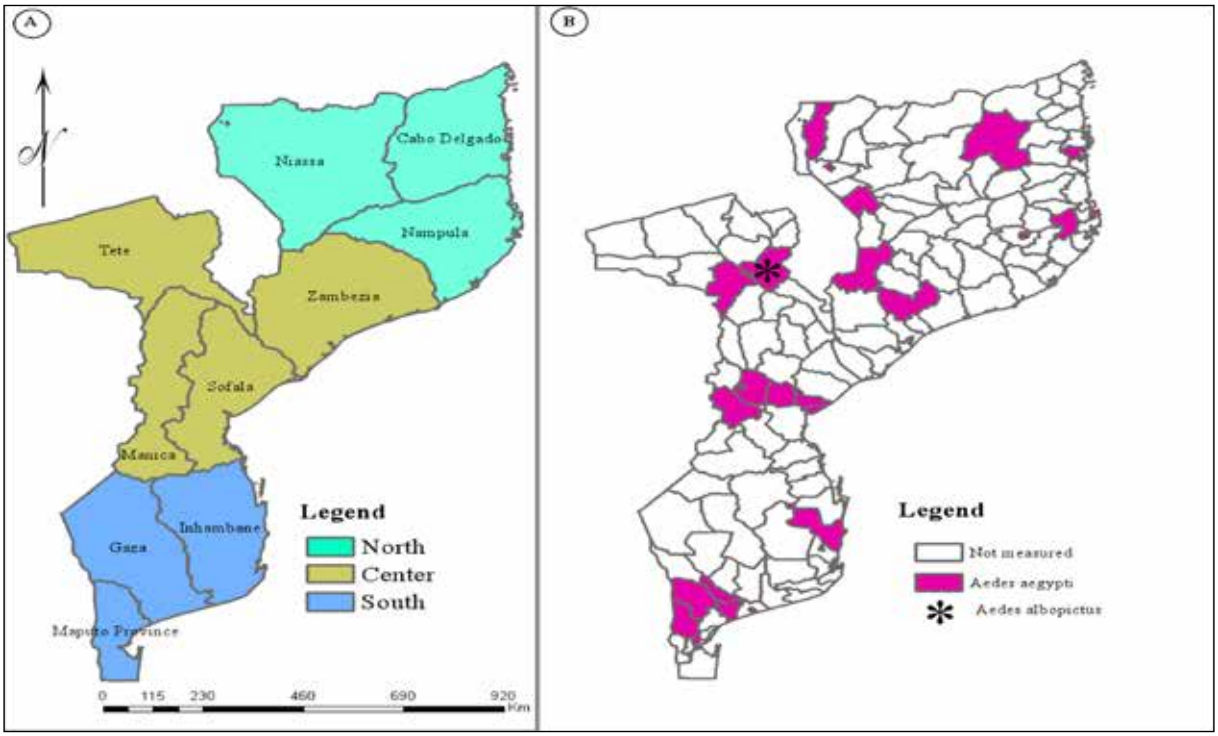

Figure 12. Map showing main regions (A) and sampling location (B) of Mozambique.

1.1. Breeding sites of *Ae. aegypti* and *Ae. albopictus*

Used tires were the most frequent type of containers, followed by flower pots, jar/pots, cement tanks, buckets, disposed cans and bottles. A total of 2,807 potential breeding containers sub-divided into nine different groups were sampled. The highest *Ae. aegypti* immature stages positivity rates were found in used tires (35.3%; 448/1268), cement tanks (32.3%; 20/62) and drums (22.1%; 21/95). On the other hand, cans (9.5%; 14/146), bottles (9.4%; 7/74) and flowerpots (6.3%; 36/576) had a lower infestation. The Ae. albopictus larvae found Moatize district, Tete Province, came from a used tire.

1.2. STUDY I (Paper II)

A total of 92 adult mosquitoes emerged from collected larvae and pupae; of these, 16 were tentatively identified as *Ae. luteocephalus* (12 females and four males) based on morphological features. The remaining specimens were identified as *Anopheles (Celia) garnhami (n = 1), Ae. (Aedimorphus) vittatus (n = 24), Ae. aegypti (n = 4), Culex (Culiciomyia) nebulosus (n = 28), Eretmapodites subsimplicipes (n = 18) and Toxorhynchites brevipalpis (n = 1). Ten females of Ae. luteocephalus* collected in this survey were deposited in the insect depository of Instituto Nacional de Saúde (INS) in Maputo Province, Mozambique, stored in individual Eppendorf® tubes (accession numbers MZ113-a1.2, a1.4-a1.12) and six specimens (two females and four males) deposited in the Entomoteca (Insect collection) of the Institute of Hygiene and Tropical Medicine (IHMT), Lisbon, Portugal (accession numbers MZ113-a1.1-a1.3 and MZ113-a2.1-a2.4).
All 16 larvae, which gave rise to the adults *Ae. luteocephalus and 5 Ae. vittatus* were found cohabiting in a rock-pool of clear water, with approximately 20 x 15 cm located at the Luaui riverbank and exposed to sunlight. Other species including the remaining 19 *Ae. vittatus* were obtained from other breeding sites, namely the tires that were placed as "ovitraps", while no specimens of *Ae. luteocephalus* were obtained from any other breeding site.
Preliminary analysis of the nucleotide sequences of cox1 mtDNA obtained from the four males revealed completely identical sequences.
All *Ae. luteocephalus* specimens collected in this study had a distinct middle longitudinal yellow stripe of thin scales in the scutum region; scutellum with wide white scales on lateral lobes; basal pale band on terga II-VI more yellow; and hind femur anteriorly with a huge light band at the base and alongside two sizable white spots on median and apical regions (Fig. 13). These characteristics are similar to those described by Huang (Huang 1990) and Jupp (Jupp 1996).

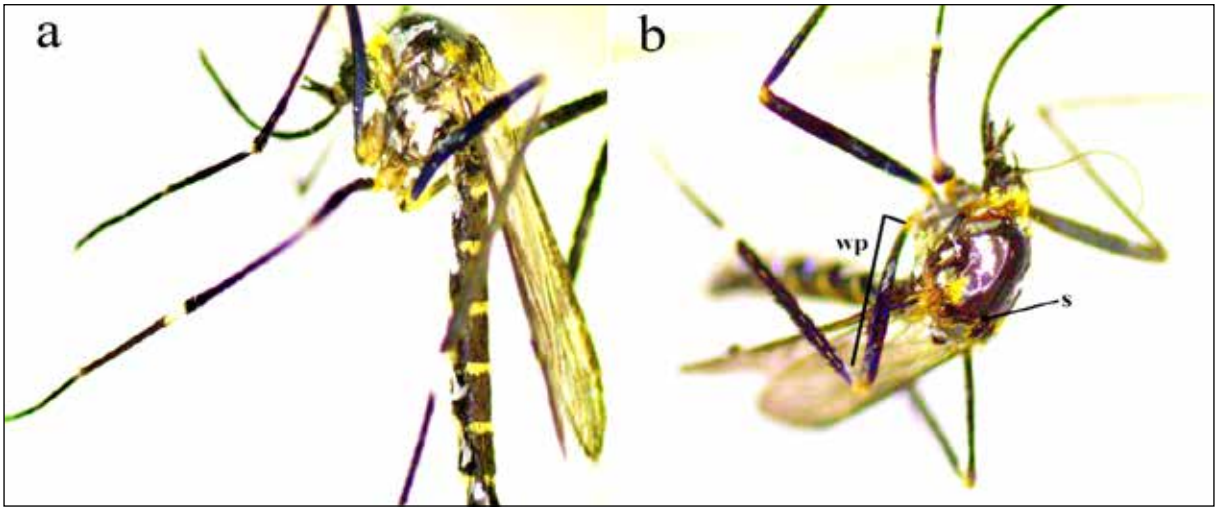

Figure 13. Adult female of Aedes luteocephalus specimen showing general characters (a). Specimen adult highlighting the main diagnostic features including for the scutum (with median-longitudinal yellow stripe) (s in b) and hind tarsomere anteriorly with large pale band at base and two large white patches on median and apical areas (wp in b), both images at 20× magnification.

All four dissected male genitalia showed gonocoxites with gonostylus simple with few setae in the apical quarter and a long slender gonostylar claw, claspette large, lobed, with distal expanded portion, oval in dorsal view, with numerous simple setae on the apicolateral portion, and with some short setae on the apicomesal portion (Fig. 14). These are considered the most important distinctive features that separate the species from other members of the *Africanus* group, namely, *Aedes (Stg.) africanus*, to which it belongs (Huang 1990; Jupp 1996).

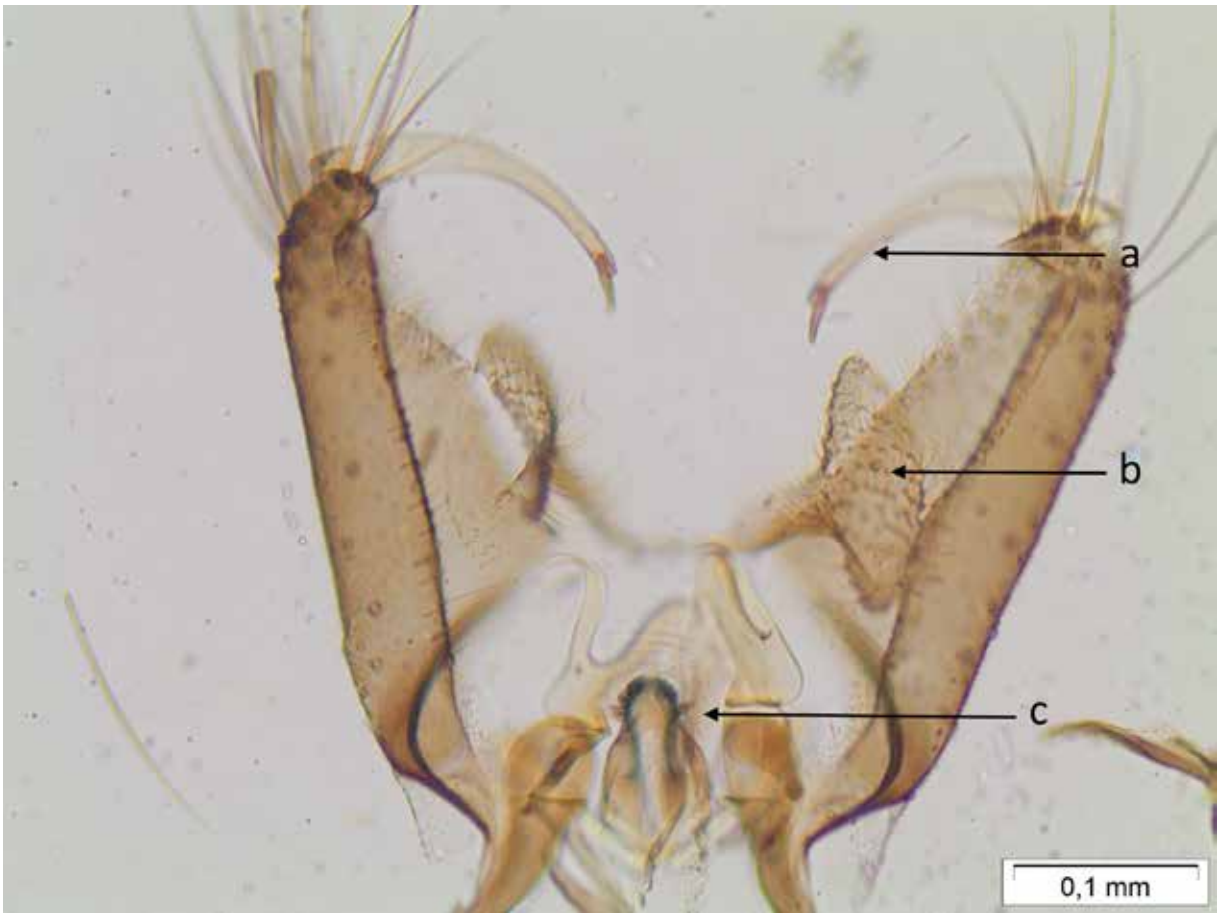

Figure 14. Dissected male genitalia of Aedes luteocephalus showing gonocoxites with gonostylus (a) with gonostylar claw and claspette (b) large, lobed with distal expanded portion, oval in dorsal view, with numerous simple setae on the apicolateral portion, and with some short setae on the apicomesal portion and aedeagus (c) at 100× magnification. Scale-bar: 100 µm.

Barcode gene sequences of all specimens analyzed displayed 97.65–98.12% sequence identity with homologues using BLAST (MegaBlast option) and 97.82–98.26% identity in the BOLD SYSTEMS database with sequences of *Ae. luteocephalus* from Tanzania and Kenya, thereby confirming its taxonomic identity (Muspratt 1956; Germain *et al*., 1981; Mutebi *et al*., 2012). Additionally, phylogenetic reconstruction analysis was carried out based on the dataset of multiple *Aedes* species of the subgenera *Stegomyia, Aedimorphus, Neomelaniconion and Ochlerotatus*, clearly placed the *cox1* sequences obtained in the course of this study in a topologically stable monophyletic cluster that only included *Ae. luteocephalus* reference sequences (Fig. 15). This further confirms the morphological, barcode and sequence similarity-based identifications presented above.

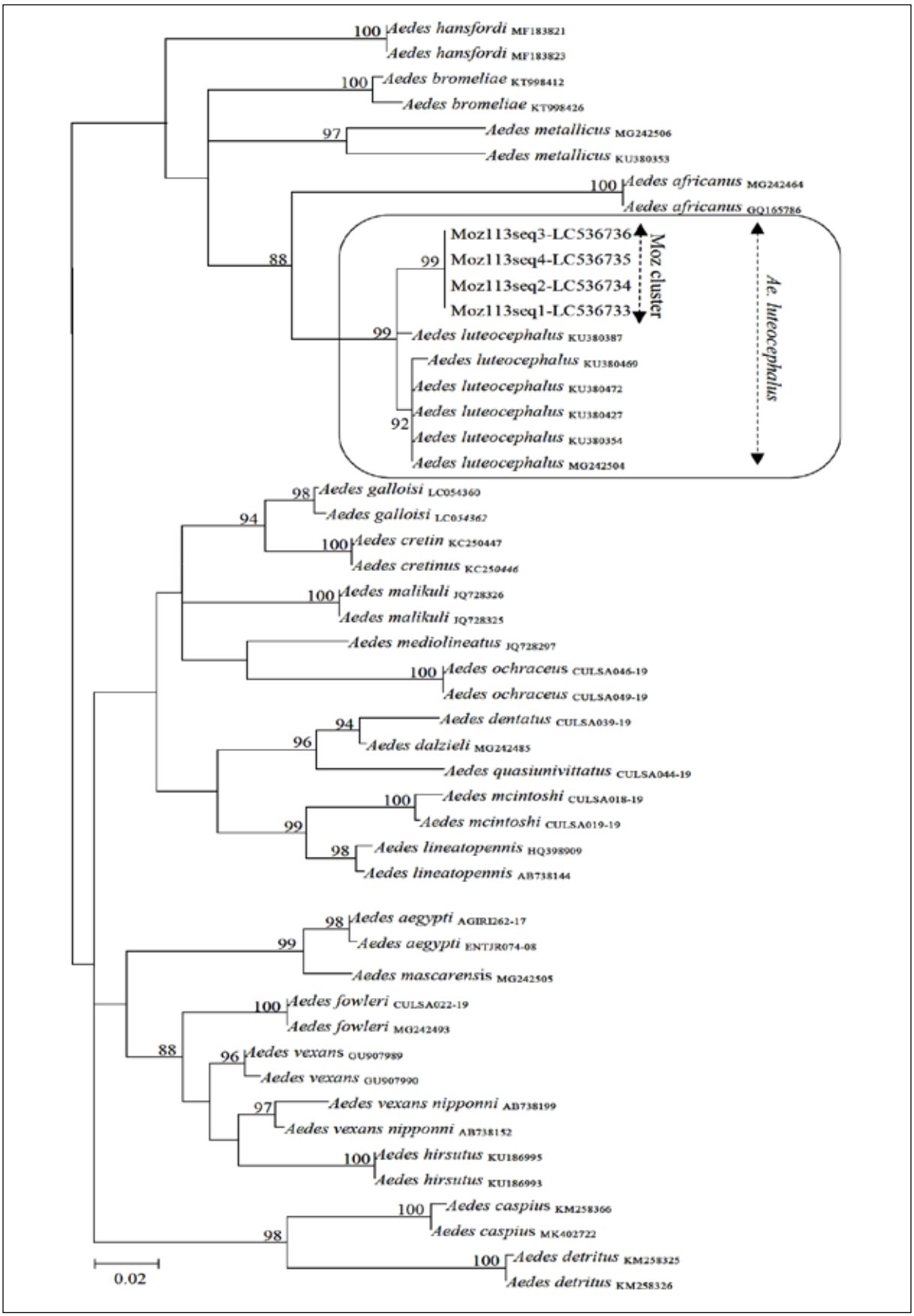

Figure 15. Maximum likelihood phylogenetic tree based on partial Aedes cox1 sequences. Nodal support values ≥ 75 are shown. The reference sequences used are indicated with either their GenBank accession number or BoldSystems code. The sequences generated in this study are indicated in bold by their laboratory code and accession numbers and are grouped in a monophyletic cluster indicated as Moz cluster. The scale-bar indicates the number of nucleotide substitutions per site.

2. STUDY II (Paper III)

2.1. Mosquito abundance and species diversity

A total of 33,621 mosquitoes were collected during the study period, of which 86.6% (29,109/33,621) were collected in the Mopeia district and 12.2% (4,092/33,621) in the Goba district (Fig. 16). The remaining 1.2% (1421/33,621) was collected from complementary surveys carried out in Maputo (n=98) and Massingir (n=322) districts. In the Mopeia district, *Culex (Cux.) antennatus, Mansonia (Mnd.) africana, Anopheles (Cel.) funestus and Ma. (Mnd.) uniformis* were the four most abundant (65.8%) mosquito species found accounting for 27.5% (8,005/29,109), 21% (6,113/29,109), 17.7% (5,140/29,109) and 15.6% (4,534/29,109), respectively. Similarly, in Goba, *Ma (Mnd.) africana, An (Cel.) funestus and Ma (Mnd.) uniformis*, were the most frequent, accounting for 34.8% (1,423/4,092), 32.9% (1,346/4,092) and 8.3% (339/4,092), respectively (amounting to 76%) (Fig. 16). Due to logistic hindrances, collections were not feasible in Goba from February to May 2015.

Regarding samples from sporadic collections, *Cx (Cux.) pipiens and An (Cel.) pharoensis*, were the most dominant mosquito species in samples from Maputo, Massingir districts, accounting for 72.4% (71/98), 30% (97/322), respectively (Fig. 17). This survey confirmed the presence of *Ae. albopictus* (5%) in Maputo city.

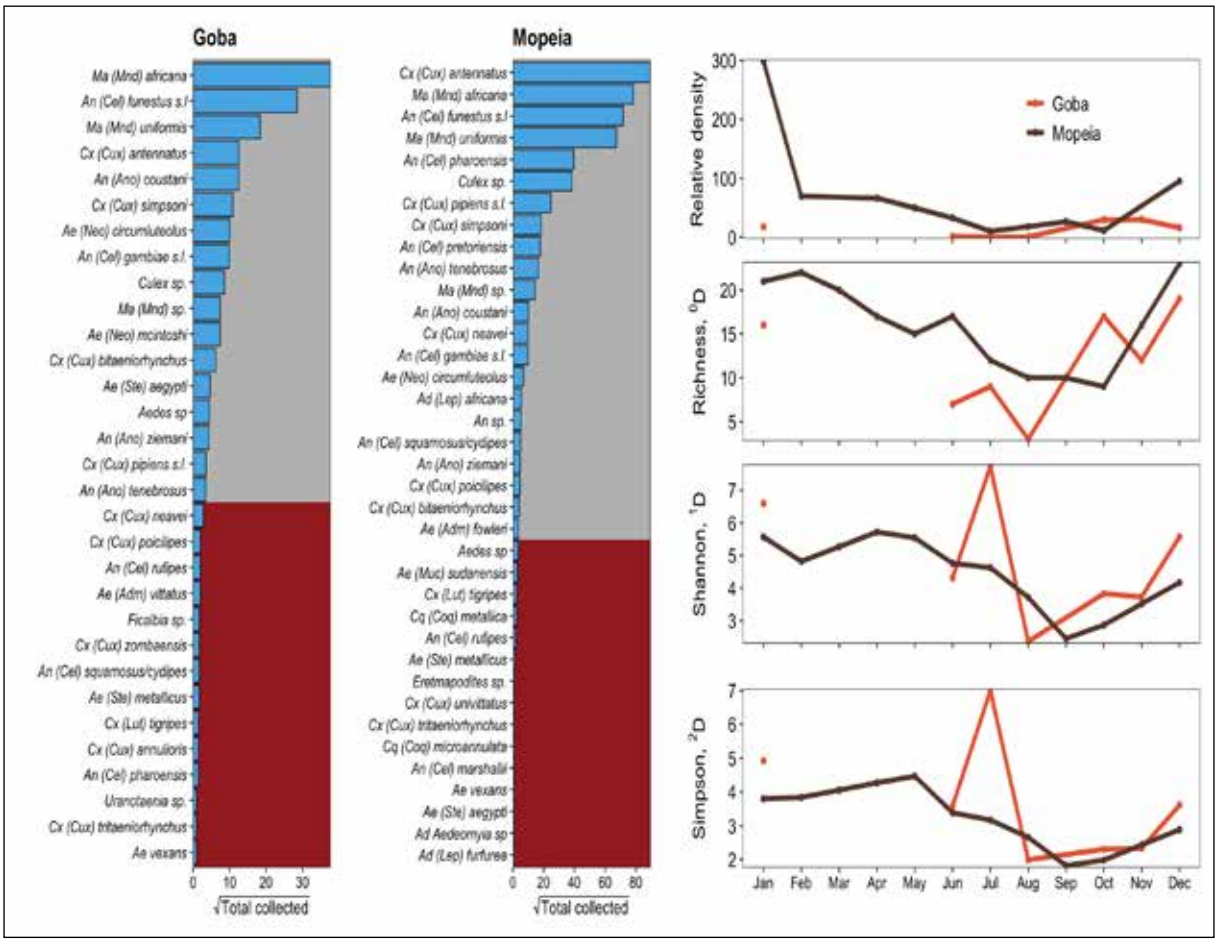

Figure 16. Structure and dynamics of mosquito communities of Goba and Mopeia districts. Bar charts show the abundance and composition of two regions mosquito communities highlighting the dominant (grey background area) and rare (brown background area). Line plots depict temporal dynamics of overall mosquito abundance, net species richness and the effective number of equally common species (^1D) and the most abundant species (^2D) in Goba and Mopeia district.

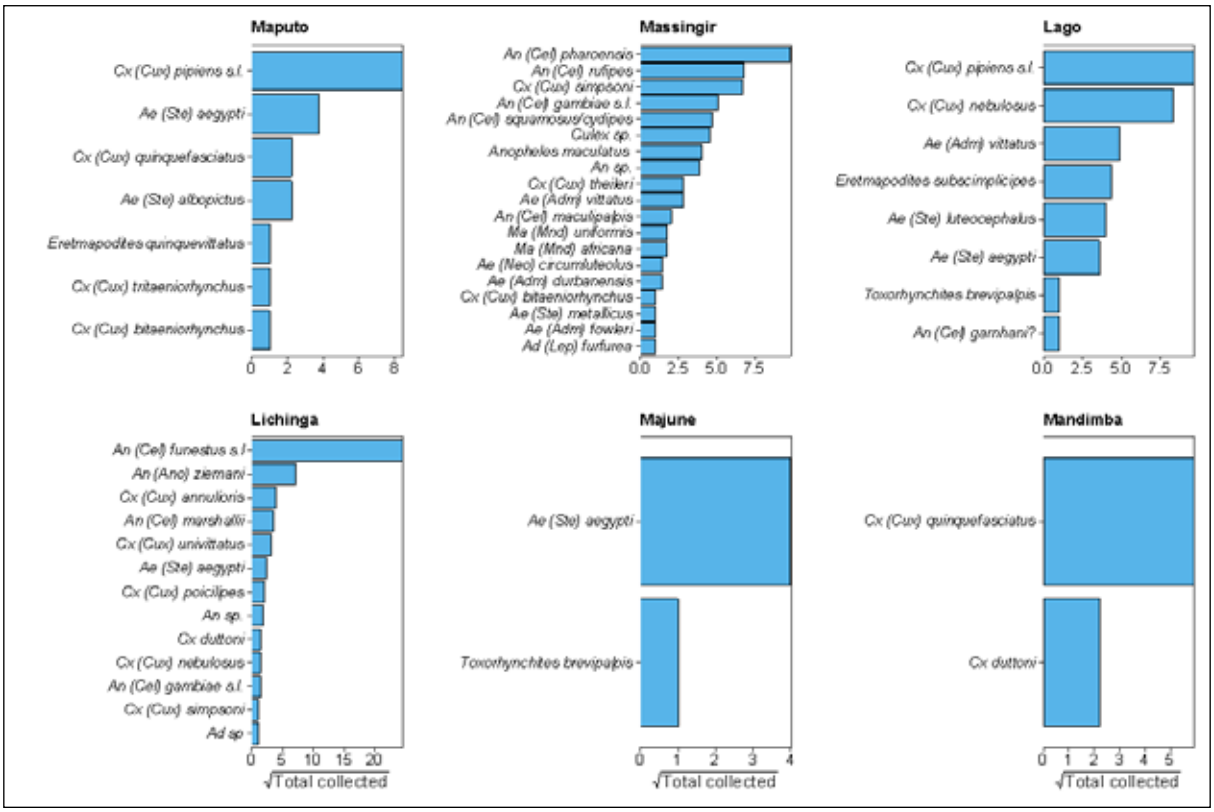

Figure 17. Abundance and composition of extra mosquito samples from Maputo city and Gaza Province (Massingir district).

Forty-three (n=43) mosquito species were identified during the study period, 31 of which were found in Goba and 37 in Mopeia districts. Four species and two genera *(Ae. mcintoshi, Ae. vittatus, Cx. zombaensis, Cx. annulioris, Ficalbia sp and Uranotaenia sp) were exclusively found in Goba, whereas nine species and one genus (An. pretoriensis, Ad. africana, Ad. furfurea, Ae. (Aedeomyia) fowleri, Ae sudanensis, Coquillettidea metallica, Cq. microannulata, An. marshallii, Cx. (Culex) univitattus, Eritemapodites sp.)* where only found in the Mopeia district. Chao1 bias corrected species richness estimator indicates that of the 31 species identified in Goba, 14 rare species and 17 were dominant species. Similarly, of the 37 species identified in the Mopeia district, 15 are rare species and 22 are dominant (Fig. 16). Overall mosquito abundance and species richness peak in December and January (Fig. 16). In both regions, the number of equally abundant (1D) and most abundant (2D) species remains high from January to July. These types of diversity reduce sharply untill reaching the minimum number in October. Chao1 species estimator also indicated that nearly 31.8 (31.1 - 39.4) mosquito species occur in Goba and 55 (41 – 118.3) species in the Mopeia district. Species-accumulation curve (Fig. 18) indicates that the sampling strategy was able of detecting nearly all mosquito taxa expected to be found in Goba but failed to estimate the true number of species occurring in the Mopeia district (Fig. 18). The overall mosquito abundance, richness, and diversity found in Goba and Mopeia are summarized in Tab. 3.

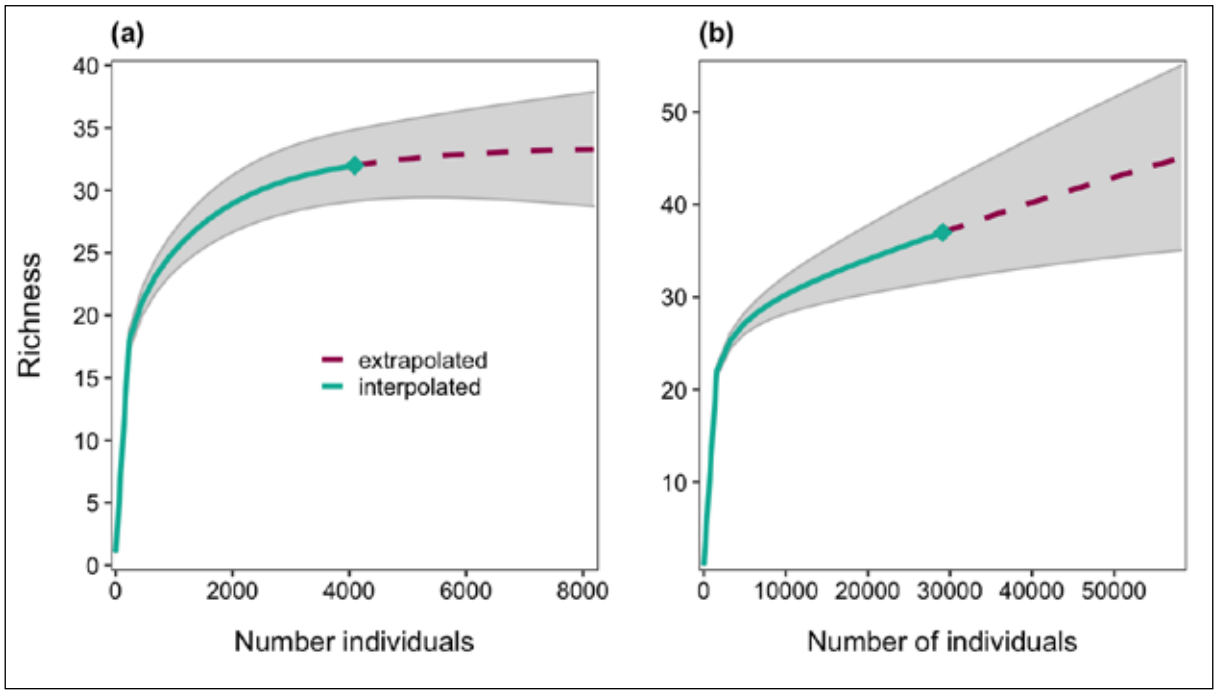

Figure 18. Specie-accumulation curves of mosquito samples from Goba (a) and Mopeia (b). The plateau in Goba curve indicates that the sample size was able to detect all possible mosquito species occurring in the region. Mopeia curve shows an increasing trend indicating that more samples needed to be taken estimates the true species richness (total number of species occurring in the region). The shaded grey area represents the 95% confidence intervals obtained by bootstrap method based on 200 replicates.

Table 3. Total number of specimens, richness and estimated species diversity of mosquito found in Goba and Mopeia. Shannon-Wiever and Simpson diversity were, respectively, transformed into the effective number of equally abundant species (^{1}D) and effective number of highly abundant species in the community (^{2}D).

Location	Total	Observed richness	Estimated richness (± 95% CI)¥	^{1}D ± 95% CI**	^{2}D ± 95% CI**
Goba	4,090	31	31.8 (31.1 - 39.4)	6.67 (6.63 - 6.92)	4.14 (4.13 - 4.28)
Mopeia	29,109	37	55 (41.0 - 118.3)	7.16 (7.16 - 7.24)	5.51 (5.51 - 5.57)

¥Calculated with Chao1 estimator; **Converted to effective number of species (Hill´s number).

2.2. Variation in mosquito composition, abundance and correlates

There was significant variation in mosquito community abundance and composition according to season, location, and changes in climate variables (Tab. 4). Season-to-season variations were mostly due to changes in *Cx. (Culex) bitaeniorhynchus, Cx. pipiens s.l., Cx. antennatus, Cx. poicilipes, Ae. metallicus, Ae. sudanensis and Anopheles sp.* abundances, as indicated by their contribution to overall deviances of season effect (Tab. 4). Similarly, location-to-location variability was largely caused by variations in *Ae. mcintoshi; Ma. uniformis, An. pretoriensis, Ma. africana; Ae. sudanensis and Anopheles sp* abundance. Regarding the effect of climate variables, rainfalls and average maximum temperature were the two climate factors that significantly influenced mosquito community composition. In general, overall mosquito community abundance and species richness increased nonlinearly with an increase in temperature and rainfalls (Fig. 19). However, many GLM tests indicate that variation of rainfalls only significantly affected *Ae. sudanensis, Ae. mcintoshi and Cx. simpsoni* abundance compared to other species in the communities, as indicated by those species' contribution to total deviance (Tab. 4). Similarly, the effect of temperature was only significantly observed in *Ae. fowleri, Ae. metallicus, Anopheles spp., Cq. metallica, Cx. annulioris, Cx. zombaensis*.

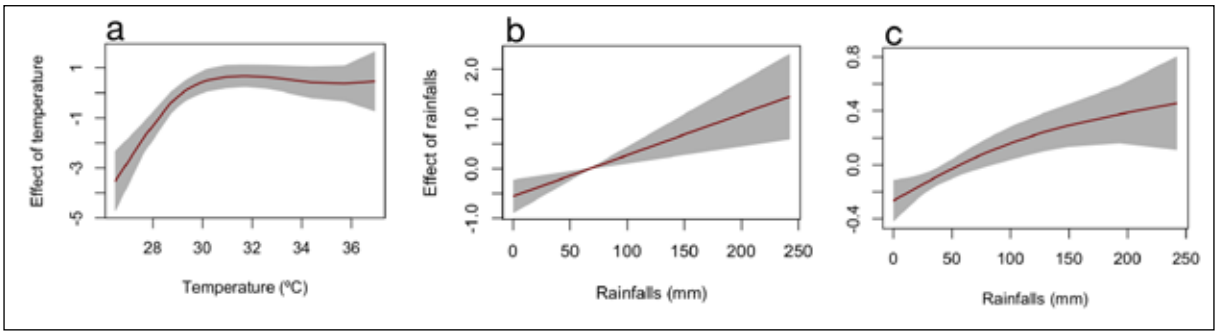

Figure 19. Association between variation of average maximum temperature and precipitation on overall mosquito community abundance (a and b) and richness (c).

Table 4. Model-based community similarity analysis showing the effect of season, location (municipality) and climate variables on mosquito community abundance and composition. Only significant variables and species that contributed significantly (at 5% level) for total variability of abundance are shown. Percentages of mosquito species contribution to total model parameters deviance are shown in brackets.

Parameters	Deviance	Residual df	*p-values*	Species with significant contribution to total deviance
Season	106.2	22	0.001	*Culex bitaeniorhynchus* (13.5%) *Culex pipiens* (10.6%) *Culex antennatus* (9.8%) *Aedes metallicus* (8.4%) *Culex poicilipes* (7.8%) *Aedes sudanensis* (5.8%) *Anopheles sp* (5.7 %)
Municipality	86.2	21	0.009	*Anopheles pretoriensis* (10.2%) *Mansonia uniformis* (9.4%) *Aedes mcintoshi* (8.5%) *Mansonia africana* (5.4%) *Aedes sudanensis* (5.2%) *Anopheles sp* (5.0%).
Precipitation	77.8	20	0.033	*Aedes mcintoshi* (10.4%) *Aedes sudanensis* (8.6%) *Culex simpsoni* (5.3%)
Temperature (maximum)	86.6	19	0.006	*Anopheles sp* (12.1%) *Aedes fowleri* (11.0%) *Culex zombaensis* (8.7%) *Coquillettidea metallica* (7.6%) *Aedes metallicus* (5.4%) *Culex annulioris* (4.6%)

3. STUDY III (Paper IV)

The results presented in this work were based on the analysis of a total of 14,519 mosquitoes, collected in 3 districts (Goba in Maputo Province, Mopeia in Zambézia Province and Massingir in Gaza Province) from Mozambique during 12 successive collection campaigns carried out between November/2014 and December/2015. The majority, 45.55 % (n=6614/14519) of the screened mosquitoes, were classified as *Culex spp.*, followed by *Anopheles spp*. 27.16 % (n=2943/14519) and *Mansonia spp*. 25.22 % (n=3662/14519). Mosquitoes were grouped into 351 pools, ranging from

1 to a maximum of 128 specimens, with an average of (approximately) 41 mosquitoes each. These were subsequently processed by RT-PCR to detect specific viral agents (such as RVFV) or groups of viruses (such as alphaviruses and flaviviruses).

3.1. Analysis of *flavivirus* sequences

The genomes of flaviviruses were targeted using the primers previously described by Vázquez *et al*., (2012), which reveal an amplicon with the expected mass of ≈1 kbp in the cDNA extracts prepared from 45/351 pools (12.8%). The results indicated the presence of flavivirus genome in 9 different species of mosquitoes from possibly four genera (*Anopheles, Culex, Coquillettidia, and Mansonia*). A sample (n=20) of these amplicons was sequenced, and BLASTn/x similarity searches unambiguously confirmed they had a *flavivirus* origin. Similarly, the mosquito species of the pool of origin was confirmed by analysis of COI sequences in all but five pools, for lack of a PCR product. These corresponded to three of *Ma. africana*, and two of *Ma. uniformis*, all of which are very distinctive and clearly identifiable *taxa*.

To further extend the characterization of the viral sequences obtained, a phylogenetic analysis was carried out using different methods. In all cases, the obtained phylogenetic trees indicated that none of the analyzed sequences had been amplified from *bona fide* arboviruses. Indeed, this is clearly revealed by their exclusion from the monophyletic cluster that assembles mosquito-borne and tick-borne flaviviruses in phylogenetic trees (cluster A in Fig. 20), the composition of which is shown in detail in the dotted box (indicated by the arrow). Conversely, all the sequences obtained in this study are segregated within the large monophyletic group that assembles the so-called classical insect-specific flaviviruses, or cISF (Bolling *et al*., 2015). Furthermore, the analysis of the tree topologies obtained clearly suggested they did not group together in a single cluster, but rather segregated (i) either with other known viral sequences or (ii) formed independent genetic lineages. One of these lineages includes only sequences amplified from *Anopheles spp*. mosquitoes, while two others, also sharing a common ancestry, were mostly found in *Mansonia spp*. Unexpectedly, one of these sequences (LC462017) was obtained from a pool of mosquitoes identified as *Cx. antennatus* (pool Moz 182). However, the association of an apparently *Culex-derived* viral sequence with this group was considered debatable given its high similarity with the viral sequences amplified from *Mansonia*. The above-mentioned lineages of cISF include the Cuacua virus, previously identified in *Mansonia spp*. (Cholleti *et al*., 2016). In this work, NS5-coding sequences 98%-100% identical to those of the Cuacua virus were described both in *Ma. africana and Ma. uniformis*.

One of the other lineages of cISF identified is represented by a viral sequence obtained from *Cq. metallica*, clustered with that of Nienokoue virus (NC_024299) from *Culex spp*. However, these sequences share only 76.1% of sequence identity (as defined by Blast2 sequence comparison), clearly below the 84% limit defined by Kuno and others (Kuno *et al*., 1998) and, therefore, indicating that they represent distinct viral species. The remainder of flavivirus lineages were detected in pools of Anopheles mosquitoes, four of which could be classified to the species level as *An. pretoriensis and An. coustani.*

Curiously, the PCR amplification profiles of the flavivirus RT-PCR reactions frequently revealed (in agarose gels) the presence of an amplicon with approximately 0.5 kbp. This amplicon was observed in association with 31/351 (8.8%) of the pools analyzed, by itself in 9/351 (2.6%) or in combination with the expected one kbp DNA fragment in 22/351 (6.3%). However, given its size, it would correspond to a deleted form of the NS5 coding gene suggesting (i) that it might have been amplified from defective viral genomes and/or (ii) rearranged forms of retro-transcribed viral DNA, possibly integrated into mosquito genomes as previously observed (Crochu *et al*., 2004; Roiz *et al*., 2009; Roiz et al., 2012), and/or their resulting transcripts. The association of these smaller sequences with a flavivirus origin was clearly confirmed both by sequence homology searches (using BLASTn) and the reconstruction of phylogenies. All six 0.5 kbp amplicons that had been apparently obtained after

amplification by RT-PCR from total RNA extracted from mosquito pools were not only clearly part of the cISF radiation but also clustered together in a single and highly stable monophyletic cluster that subdivides into two subclusters. Moreover, these same 0.5 kbp amplicons could also be obtained when total DNA was used as a template for PCR amplification, and no reverse-transcription had been performed, but when a DNase I treatment preceded reverse-transcription, no 0.5 kbp amplification product was obtained. These results show that the origin of the frequently observed 0.5 kbp fragment was not cDNA, but rather corresponded to viral DNA forms (vDNA) contaminating the RNA extracts. Three of these amplified from mosquito DNA pools of Ma. africana, *Ma. uniformis and Cx. antennatus*, were cloned and sequenced. Once again, the obtained sequences fell within the same monophyletic cluster. Moreover, when the structure of these DNA fragments was investigated, all of them revealed a similar architecture, combining both different sized-deletions and point mutations.

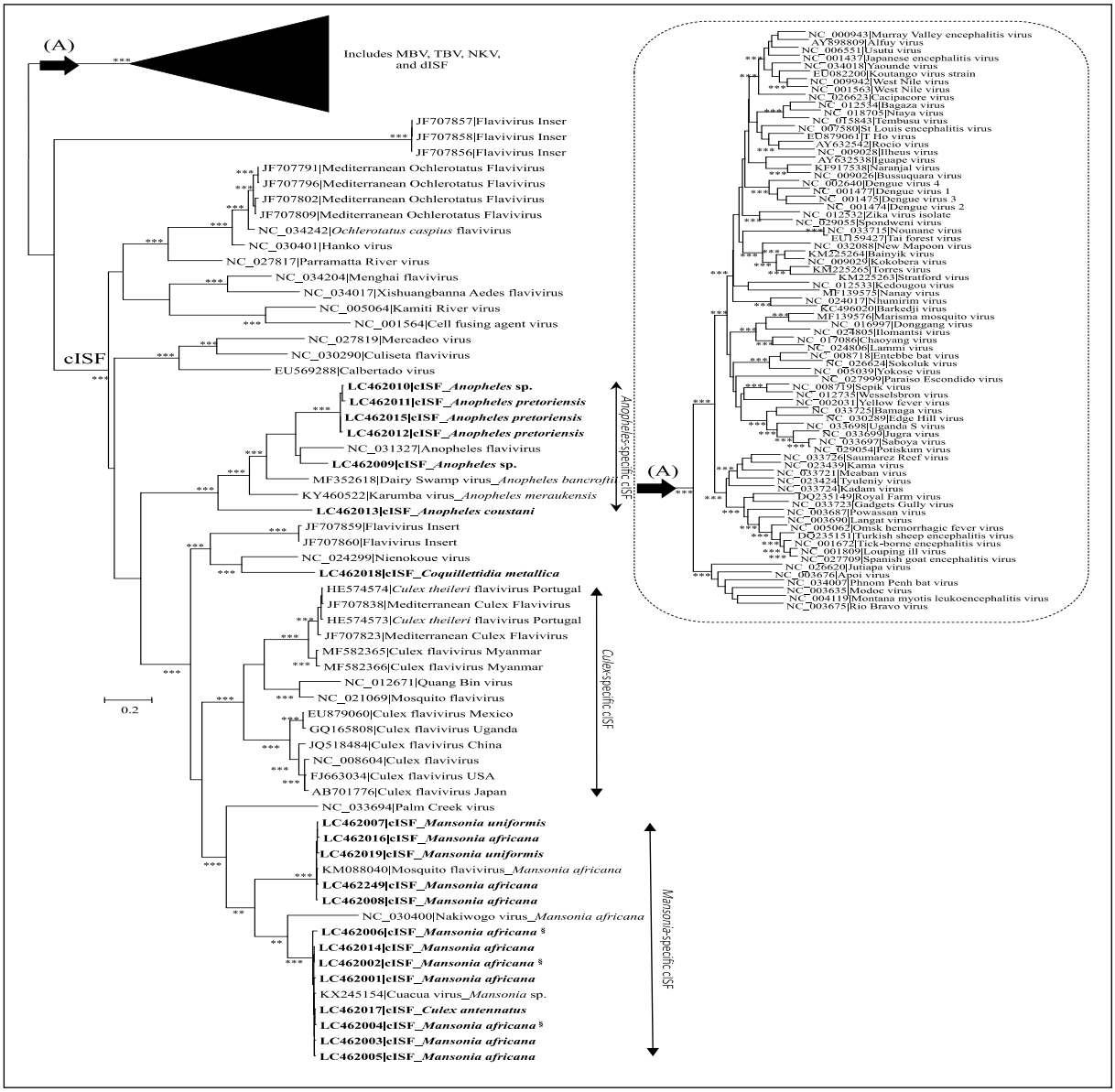

Figure 20. Phylogenetic analysis of flavivirus NS5 nucleotide sequences (≈1 kbp per sequence) (A). At specific branches, the number of "*" indicates the branch-support as revealed by the different phylogenetic reconstruction methods used, and assuming as relevant bootstrap values ≥75% (using 1000 resampling of the sequence data in maximum likelihood analysis) and posterior probability values ≥0.80 (when Bayesian approaches were used). One, two or three "*" would indicate that a given branch had been supported by one, two, or all the phylogenetic reconstruction approaches used in the analysis (ML and Bayesian analysis using two sets of demographic priors). At the top of the tree, the collapsed monophyletic group including reference sequences from mosquito-borne viruses (MBV), tick-borne viruses (TBV), no known vector viruses (NKV), and dual-host associated insect-specific viruses (dISF), while the branches shown comprise the so-called classical insect-specific flaviviruses (cISF), is expanded at the right (B). The sequences described in this work are indicated in bold-face. All the sequences used are designated by their respective accession numbers virus name. The size bar indicates the number of nucleotide substitutions per site. §-Mosquito species could not be confirmed by COI sequence.

3.2. Screening of alphaviruses and bunyaviruses, and analysis of phenuivirus L-sequences

Different results were obtained when either Alphavirus-specific primers (Sanchez-Seco *et al*., 2001) or those targeting conserved sequences in the RVFV NSs coding-region (Sall *et al*., 2002) were used. In fact, neither of these sets of primers revealed the presence of the genomes of these viruses in any of the 351 pools of mosquitoes analyzed. Frequently, the use of the RVFV primers did result in the non-specific amplification of different sized PCR products, many of which were cloned and sequenced. In all cases (results not shown), the obtained sequences confirmed the non-viral origin of these amplicons.

On the other hand, given the overwhelming diversity of the viruses that compose the recently proposed Order *Bunyavirales*, a decision was made not to restrict the screening of bunyaviruses to RVFV but to extend it to a smaller subset (43/351) of the pools of mosquitoes collected in different geographic areas of Mozambique, using *Phlebovirus and Orthobunyavirus* primers (Matsuno *et al*., 2015; Silva *et al*., 2019).

Whereas the obtained results failed to reveal the presence of Orthobunyavirus genomes in 5 pools, two of *An. coustani* (sequences LC461999, LC462000), two of *An. pretoriensis* (sequences LC461996, LC461997, and LC461998) and one of *Cx. tritaeniorhynchus* (sequences LC 461994 and LC46195) mosquitoes, a DNA fragment with the expected size, was, indeed, amplified. All these amplicons were sequenced, but while BLASTn/x sequence searches did indicate a viral origin, unexpectedly they did not seem to have derived from bona fide *Phlebovirus* genomes, and this was confirmed by phylogenetic analysis using an assemblage of *Phlebovirus, Bandavirus, Banyangvirus, and Goukovirus* reference sequences. Regardless of the fact that the *Phlebovirus* group was paraphyletic, the sequences obtained from the analyzed mosquitoes from Mozambique did not cluster in any of the viral taxa in the tree but rather formed three independent genetic lineages. The origin of these viral sequences was investigated using phylogenetic analysis of aligned amino acid sequences of the viral L protein from viruses classified within the different families in the Order *Bunyavirales*. The obtained results (Fig. 21) showed that, while all these sequences were clearly placed within the family Phenuiviridae, only two of them clustered with previously known viral references (Chandler *et al*., 2015; Li *et al*., 2015; Sadeghi et al., 2018), yet in a cluster with no assigned designation. The other five sequences, two amplified from a pool of *Cx. tritaeniorhynchus*, and three others from pools of *An. pretoriensis and An. coustani* formed isolated genetic lineages, probably representing new unassigned genera.

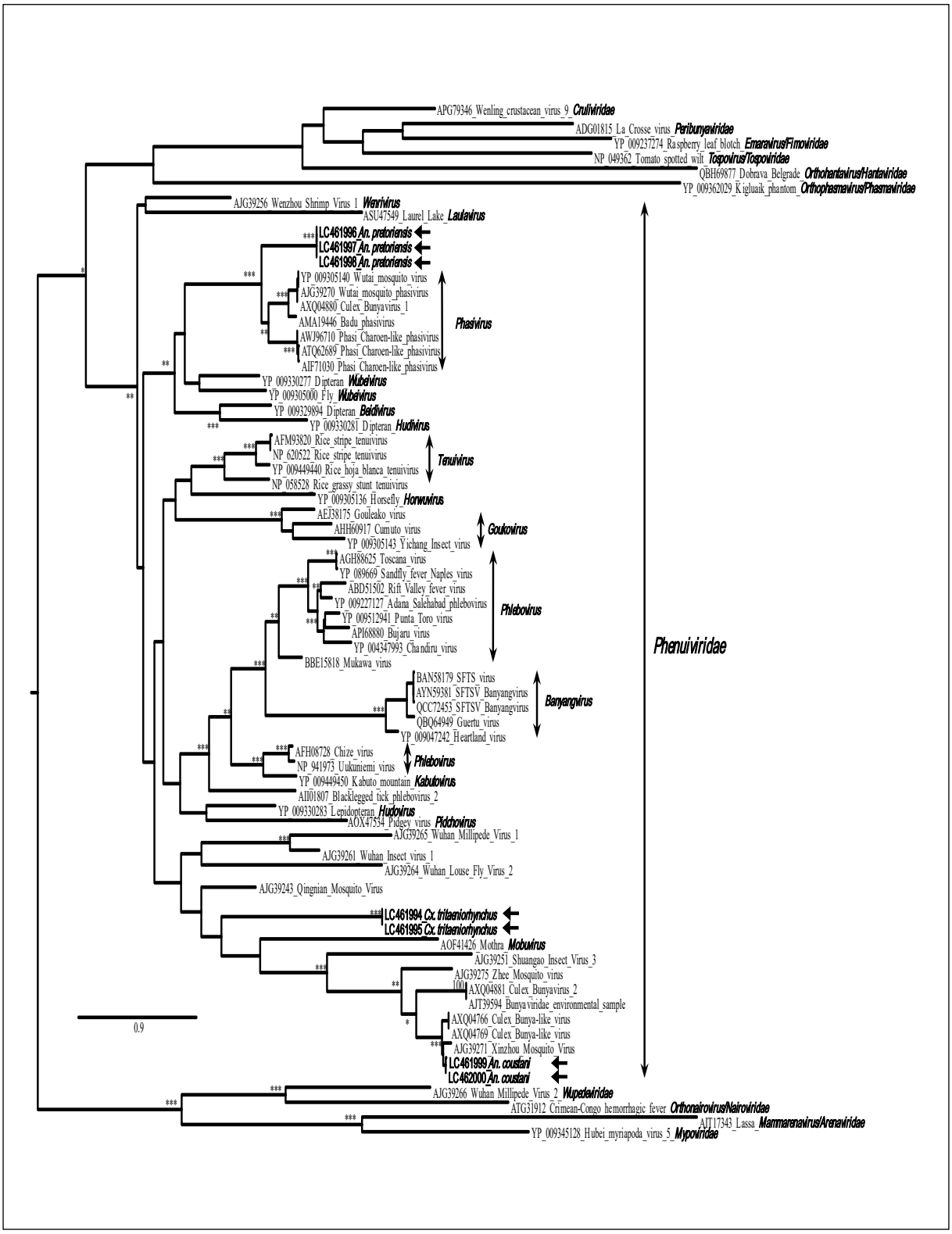

Figure 21. Phylogenetic analysis of partial amino acid sequences of the viral-encoded RNA polymerase of viruses within the Order Bunyavirales. At specific branches the number of "*" indicates the support revealed by the different phylogenetic reconstruction methods used, assuming as relevant bootstrap values ≥75% and posterior probability values ≥0.80. The reference sequences used are indicated by their accession number virus name. The sequences described in this work are indicated by their accession numbers, by the horizontal arrows and in boldface. The size bar indicates the number of amino acid substitutions per site.

CHAPTER 5

DISCUSSION

In this thesis, we investigated the entomological characterization of potential vectors and the arbovirus survey, in mosquitoes from Mozambique. Furthermore, updated field and laboratory-based evidence on the occurrence and seasonal dynamics of the mosquito population associated with the transmission of arboviral diseases in selected settings of Mozambique has been obtained.

Our findings indicated that:

i) *Aedes aegypti o*ccurs in the whole country while *Ae. albopictus* is limited to Maputo and Tete Provinces.
ii) additionally, the occurrence of *Ae. luteocephalus* was discovered in Niassa Province.
iii) the abundance and diversity of mosquitoes from Mopeia and Goba, in central and southern localities from Zambézia and Maputo Provinces, respectively, seem greatly influenced, among other factors, by variation of rainfall and high temperatures.
iv) novel insect-specific flaviviruses and novel phenuiviruses, and previously detected *flavivirus*-like viral DNA forms, were identified in several widely known vector species, representing a unique large-scale survey of virus screening conducted in mosquitoes from three different provinces of Mozambique.
v) despite the high coverage of malaria, there are still gaps for other important VBD, as in the case of arboviruses. This limitation is a consequence of the lack of evidence on the occurrence of important arboviruses and their vectors;

Here now follows an interpretation of the results for each of the research objectives addressed by the three studies regarding the present state-of-the-art of knowledge. Also highlighted are the limitations of the study design and results, and the public health implications thereof.

1. Occurrence and Distribution of Mosquito Species with Potential for Transmitting Arboviruses in Mozambique

The current expansion of arboviruses poses major threats to public health across the world. Thus, sub-Saharan Africa is at a particularly high risk of the occurrence and spread of several mosquitoes that transmit pathogenic arbovirus (Braack *et al*., 2018). In recent years, growing evidence has accumulated concerning the circulation of arbovirus in Mozambique (Gudo *et al*., 2015b; Kraemer *et al*., 2015; Fafetine *et al*., 2016; Gudo *et al*., 2016b; Massangaie *et al*., 2016; Aly *et al*., 2017). These results provide a substantial indication that the transmission risk might be greater than predicted. Numerous biotic and abiotic factors might also increase the transmission risk of *Aedes*-borne arboviral diseases in Mozambique (Higa *et al*., 2015). The country is the third most vulnerable to climate and extreme environmental events, such as floods and droughts in Sub-Saharan Africa (MICOA 2005). The frequency of unusually long periods of droughts has increased in the last decades leading people to recur to water storage practices which might increase the number of sites suitable for *Aedes spp.* larvae (Ambiental 2005).

The knowledge about the distribution and ecology of *Aedes spp.* mosquito species in the country remained limited. Previous records from the 1960s reported the presence of *Ae. (Stegomyia)* species in Northern to Southern regions, with the highest densities in coastal areas (Simard *et al.,* 2005). However, the pattern of distribution may have changed. Therefore, knowing the occurrence and distribution of existing and the new important vectors species is crucial for devising systematic transmission surveillance and vector control approaches.

One of the most competent arboviruses vectors is *Ae. aegypti*, which has originated in Africa, and presently is found in more than 120 countries worldwide (Brown *et al.*, 2014; Kraemer *et al*., 2015; Ngoagouni *et al*., 2015), including countries situated in temperate regions (Roiz *et al*., 2011; Kampen *et al*., 2013; Equihua *et al*., 2017). In this survey, *Ae. aegypti* was collected in every district sampled, indicating that it is either endemic to the country or that it may have already spread to colonize the entire country. This condition can explain the transmission of DENV, CHIKV and other arboviruses in many parts of Mozambique (Gubler *et al*., 1986; Gudo *et al*., 2015b; Gudo *et al*., 2016b; Massangaie *et al*., 2016; Aly *et al*., 2017; Oludele *et al*., 2017). The heterogeneity of abundance and distribution of *Ae. aegypti* shown in the present study has previously been suggested by Kraemer and others in their mathematical modelling (Kraemer *et al*., 2015). Similar findings were observed in Cameroon (Simard *et al*., 2005) and in a prior study conducted in four cities of Mozambique in 2014 (Higa *et al*., 2015). Consequently, the risk of arbovirus transmission is also likely to be heterogeneous across the country, suggesting that vector control activities should prioritize the regions with higher *Ae. aegypti* infestation levels.

The present survey showed that the preferred breeding sites of *Ae. aegypti* were used tires, cement tanks and drums. This was not surprising, considering that *Ae. aegypti* is highly synanthropic. Old tires are commonly used in Mozambique for fencing in peri-urban and rural households, to weigh down the tin sheeting used for roofing material in some houses and to control soil erosion (Higa et al., 2015). Furthermore, used tires are frequently sold along the main public highways, where they usually remain unattended and exposed to rainfall and sunlight for long periods. Cement tanks and drums are the most frequently found water-storage containers in communities with intermittent or deficient water supply. Data from Cameroon, India and Vietnam (Katyal *et al*., 1998; Simard *et al*., 2005; Tsunoda *et al*., 2014; Ferdousi *et al*., 2015), also showed that water storages for domestic use in cement tanks and drums are among the most productive breeding sites of *Aedes* mosquitoes.

The lower abundance of *Ae. aegypti* in the Southern region of the country, found in this study, might be due to a lower amount of rainfall (MOZAMBIQUE 2015; NCEA 2015), relatively good environmental sanitation and a consistent water supply system, which reduces the number of putative anthropophilic as such *Ae. aegypti* breeding sites. In contrast, the high CI in Northern Mozambique may be due to the high annual precipitation (NCEA 2015), a poor water supply system (leading to an increase in water storage containers) and poor environmental sanitation, which increases the number of putative breeding sites such as, disposed of cans and abandoned used tires. These findings are in accordance with an exploratory investigation conducted in four districts during the dengue outbreak in 2014 in Mozambique (Higa *et al*., 2015) and could explain why most of the arbovirus outbreaks reported so far occurred in the Northern region (Gubler *et al*., 1986; Massangaie et al., 2016; Aly *et al*., 2017). A similar pattern has been observed for malaria prevalence in Southern regions, having lower prevalence rates than in Central and North regions of the country (NCEA 2015; INS 2017). This profile suggests once again that arboviral vector control activities should prioritize the Northern and Central settings of the country, regions with considerable poor environmental sanitation that contributes to high infestation levels of *Ae. aegypti*.

It is well known that unplanned urbanization represents an important driver of anthropophilic *Aedes spp*. expansion in sub-Saharan Africa (Zahouli *et al*., 2017). The rate of unplanned urbanization in Mozambique is high, favouring the presence of high population densities associated with artificial breeding sites for the mosquitoes (UNITED NATIONS 2017). This pattern is likely to exacerbate the problem. According to the World Urbanization Prospect report, the urban population in Mozambique rose from 7.0% in 1970 to 32.8% in 2017, and it is predicted to be 50.0% by 2050 (UN 2014). It, therefore, becomes increasingly important that control and monitoring start soon.

On the other hand, *Aedes albopictus* is well known as an oriental arbovirus vector, native to SE Asia and islands of the western Pacific and Indian Ocean (Bonizzoni *et al*., 2013), that is positively affecting

tropical and temperate territories globally. These particular species were first recorded recently in Maputo city (Kampango & Abilio 2016), found, in the Moatize district, situated in Tete Province, in the central region, as part of this study. Additionally, this study confirmed the presence of these species in Maputo city, suggesting that they have already established themselves in some urban settings of the country. These samples were collected as immature found in used tires in Changara district. Other specimens were collected as adult mosquitoes, in enclosure of the Veterinary Faculty of Eduardo Mondlane University, Maputo city. This result corroborates with the study conducted in 2016, where specimens of Ae. albopictus were collected outdoors of the house in Maputo city (Kampango & Abilio 2016). Thereby, all *Ae. albopictus* collections from this study were performed in the peridomestic environment confirming its anthropophilic preference (Reiter & Sprenger 1987; Lounibos 2002; Madon *et al.*, 2002).

Another important arbovirus vector addressed in this thesis is *Ae. luteocephalus*, a mosquito species native to Africa, reported in circa twenty countries, particularly in the western and central regions of the continent, as well as in southern African countries such as Botswana and Zimbabwe [15–22]. *Aedes luteocephalus* has been uncovered for the first time in an exploratory analysis carried out on the samples from adhoc surveys in Niassa Province of Mozambique, confirmed by molecular and phylogenetic analyses. *Ae. luteocephalus* is a competent vector of yellow fever virus and dengue fever virus in Africa and has also been reported in other countries such as Angola, Burkina Faso, Cameroon, Nigeria, Senegal, and Zambia (Newstead *et al*., 1907; Germain *et al*., 1982; Huang 1990, 2004; Mutebi *et al*., 2012; Diagne *et al*., 2015). In fact, laboratory experiments confirm that *Ae. luteocephalus* can transmit yellow fever with an efficiency comparable to *Ae. aegypti* (Bauer 1928), readily bites humans and is involved in the transmission of YFV in West and Central Africa, and chikungunya virus (ZIKV) and DENV2 have been isolated from it in West Africa (Huang 1990).

Specimens of *Ae. luteocephalus* collected in this survey were found in rock pools corresponding to its natural range of tropical forest habitats. Indeed, *Ae. luteocephalus* can be found in forests, savannah, mangrove gallery forests and in intermediate landscapes between sylvatic and urban areas [15, 17, 20]. Bionomically the species utilize a varied range of breeding places such as rot holes, tree holes, rock holes, bamboos, bamboo stems, tree forks, plastic bottles and artificial containers in heights up to 9 meters [15–20]. Although *Ae. luteocephalus* habitat has been essentially rural and sylvatic, increasing demographical expansion and human pressure on forest resources for logging and farming, has been observed in the studied place. This situation might also intensify the likelihood of vector-human contact and, therefore, the risk of rural arbovirus epidemics. Hence, further investigations are urgently required to explore the effect of anthropogenic activity on arboviruses transmission risk in Mozambique.

The potential spread of *Ae. aegypti*, and its cohabitation with the highly frequent *Ae. albopictus,* coupled with the discovery of *Ae. luteocephalus* in the country, raises serious concerns as it may enhance the transmission risk of arboviruses, since these mosquitoes have been incriminated as competent vectors of at least 22 viruses affecting humans and animals, including dengue, chikungunya, Zika, yellow fever, Japanese encephalitis and Rift Valley fever (Gratz 2004; Simard *et al*., 2005; Zhang *et al.*, 2018b). Therefore, the occurrence of *Ae. aegypti, Ae. albopictus and Ae. luteocephalu*s underlines the need for thorough and permanent surveillance of mosquito populations occurring in Mozambique, from North to South, in order to assess the risk of arbovirus outbreaks, and the establishment of early detection systems for their introduction.

While *Ae. luteocephalus* occurrence is somehow reported in Mozambique for the first time in this thesis, the geographical distribution of *Ae. albopictus and Ae. aegypti* has expanded worldwide over the past three decades, with several countries reporting its presence for the first time (Lowenberg-Neto & Navarro-Silva 2002; Toto *et al*., 2003; Villegas-Trejo *et al*., 2010; Fernandez Mdel *et al*., 2012; Benallal *et al*., 2016; Muller *et al*., 2016; Reis *et al*., 2017). Climate change has been pointed

out as a major determinant of main arbovirus vector expansion (Roiz *et al*., 2011; Proestos *et al*., 2015). However, additional studies must be encouraged to systematically better understand the *Ae. aegypti, Ae. albopictus and Ae. luteocephalus* distribution, ecological features and the effect of anthropogenic activity on arboviruses transmission risk for better support of effective vector control strategies under the local conditions. Thus, *Ae. aegypti, Ae. albopictus and Ae. luteocephalus* control programs should concentrate their interventions on the education and engagement of residents inappropriate use, disposal of old tires, covering of water drums and tanks and also for improvement on individual protection.

2. Abundance, Diversity, and Seasonal Assemblages of Mosquito Species Potentially Associated with Arbovirus Transmission in Mozambique

Vector-borne diseases remain one of the largest contributors to human and veterinary disease burden. It has been estimated that more than half of the global population is at risk of MBDs, with particular emphasis on tropical regions (WHO 2017; Wilder-Smith *et al*., 2017). Despite considerable control efforts, the prevalence of MBDs has shown a dramatic increase in endemic regions (Paixão *et al*., 2018; WHO 2020). Malaria, for instance, caused 405,000 deaths in 2019, after years of astonishing reduction in incidence and mortality rate (Bhatt *et al*., 2015; Gething *et al*., 2016), followed by stagnation, and a recent rising, in the number of cases again (WHO 2020). On the other hand, 104,771 cases of dengue, and 4,050 deaths have been reported over the last two decades (Disease *et al*., 2018). Furthermore, the prevalence of dengue in individuals residing in Africa also increased during the same period (Simo *et al*., 2019). Concomitantly, has been observed an unprecedented increase in frequency and severity of several other mosquito-borne arboviral disease outbreaks, including Zika, chikungunya, yellow fever, Rift Valley fever, West Nile fever (Wilder-Smith *et al*., 2017). This has been accompanied by the spread of vectors and pathogens to formerly non-endemic regions (Wilder-Smith *et al*., 2017; Kraemer *et al*., 2019). Rapid demographic expansion coupled with environmental changes due to unplanned land use, deforestation and habitat fragmentation, climate changes, and reduced susceptibility of vector populations to conventional insecticide-based control measures, has been agreed as being the main drivers of the observed surge in VBDs incidence worldwide (Norris 2004; Sutherst 2004; Ryan *et al*., 2019).

Strategies for surveillance and control of VBDs depend on the accurate knowledge of mosquito community composition, seasonal dynamics, ecology and determining factors of a spatiotemporal assemblage of local vector species. This study investigated the occurrence, structure and dynamics of mosquito communities from two different eco-geographical regions of Mozambique, namely Goba and Mopeia, in the locality of southern and central regions of the country. Overall, were identified 31 mosquito species in Goba and 37 species in Mopeia localities of Maputo and Zambézia Provinces, respectively. Despite an apparent difference in the number of species, the core group of the ten most frequent ones occurred in both Goba and Mopeia. The number of dominant taxa comprised those that were common to both regions. Species-rarefaction analysis showed that no information regarding new taxa could be added to the data after we had reached a sample size of 4,000 individuals in Goba, indicating that the sampling routine was able to detect all possible mosquito species occurring in the studied settings, even despite the lesser number of months of collections in this site. Differently, species-rarefaction curve analyses on Mopeia data have indicated an estimate of 55 mosquito taxa that may occur in the studied settings, contrary to the 37 observed. Thus, about 18 species remained undetected. Host-seeking mosquitoes were mostly sampled by CO2-baited light-traps. Several factors may affect the likelihood of mosquito detection by traps, such as

differential response to type and source of the odour and trap visual components, prevailing wind speed during trapping time, moonlight, and landscape features (Bidlingmayer 1967; Snow 1970a; Snow 1970b; Service 1980). Regarding olfactory stimuli, although CO2 is a powerful long-range generalist attractant (Gibson & Torr 1999), and it has been shown that some CO2 outputs can either attract or repel certain mosquito species (Gibson & Torr 1999). Accordingly, wind speed above three m/s can inhibit the flight activity of several mosquito species and, therefore, reduce the likelihood of detection by traps (Bidlingmayer *et al*., 1995). The use of BG sentinel traps, with special odours attractants/bait and a design for aedine mosquitoes, might have improved collections had we had access to them and certainly should be considered in future studies (Li *et al*., 2016).

Moreover, moonlight has long been known that to strongly influence the size and composition of mosquito catches by light traps (Ribbands 1946; Birley & Charlwood 1989; Kampango *et al*., 2011). Landscape features can also influence the probability of mosquitoes finding the trap. Bidlingmayer (Bidlingmayer 1974) reported that traps deployed in the forest collected more mosquitoes than those deployed in the open land. Another factor that may have contributed to the failure in detecting all mosquito species may be the limited number of sampling days performed per month, as well as the atmospheric conditions on those particular days the collections took place.

Goba, Mopeia, Maputo and Massingir mosquito communities contain known vectors of important pathogens, including malaria vectors (e.g., *An. gambiae s.l, An. funestus s.l, An. coustani, An. pharoensis*) and several known arboviruses vectors of medical and veterinary relevance as Rift valley fever, dengue, chikungunya, yellow fever, O'Nyong nyong, West Nile and Zika (e.g., *Ae. aegypti, Ae. albopictus, Ae. mcintoshi, Ae. simpsoni, Ae. vittatus, Ae. mettalicus, Ae. durbanensis, Cx. tritaeniorhynchus, Cx. antennatus, Ma. africana, Ma. uniformis*). This corresponds to the third time *Ae. albopictus,* a second important arbovirus vector, is collected in the country, confirming its establishment in the Mozambican territory. Apart from that, it is known that *Ae. Aegypti*, one of the main vectors of arbovirus, was likely to occur in the whole country. However, recent evidence was only produced in this thesis (Abilio *et al*., 2018). While, *Ae. albopictus,* another important vector for at least 26 arboviruses (Paupy *et al* 2009) tends to expand (Kampango & Abilio 2016; Abilio *et al*., 2018), *Ae. luteocephalus* an important vector of dengue, yellow fever and Zika in Africa were recently confirmed to occur in Mozambique territory (Abílio *et al.*, 2020). Additionally, the two existing reports of screening for different groups of RNA viruses in mosquitoes from Mozambique, one of which as mentioned, as part of this study, discloses the presence of several insect-specific flaviviruses and phenuiviruses in several widely known vector species from genera of *Anopheles spp, Culex spp, Coquillettidia spp, and Mansonia spp*. Hence, the presence of the most important arbovirus vector and the circulation of different RNA viruses present in mosquitoes from Mozambique coupled with the high density of the most abundant mosquito species vectors obtained in this study, clearly suggests a high risk for pathogenic arbovirus transmission. Recognizing that Goba and Mopeia correspond to rural settings close to the sylvatic areas, improvement of habitation and individual protection such as indoor residual spraying (IRS), use of insecticide-treated nets (ITNs), animal vaccination coupled with systematic surveillance is encouraged to tackle the risk of arbovirus transmission.

Results also indicate that most of the vector's population usually occurs all year round in the studied setting. Further investigations are encouraged to determine the role of the aforementioned potential arbovirus vectors in maintaining local transmission of mosquito-transmitted arboviral agents. The overall mosquito population abundance has usually peaked in the middle of the summer/rainy season. However, Goba results also indicated that high mosquito abundance erupts in June. Contrarily, the peak of mosquito richness, effective number of equally common species, and highly abundant species remain nearly constant for nearly six months (from January to June). A similar pattern of temporal dynamics of abundance and diversities has been reported elsewhere (Franklin & Whelan 2009). Results also suggest that climate factors, particularly, maximum temperature and average rainfalls are the main drivers of mosquito abundance and diversity at studied sites. Overall mosquito abundance and

community richness increased with the increase of maximum temperature and amount of rainfall. Conversely, Shannon and Simpson diversities tend to respond negatively to higher temperatures. These findings suggest that in general, the mosquito community from Goba and Mopeia might successfully thrive under extreme climate events, such as a rise in temperatures as a consequence of climate changes. It has been argued that climate change may turn the African continent much more suitable for arboviruses rather than malaria (Mordecai *et al.*, 2020). Our results also suggest that the higher risk of exposure to pathogens potentially transmitted by vector populations identified, concerning their densities, might extend for at least eight months a year, that is, from November to June in Mopeia. Unfortunately, the impossibility of collections all year round in Goba, Maputo Province, prevented us from estimating the season of higher transmission risk (Fig. 16). However, it is very likely that the trajectory abundance curve, depicted in Fig. 16, may be mostly due to variations of dominant species abundance, particularly *Ma. africana, Ma. uniformis, Cx. antennatus, An. funestus* that breed in permanent larval habitats and, therefore, do not show dramatic seasonal variations across the range of their occurrence (De Meillon 1956; Laurence 2009). Interestingly, the peak of mosquito richness and an effective number of species (equally abundant and rare) last for nearly four to five months, suggesting great stability of larval habitats even during the dry season (e.g., April – June). We observed significant variation in community composition between sites and seasons. Similarly, mosquito composition was significantly affected by temperature (maximum) and rainfall fluctuation. However, the effect covariates on community composition were species-specific. The heterogeneity in the covariate effect on mosquito composition may reflect some degree of overlap in ecological niche requirements between groups of species. With the exception of *Ma. africana and Ma. uniformis*, species that were significantly affected by variation of rainfall inputs and temperature are essentially container exploiting mosquito species and species that usually breed in marshes, swamps or other types of periodically flooded environments, namely, *Ae. sudanensis, Ae. mcintoshi, Ae. fowleri, Ae. metallicus, Cx. annulioris, Ae. pretoriensis* (Edwards 1941; Lambrecht & Peterson 1977). It is also well known that the higher risk for arbovirus transmission occurs at the end of the rainy season, as mosquito populations get older and are likely more prone to higher infection rates. Therefore, future studies should aim at understanding the real risk these vector species impose on residents, as well as, determine the extent to which variations of local environmental and climate factors regulate the spatiotemporal vector´s assemblages and pathogens transmission exposure in Goba and Mopeia in southern and central localities from Zambézia and Maputo respectively.

3. Arboviruses of *Flavivirus, Alphavirus* and *Phlebovirus* Groups in Mosquito Populations from Different Regions of Mozambique

Our knowledge of the diversity of the viral world has significantly expanded over the last decade. During this period, a large number of studies have shown that viruses are the most abundant biological entities on the planet and display a remarkable degree of genetic diversity and genomic plasticity (Desnues & Raoult 2010; Zhang *et al.*, 2018a), and have also allowed us to bridge apparent phylogenetic gaps in the virosphere. This is especially true when viral surveys focus on rarely sampled *taxa* or infrequently visited biotopes, and reveal novel or divergent viral groups (Li *et al.*, 2015; Shi *et al.*, 2016; Abrahao *et al.*, 2018; Kauffman *et al.*, 2018; Zhang *et al.*, 2018b).

Invertebrates are among the animals most frequently sampled in recent viral surveys, and their viromes seem to include a large number of genetically diverse viruses (Shi *et al.*, 2016). Mosquitoes (Diptera: Culicidae) are clearly the invertebrates most commonly studied due to their role as vectors

of pathogenic viruses to humans and other animals (Gould *et al.*, 2017). However, the viromes of mosquitoes have been shown not to be limited to the latter, many of which (e.g. dengue, yellow fever or Zika viruses) have become household names in recent times. In fact, mosquitoes also host a profusion of viruses that only infect invertebrate cells and are, therefore, regarded as insect-restricted (Junglen & Drosten 2013; Bolling et al., 2015; Calisher & Higgs 2018). On the other hand, viral surveys are still frequently carried out in association with disease outbreaks, or when identifiable factors increase the probability for an arbovirus to (re)emerge and/or rapidly disperse (Gould *et al.*, 2017). Moreover, since there is limited knowledge on the genetic diversity, and ecology, of viruses in their natural enzootic maintenance cycles, little is also known regarding the adaptive constraints ruling the evolutionary steps that determine arbovirus emergence from their sylvatic niches (Marklewitz & Junglen 2019).

Mozambique is located in a region suitable for arbovirus outbreaks, and in recent times the country has been affected by two dengue virus outbreaks, which occurred in the northern regions (Higa *et al.*, 2015; Massangaie *et al.*, 2016). Increasing serological evidence also suggests that the country may be endemic to other debilitating and life-threatening arboviral threats, including RVFV in animals and humans (Fafetine *et al.*, 2007; Fafetine *et al.*, 2013a; Gudo *et al.*, 2016c), DENV (Gubler *et al.*, 1986; Bhatt *et al.*, 2013; Higa *et al.*, 2015) and CHIKV (Gudo *et al.*, 2015a; Mugabe *et al.*, 2018). Moreover, historical and global risk projections have suggested that the country may also be suitable for the establishment of ZIKV (Bogoch *et al.*, 2016; Gudo *et al.*, 2016a; Samy *et al.*, 2016), a virus recently linked to cases of microcephaly as well as many other neurological abnormalities in newly born infants (Cugola *et al.*, 2016). Recently, in the neighbouring country of South Africa, several arboviruses such as *Alphavirus*, namely Sindbis, Middelburg and Ndumu virus, *Orthobunyavirus*, namely Shuni virus (an associated with neurological and febrile illness in animals and humans), and also Flavivirus, such as WNV, have been detected in several wild and domestic animals and people, but also mosquito species of the *Culex and Aedes* genera (Guarido *et al.*, 2021; Motlou & Venter 2021). This study showed that arboviral activity is concentrated in peri-urban, rural and conservation areas, suggesting a role for animals as amplifying hosts (Guarido et al., 2021). Additionally, in the Ndumo reserve of tropical north-eastern KwaZulu-Natal, South Africa, bordering with Mozambique, it was reported the presence of RVFV in *Ae. (Aedimorphus) durbanensis* mosquitoes tested for nucleic acid using RT-PCR (Peter Thompson personal communication). The isolation/detection of arbovirus in animals, humans and their vectors clearly proves its circulation in the southern African region (Venter 2018; Motlou & Venter 2021).

The increasing evidence indicating the circulation of public health-relevant arboviruses in the country and its neighbours, coupled with the circulation of more than a hundred potential arbovirus vectors from the main mosquito genera *Aedes spp, Culex spp, Mansonia spp and Anopheles spp* (Worth & De Meillon 1960; Cholleti *et al.*, 2016; Kampango & Abilio 2016; Abilio *et al.*, 2018) clearly shows that systematic studies are necessary to understand the epidemiology of arbovirus transmission that includes the role of the vector in maintaining arboviruses in nature.

In this report, a screening for different groups of RNA viruses targeting the detection of some of those previously shown (genome detection) or suggested (seroprevalence studies) to circulate in Mozambique was performed (Cholleti *et al.*, 2016; Fafetine *et al.*, 2016; Gudo *et al.*, 2016b; Mugabe *et al.*, 2018). This analysis was carried out based on a one-year sampling effort, that amounted to the screening (for viral genomes) of 14,519 mosquitoes from three regions of the country. As only female mosquitoes may serve as vectors of viruses to vertebrates, male mosquitoes were excluded from this viral screening. Although the detection of viral agents is facilitated when their presence is associated with visible clinical signs/symptoms in vertebrates, their screening in their natural hosts/ vectors may have the advantage of signalling their circulation before any cases of clinical disease or seroprevalence are detected. Moreover, a viral screening effort based on the identification of *foci* of disease cases only discloses the circulation of pathogenic viruses, and these have been shown to

represent only a part of the virome of mosquitoes (Junglen & Drosten 2013; Bolling *et al*., 2015; Gould *et al*., 2017; Calisher & Higgs 2018).

The molecular screening that was carried out did not reveal the presence of RVFV or any recognizable pathogenic alphaviruses, flaviviruses and bunyaviruses. These include viruses such as DENV, ZIKV, CHIKV, o'nyong nyong, Sindbis or Middelburg (Sanchez-Seco *et al*., 2001). While the absence of alphaviruses in this viral screening may be intriguing, we must bear in mind that unlike other virus groups (e.g. flaviviruses), alphavirus ISVs have, with exceptions (Nasar *et al*., 2012; Hermanns *et al*., 2017), been less frequently reported in viral surveys, while pathogenic alphaviruses such as CHIKV are usually associated with *Aedes* mosquitoes which were clearly underrepresented in our screening. Furthermore, no *Orthobunyavirus* sequences were ever detected in a small sample of the pools analyzed (n=43; the same subset of pools where a survey for *Phlebovirus* genomes was also carried out).

On the contrary, the use of a highly degenerate flavivirus-specific primer set (Vazquez *et al*., 2012) confirmed the presence of multiple genetic lineages of flaviviruses in a large number of pools of mosquitoes. Despite the fact that not all of the obtained amplicons were sequenced, those for which a sequence was obtained were found to segregate in the cISF radiation.

The different genetic lineages of viral *NS5* sequences were apparently associated with multiple species from 4 genera, supporting the perception that cISF are widespread in the natural populations of mosquitoes. Some of these NS5 sequences seemed to segregate away from previously described viral assemblages and formed isolated branches in phylogenetic trees. Others were joined in clusters with multiple operational taxonomic units that were never exclusively associated with a single species of mosquitoes, adding to the possibility that cISF may not host species-restricted (Cook & Holmes 2006; Cook *et al*., 2012). However, while phylogenetic analysis did suggest a *Culex* origin for one of the sequences (LC462017), given the fact that it was almost identical to many others amplified from *Mansonia* mosquitoes, its association with *Culex* mosquitoes is disputable. Furthermore, although a molecular confirmation of the identity of these mosquitoes was obtained by *COI*-sequence analysis, the sequencing strategy used (Sanger) is a population-based approach that only reveals the sequence of the most abundant molecular form in a PCR product, while minor variants fail to be detected. Therefore, we cannot formally exclude the possibility that sequence LC462017 may have been derived from one/a small number or even body-parts of non-*Culex* mosquitoes (possibly *Mansonia*) originally present in the pool in question.

Surprisingly, in a high number of pools of *Mansonia spp*. (n=29) in one pool of *Anopheles sp.* and another of *Culex sp*. mosquitoes, the flavivirus-specific primers used generated a smaller than expected PCR product, with approximately half the size (≈0.5 kbp). The analysis of some of these smaller amplicons showed that they corresponded to defective versions of the RdRp coding sequence, and their origin was found to be DNA (vDNA), rather than RNA. For all those cases where a nucleotide sequence could be obtained, a shared ancestry between the latter and *bona fide* viral *NS5* sequences (obtained by RT-PCR) was also revealed.

While we cannot ascertain, at this stage, whether the flavivirus vDNA forms are present as part of the host genome (endogenized), or whether they exist in the form of a stable extra-chromosomal DNA element, flavivirus-like sequences have been known to occur in the genome of mosquitoes for over a decade, especially in association with *Aedes* mosquitoes (Crochu *et al*., 2004; Roiz *et al*., 2012). While the sequence of a vDNA amplicon amplified from *Anopheles* could not be obtained due to technical difficulties, the fact that virtually identical vDNA sequences could be amplified from DNA extracts of *Mansonia and Culex* mosquitoes is hard to explain given the evolutionary divergence of these *taxa*. Moreover, while these vDNA forms could result from exposure of these mosquitoes to a common source of viruses, the possibility of contamination of pools of *Culex* mosquitoes with even a limited amount of the highly abundant *Mansonia* specimens cannot be discarded.

Whereas the presence of bacterial symbionts of mosquitoes can alter the competence of mosquitoes for transmission of pathogenic viruses (Hegde *et al*., 2015), to what extent the same applies to the persistent presence of insect-specific viruses in insect cells is still open to discussion. In fact, evidence demonstrates that their position in phylogenetic trees indicates that they represent a useful model for evolutionary steps as ancestral viruses from which pathogenic humans originated (Cook & Holmes 2006). This point offers valuable evidence on the evolution and genetic discrepancies of distinct viral species, and molecular bases of transmissibility and pathogenesis. Furthermore, there are contradictory conclusions about whether the presence of ISV enables (Öhlund *et al*., 2019) to suppress (Hobson-Peters *et al*., 2013) or has no effect on the vectorial capacity of mosquitoes to transmit the pathogenic virus (Talavera *et al*., 2018). Thus, the discovery and description of novels insect-specific viruses will not only enrich our understanding of the mosquito virome but will also enrich our understanding of virus transmission, ecology and vector microbial diversity that is important for vector control strategies. The latter allows us to explore viral interference and exclusion or the vector's immune system regulation to prevent the replication of pathogenic viruses. Nevertheless, the large contradiction mentioned above reveals the urgent need for more research on ISVs in order to clarify this important gap, probably with the potential for identifying and modifying the usefulness of ISVs in the context of disease control tools and other different applications in the global context and in Mozambique.

Given the a priori specificity of the primers used for the screening of *Phlebovirus* sequences, the observation of a specific amplicon in association with five pools of two different species of *Anopheles (An. coustani and An. pretoriensis)* and one species of *Culex (Cx. tritaeniorhynchus)* mosquitoes suggested that these viruses might have been detected. However, the different phylogenetic analyses were congruent in showing (i) their inclusion in the *Phenuiviridae* family, (ii) their exclusion from the *Phlebovirus* genus, (iii) and their separation into three genetic lineages. Two of these sequences did segregate in a stable monophyletic cluster defining a genetic lineage with no assigned designation, but that included sequences previously detected in other studies (Chandler *et al*., 2015; Li *et al*., 2015; Sadeghi *et al*., 2018), while the other five formed two genetic lineages with no associated references.

Although no recognizable pathogenic viruses were identified in the course of this work, this may result from a combination of multiple factors that include sampling bias. In fact, collections did not focus on settings where DENV/ZIKV/CHIKV were previously known to circulate in Mozambique (Fafetine *et al*., 2016; Gudo et al., 2016b; Mugabe *et al*., 2018), but rather on areas where RVFV had been detected (Fafetine *et al*., 2007; Fafetine *et al*., 2013a). On the contrary, *Mansonia and Culex* mosquitoes clearly dominate the collections in the three provinces of Mozambique, which were the focus of this study. However, pathogenic flavivirus, such as the Spondweni virus (the closest known relative to ZIKV), has been isolated from *Ma. africana and Ma. uniformis* (Gould *et al*., 2017), as well as from *Cx. quinquefasciatus* mosquitoes in Haiti (White *et al*., 2018). Association of other pathogenic flaviviruses with *Mansonia sp*. mosquitoes include the S. Louis encephalitis and West-Nile viruses (which also use *Culex sp*. for their natural maintenance), alphaviruses (including Venezuelan equine encephalitis virus), orthobunyaviruses (Beranek *et al*., 2018), and phleboviruses, including RVFV (Gould *et al*., 2017). While sampling bias may partially explain the absence of some of the arboviruses that have been previously shown to circulate in Mozambique, other factors may also explain the results obtained. These include a low natural incidence of arboviruses in the areas where mosquitoes were collected or the concurrent absence of recorded cases of human/ animal disease cases associated with the circulation of viruses such as RVFV during the mosquito collection periods. Furthermore, a technical limitation of this study is associated with the use of a less technologically advanced virus detection approach based on convectional RT-PCR, as opposed to addressing viral screening with a bona fide metagenomic experimental design combined with the use of NGS sequencing methods. To the best of our knowledge, this study and the previous

detection of ISF in *Mansonia spp* (Cholleti *et al*., 2016), are the only recent virus surveys using mosquitoes from Mozambique, and clearly demonstrate the dire need for such surveys that might clarify their epidemiology. Despite that, this survey did not disclose the circulation of pathogenic arboviruses. Taking into account existing evidence of pathogenic arbovirus circulation in animals and/or humans, either in the country or in neighbouring countries, combined with non-existent physical borders between them within the Southern African region, that consequently contribute to intense movements of people, animals and goods in the region, and particularly trans-frontier national parks (South Africa 2021) of which Great Limpopo with Mozambican territory (Mozambique *et al*., 2021), with wide movements of wildlife, associated with the fact that Southern Africa is a tropical region with a climate that favours for the occurrence and maintenance of high densities of arbovirus vector populations confirmed their occurrence in this study, it is discernible that considerable work is necessary. This effort must be focused on systematic well-structured surveillance that can easily detect outbreaks, alert and support the national health system on effective control measures in a timely manner, and in a One Health approach.

CHAPTER 6

CONCLUSIONS AND RECOMENDATIONS

1. CONCLUSION

1. Overall findings suggest that important vector species occur in the studied sites. The data also showed that *Ae. aegypti* is present nationwide since it occurred in every sampled district. The survey revealed that the preferred breeding site of *Ae. aegypti* were used tires, cement tanks and drums. Cans, bottles and flowerpots also contributed to the infestation of *Ae. aegypti. Aedes albopictus* showed to be successfully established in the country, although with limited distribution. This study has also uncovered the occurrence of *Ae. luteocephalus*, a competent vector species of yellow fever virus (YFV), Zika virus (ZIKV) and dengue virus (DENV) in Africa, in Mozambican territory. The occurrences of these three very important potential arbovirus vectors in Mozambique clearly indicate that the risk of transmission of Rift Valley fever, dengue, chikungunya or other arboviruses is likely to have been underestimated in Mozambique so far.

2. The study has also shown a high diversity of vector species in mosquito communities from Goba and Mopeia. The two-mosquito communities show predictable annual cycles, modulated by variations in the amount of precipitation and variations in high temperature. The period of highest risk of mosquito exposure is December and January since the two months have coincided with the period of the high peak of mosquito abundance and diversity.

3. This study reports novel insect-specific flaviviruses and phenuiviruses, and frequent flavivirus-like viral DNA forms in several widely known vector species. While a large diversity of ISVs has been found on a global scale in association with a plethora of insect hosts, this work extends the results of the sole study that had, up to the present day, revealed their presence in Mozambique. Although this survey did not disclose the circulation of pathogenic arboviruses, it has confirmed the circulation of different RNA viruses present in mosquitoes from Mozambique.

2. RECOMMENDATIONS

The results represented in this thesis express the professional effort to help to elucidate and provide higher resolution information on arboviruses vectors hotspots, transmission dynamics and routes in Mozambique. However, for VBD control, as in the case of arbovirus, major investments should be boosted in order to gather basic and relevant information that is still missing as such:

1. The need to establish a national entomological surveillance system for arbovirus in Mozambique and other countries within a regional context sharing the same pattern to better understand vector bionomics and to support the development of informed, effective vector control strategies.

2. More studies are needed to understand the distribution of all mosquito species including *Ae. Luteocephalus*, in the three administrative regions and/or all provinces of Mozambique.

3. More studies are needed to further investigate the systematics of the less known mosquito species in Mozambique, particularly the morphological and molecular aspects and phylogenetic relationships, of members of the *Culex and Aedes* genera that are often only differentiated as males. In this sense, this thesis has, been given material for such studies, being already followed by an ongoing master thesis at IHMT-NOVA in collaboration with UEM.

4. Further systematic studies are required to determine the degree of ecological association between the potential arbovirus vector, *Ae. aegypti, Ae. albopictus and Ae. luteocephalus*.

5. It's of high importance to determine the contribution to the transmission of RVF and other arboviral diseases such as dengue, chikungunya, etc., and to have an approximate characterization of their circulation or co-circulation, among different vector populations in Mozambique.

6. Given the present revelation of a significant number of insect-specific viruses (ISVs) in this study coupled with those identified elsewhere, it is urgent to deeply investigate their importance in the transmission of pathogenic arboviruses in Mozambique and the global context.

7. Since Mozambique has a well-established sentinel surveillance system for malaria vectors, a recommendation for *Aedes* surveillance should be integrated into the existing surveillance system for malaria vectors being carried out in urban and rural areas of the country. The surveillance for *Aedes* should be enhanced in urban areas where *Ae. (Stegomyia)* mosquitoes are more frequent, in order to ensure their sustainability and optimize the use of scarce resources.

8. *Aedes aegypti and Ae. albopictus* control strategy should concentrate its interventions on the health education and engagement of the population in appropriate use and disposal of old tires and covering of water drums and tanks, and all other disposed items that can be a source of collected rainfall water.

9. For rural and sylvatic areas, the control measures should focus on the improvement of habitation that includes nets in windows and protection such as indoor residual spraying (IRS), use of insecticide-treated nets (ITNs), animal vaccination coupled with continued entomological surveillance, involving viral screens on mosquito pools particularly at low transmission season (also in order to reduce costs), preferably included in a nationwide surveillance program that could be globally planned with Malaria and Filariasis surveillance/monitoring (an integrated VBD control approaches), to tackle the risk of arbovirus and other VBD transmissions.

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ANNEXES

Printed copies of articles published or under review in scientific journals as part of this Doctoral Thesis

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Distribution and breeding sites of *Aedes aegypti* and *Aedes albopictus* in 32 urban/peri-urban districts of Mozambique: implication for assessing the risk of arbovirus outbreaks

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Abstract

Background

Aedes-borne arboviruses have emerged as an important public health problem worldwide and, in Mozambique, the number of cases and its geographical spread have been growing. However, information on the occurrence, distribution and ecology of *Aedes aegypti* and *Ae. albopictus* mosquitoes remain poorly known in the country.

Methods

Between March and April 2016, a cross-sectional study was conducted in 32 districts in Mozambique to determine the distribution and breeding sites of *Ae. aegypti* and *Ae. albopictus*. Larvae and pupae were collected from a total of 2,807 water-holding containers using pipette, dipper, funnel and sweeping procedures, depending on the container type and location. Both outdoor and indoor water-holding containers were inspected. The immature forms were reared to adults and the identifications of the mosquito species was carried out with a stereomicroscope using a taxonomic key.

Results

Aedes aegypti was found in every district sampled, while *Ae. albopictus* was only found in Moatize district, situated in Tete Province in the central part of the country. Six hundred and twenty-eight of 2,807 (22.4%) containers were positive for *Ae. aegypti* but only one (0.03%) was positive for *Ae. albopictus*. The Container Index (CI) of *Aedes* was highest in densely populated suburban areas of the central region (260/604; 43.0%), followed by suburban areas in northern areas (228/617; 36.9%) whilst the lowest proportion was found in

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urbanized southern areas (140/1586; 8.8%). The highest CI of *Aedes* was found in used tires (448/1268; 35.3%), cement tanks (20/62; 32.3%) and drums (21/95; 22.1%).

Conclusion

Data from our study showed that *Ae. aegypti* is present nation-wide, since it occurred in every sampled district, whilst *Ae. albopictus* had a limited distribution. Therefore, the risk of transmission of dengue and chikungunya is likely to have been underestimated in Mozambique. This study highlights the need for the establishment of a national entomological surveillance program for *Aedes spp.* in Mozambique in order to gain a better understanding about vector bionomics and to support the development of informed effective vector control strategies.

Author summary

Dengue, chikungunya and Zika are a group of rapidly spreading mosquito-borne diseases worldwide. These arboviral diseases have received increasing attention in Mozambique as a consequence of recent dengue outbreaks, which occurred in the northern region. There has also been an increase in the number of cases of chikungunya reported in the country. Additionally, earlier evidence obtained from neutralizing antibodies against Zika revealed an overall prevalence of 4% in 249 individuals (142 adults and 107 children) sampled from 22 localities across Mozambique in the 1950's. These arboviruses are primarily transmitted by the bites of infected *Aedes* (*Stegomyia*) females, especially *Ae. aegypti* and *Ae. albopictus*. However, data on the distribution and the bio-ecology of both *Aedes* species are scarce. This lack of information is a major barrier for the implementation of public health interventions to prevent *Aedes*-borne arbovirus infections. In this study, we investigated the distribution and abundance of *Ae. aegypti* and *Ae. albopictus* in 32 districts of Mozambique. We found *Ae. aegypti* in every district sampled, although with heterogeneous abundance, while *Ae. albopictus* had limited occurrence. *Aedes aegypti* breeding sites varied among districts. The predominant containers were used tires, cement tank and drums, all present at high densities in central and northern Mozambique. This is the first study that investigates the distribution of breeding sites and abundance of *Aedes spp.* in a large number of districts in Mozambique and provides relevant baseline data for the establishment of a vector surveillance and control interventions for arboviruses in the country.

Introduction

Dengue, chikungunya and Zika are among the most important mosquito-transmitted viruses worldwide. Their global burden of these diseases has increased rapidly in the last decades [1, 2]. An estimated 390–500 million cases of dengue occur every year [1, 3]. Zika was declared a public health emergency of international concern in February 2016 [4], whilst Chikungunya virus has caused massive and severe outbreaks worldwide over the last decade [5–7]. The spread of these viruses follows the distribution of the primary vector, *Aedes aegypti* [8]. *Ae. aegypti* originated in Africa, but is now found in more than 120 countries worldwide [8–10], including countries situated in temperate regions [11–13]. Additionally, *Ae. albopictus* which

is considered to be a potential vector of several arboviruses, has also expanded its geographical distribution [14, 15]. In 2015 its presence was confirmed in Maputo, Mozambique's capital [14, 15].

Sub-Saharan Africa is at particularly high risk of occurrence and spread of *Aedes* transmitted pathogens due to its climate and environmental conditions. Recent studies presented evidence of arboviruses in Mozambique, such as the recent confirmation of a DENV-2 outbreak in 2014 during which a total of 100 confirmed/probable cases were reported [16]. Subsequently, the endemic circulation of DENV-2 was demonstrated in 2015–2016, from a total of 21 PCR-positive samples detected in northern Mozambique [17]. Anti-CHIKV IgG antibodies were found in 26.4% of the samples from a cohort of convalescent patients with acute febrile symptoms in Maputo city in 2013 and a case of severe chikungunya infection was reported in the Northern region of the country in 2014 [18]. These findings of arbovirus circulation in the country provide convincing evidence that transmission risk might be higher than expected. Several biotic and abiotic factors might also enhance the transmission risk of *Aedes*-borne arboviral diseases in Mozambique. The country is the third most vulnerable to extreme climate events, such as floods and droughts in Sub-Saharan Africa [19]. The frequency of unusually long periods of droughts have increased in the last decades leading people to opt for water storage practices which might increase the number of sites suitable for *Aedes spp* larvae [20]. In addition the rate of unplanned urbanization in Mozambique is high, favoring the presence of high population densities with associated artificial breeding sites for the mosquitoes [21]. Field studies of *Aedes* populations of sub-Saharan Africa are mostly from East, Central or West Africa [22–32] and little data is available for the Southern region of Africa. In particular in Mozambique [33], with the exception of an exploratory study conducted in four districts during a dengue outbreak in 2014 [34], there has been no systematic study concerning the distribution of *Aedes spp* populations. This is a barrier for the implementation of preventive and control interventions. This report, therefore, describes the results of the first country-wide survey of the density, distribution and breeding sites of *Aedes spp* in Mozambique.

Methods

Study area

Mozambique is situated in southeast coast of Africa with 2,515 km of coastline, and an estimated population of 27 million inhabitants [35]. The climate is tropical with two distinct seasons, namely; the rainy season from November-April and dry season from May-October. The average humidity ranges between 70–80%, with highest values being reported in Central and North regions. The average annual air temperature varies between 20˚C in the South to 26˚C in Northern regions.

Ethics statement

The study was approved by the Mozambican National Bioethics Committee (Ref #: 05/CNBS/2016). Oral consent to examine potential breeding habitats was obtained from the head of the household.

Sampling design and households selection

A cross-sectional study was conducted between March 19 and April 30, 2016, during the rainy season, in a total of 32 districts. Households were selected using a sampling approach stratified into three stages. The first stage involved the selection of all the eleven provinces of Mozambique to ensure that every province is represented in this survey. In each province, three

districts and in each district, one village or neighbourhood were selected as a second stage, on the basis of the following criteria: i) occurrence of confirmed dengue cases in the preceding months or years, and ii) climatic and socio-demographical factors (human population density and degree of urbanization) considered suitable for the occurrence and establishment of dengue vectors. The most populated and urbanized village or neighbourhood was preferentially chosen.

A spatial sampling procedure oriented to clusters of households was adopted to select households. A cluster was considered as a geographical area comprising between 10–20 households located within a radius of 50–100 metres. The selection of a household cluster was carried out following the procedure described by Troyo *et al.* [36]. According to this procedure, an administrative map of each village/neighbourhood was obtained using Google Earth Pro v. 7.3.0 (Google Inc., USA). Then, grid cells of $10km^2$ of the area were drawn on the map. The number of grid cells varied according to the size of the region. Grids were numbered starting from the cell on the upper left corner of the map. Then, a random sample of three $10km^2$ area grids was selected for the household cluster survey. In each of these grids, three clusters comprising 10–20 households were selected, based on the accessibility of the location. The clusters were at least 400 metres apart, considered to be the maximum distance of *Ae. aegypti* flight [37], to reduce the likelihood of pseudoreplication. A household was defined as a single unit of accommodation (individual household or an apartment) including the surrounding enclosure/compounds.

Entomological survey

In every household, intra and peridomestic breeding sites were inspected for the presence of immature stage (larva and pupa) of *Ae. Aegypti* and *Ae. albopictus*. All selected households were assessed indoors and outdoors. We considered as outdoors any place outside the rooms, but inside the enclosure/compound, including the rooftop, while any place inside the household was classified as indoors. The immature stages were sampled in all water holding containers following standard operating procedures for *Ae. aegypti* [38]. Containers were classified according to the presence of larvae (positive/negative). For small containers, the total number of larvae and pupae (as well as pupa carcasses) were collected using pipettes, whereas for containers $\geq$ 25 litres in volume or wells, the funnel and sweeping-net technique and dipper (500 μm of mesh diameter) were used [38, 39] and ten dips and sweeps were performed per container. Larvae were transported to the insectary and reared to adults under controlled environmental conditions of temperature (27˚C ± 2˚C). Adults were morphologically identified using the taxonomical key of Huang [40]. The identification of specimens was double checked by two-experienced entomologists. The field team at each province comprised four entomologists, two from the central level and two from the provincial level.

Mosquitoes collection, transportation, preservation and morphological identification

Water holding containers were categorized according to the type of container. All information related to each container including the presence of *Aedes spp*., and whether immature stages were sampled as larvae or pupae, was recorded in a field form. Immature forms were collected using pipette or dipper net (5 x 7 cm, 500 μm mesh) depending on container type and its location in the household [35]. All larvae and pupae were stored in a labeled specimen bottle and transported to local insectaries for growth until adult stage according to the standard procedures for rearing mosquitoes [51]. Upon adult emergence, mosquitoes were sacrificed and preserved on a 1.5 ml tube containing silica gel. All preserved samples were transported to the

Medical Entomology Laboratory (ENTMED) at National Institute of Health (INS) in Maputo for morphological identification of the *Aedes* species under a stereomicroscope using a taxonomic key [41].

Data analysis

Data were entered into a database developed using Microsoft Excel 2013 imported into Stata 13 for descriptive data analysis to determine the frequencies and distribution of *Ae. aegypti* and *Ae. albopictus*. The container index (CI) was determined using the following formula: CI = Total n° of positive container / Total n° of water–holding containers ×100% [42]. The spatial variation of CI estimates for each region was visualized in maps using ArcGIS 10.2 Software (ESRI Inc, Redlands, CA), were used to produce maps of occurrence.

Results

Geographical distribution of *Aedes spp.*

A total of 2,807 water-holding containers were inspected of which 628 (22.4%) were positive for *Ae. aegypti*. *Aedes albopictus* was only found in a single breeding site located at Moatize district (Central region), which was also positive for *Ae. aegypti* (Fig 1).

Pink coloured areas depict those districts where *Ae. aegypti* breeding sites were found. *Aedes aegypti* was found in all sampled districts. The legend key (*) indicates the only district where *Ae. albopictus* was found in this survey.

The Container index (CI) of *Aedes spp*. was higher in the Central region (43.6%; 260/596), followed by the North (36.9%; 228/617), whilst the lowest CI was found in the South region (8.7%; 140/1594) (Fig 2).

In the Northern region, the highest *Ae. aegypti* CI at the Province level was reported in Nampula (49.4%; 158/320), followed by Cabo Delgado (24.3%; 28/115) and Niassa (23.1%; 42/182) (Table 1). The districts of Nacala Porto (CI = 68.1%; 47/69) and Nampula city (CI = 46.7%; 78/167) in Nampula Province, and Pemba Metuge (CI = 42.8%; 9/21), in Cabo Delgado Province exhibited the highest infestation levels of *Ae. aegypti* (Table 1).

Regarding the Central region, the highest *Ae. aegypti* CI was registered in Manica (53.5%; 107/200), followed by Tete (46.2%; 24/52) and Sofala (38.4%; 53/138) Provinces. The lowest CI was found in Zambézia Province (35.0%; 75/214). The highest *Ae. aegypti* infestation levels were found in Milange district (CI = 62.3%; 33/53) in Zambézia Province, Changara district (CI = 61.1%; 11/18) in Tete Province and Sussundenga district (CI = 60.3%; 35/58) in Manica Province.

In South Mozambique, the highest CI was reported in Maputo city (37.5%; 15/40), followed by Maputo (16.8%; 48/285) and Gaza (13.1%; 52/396) Provinces. The lowest CI was reported in Inhambane Province (2.9%; 25/863). The districts with highest *Ae. aegypti* CI in the South were Kamachaquene (50.0%; 2/4) and Kanfumo (36.1%; 13/36) in Maputo city and Matola district (30.2%; 29/96) in Maputo Province (Table 1).

Breeding sites of *Ae. aegypti* and *Ae. albopictus*

The types of container in which larvae of *Ae. aegypti* were found is shown in Table 2. Used tires were the most frequent type of containers, followed by flower pots, jar/pots, cement tanks, buckets, disposed cans and bottles. A total of 2,807 potential breeding containers subdivided into 9 different groups were sampled. The highest *Ae. aegypti* immature stages positivity rates were found in used tires (35.3%; 448/1268), cement tanks (32.3%; 20/62) and drums (22.1%; 21/95). On the other hand, cans (9.5%; 14/146), bottles (9.4%; 7/74) and flower pots

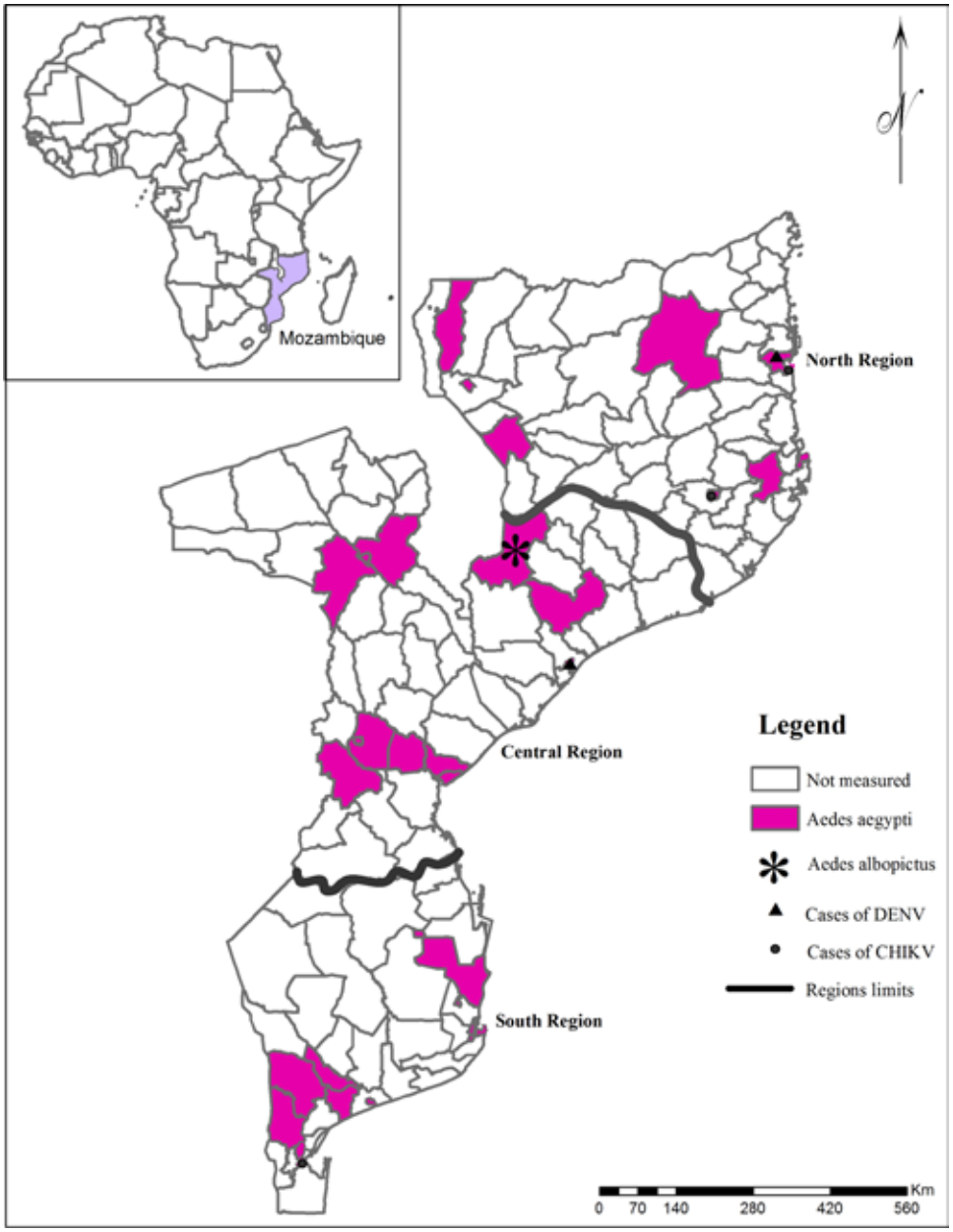

Fig 1. Map of Mozambique highlighting the three main regions of the country, and the geographical locations of the 32 districts studied.

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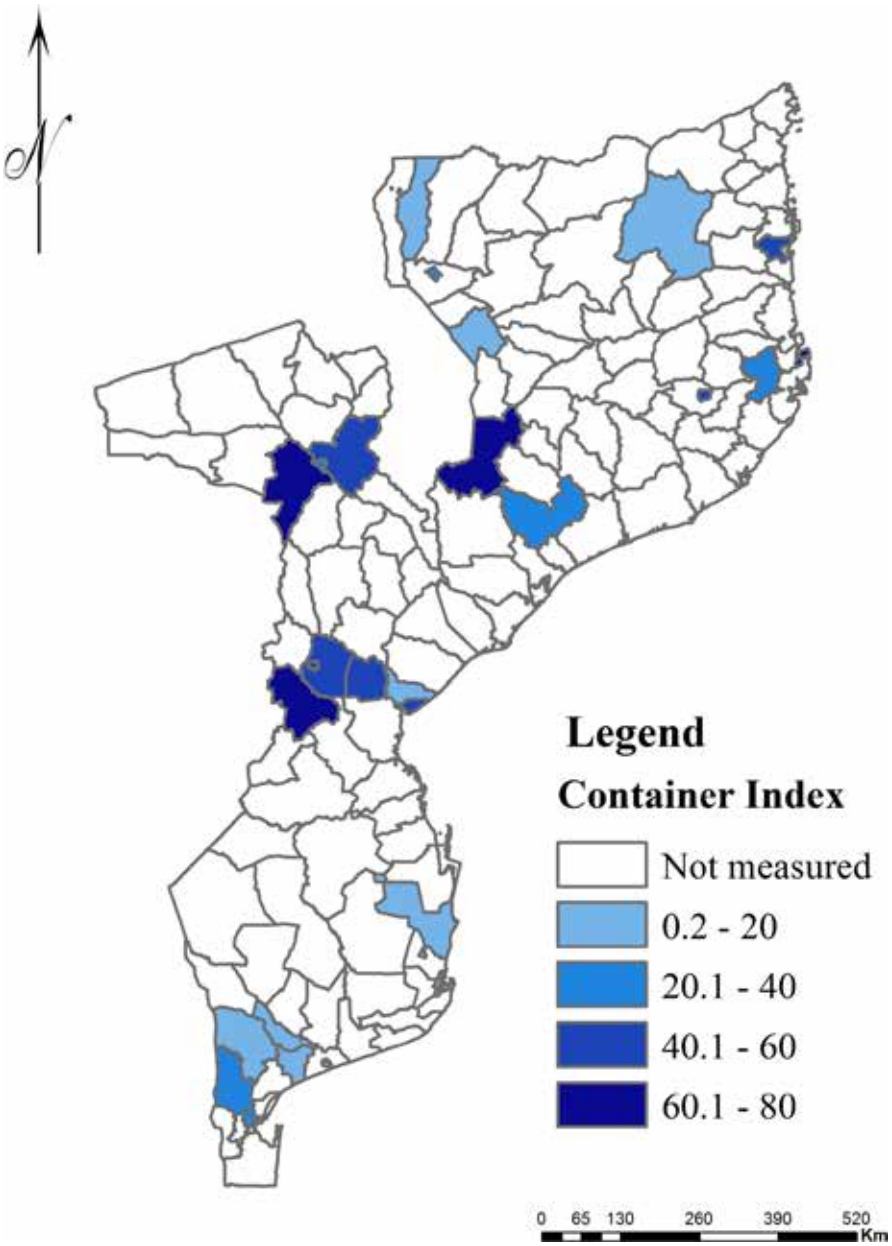

Fig 2. Infestation of *Aedes aegypti*, expressed as container index (CI), in 32 districts surveyed between March and April 2016.

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Table 1. Presence of larvae/pupae of *Aedes* (*Stegomyia*) *spp*. per container inspected stratified by region, province, district and neighborhood, March-April 2016.

Region and Province		District	Neighborhood	*# Inspected n*	*# Positive, n (%)*	
					Ae. aegypti	*Ae. albopictus*
Total				**2807**	**628 (22.4)**	**1 (0.03)**
Northern region				**617**	**228 (36.9)**	
	Cabo Delgado			***115***	***28 (24.3)***	
		Pemba Metuge	3 de Fevereiro	21	9 (42.8)	
		Motepuez	Miringe	59	7 (11.8)	
		C. Pemba	Natiti	35	12 (34.2)	
	Nampula			***320***	***158(49.4)***	
		Nacala porto	Triangulo	69	47 (68.1)	
		Monapo	Topelane	84	33 (39.3)	
		C. Nampula	Muahivire	167	78 (46.7)	
	Niassa			***182***	***42 (23.1)***	
		C. Lichinga	Nzinje	112	31 (27.7)	
		Lago	Sanjala	36	6 (16.7)	
		Mandimba	Bairro Central	34	5 (14.7)	
Central region				**604**	**260 (43.0)**	**1 (0.2)**
	Manica			***200***	***107 (53.5)***	
		C. Chimoio	7 de Abril	84	47 (55.9)	
		Gondola	Josina Machel	58	25 (43.1)	
		Sussundenga	Nhamizara	58	35 (60.3)	
	Sofala			***138***	***53(38.4)***	
		C. Beira	Munhava	75	33 (44.0)	
		Dondo	Nhamayabue	23	3 (13.0)	
		Nhamatanda	3° Bairro	40	17 (42.5)	
	Tete			***52***	***24 (46.2)***	***1 (1.9)***
		C. Tete	Filipe S. Magaia	22	7 (31.8)	
		Moatize	25 de Setembro	12	6 (50.0)	1 (8.3)
		Changara	Nhalicune	18	11 (61.1)	
	Zambézia			***214***	***75 (35.0)***	
		Mocuba	Marananeulo	63	20 (31.7)	
		Milange	25 de Junho	53	33 (62.3)	
		C. Quelimane	Floresta	98	22 (22.4)	
Southern region				**1586**	**140 (8.8)**	
	Gaza			***396***	***52 (13.1)***	
		Xai-Xai	P. Lumumba	255	40 (15.7)	
		Chokwe	1° Bairro	73	7 (9.6)	
		Bilene	6° Bairro	68	5 (7.4)	
	Inhambane			***865***	***25 (2.9)***	
		Massinga	7 de Setembro	233	7 (3.0)	
		C. Inhambane	Chalambe 2	487	1(0.2)	
		Maxixi	Chalambe 1	145	17 (11.7)	
	Maputo Cidade			***40***	***15 (37.5)***	
		Kanfumo	Malhangalene B	36	13 (36.1)	
		Kamachaquene	Polana Caniço	4	2 (50.0)	
	Maputo Província			***285***	***48 (16.8)***	
		Magude	Ricatlana	171	10 (5.8)	
		Matola	Infulene	96	29 (30.2)	
		Muamba	Cimento	18	9 (50.0)	

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Table 2. Presence of larvae/pupae of *Aedes* (*Stegomyia*) *spp*. in different breeding sites stratified by region and province, March-April 2016.

Region/Province		Total inspected (n)	Total positive, n (%)	Number of positive breeding sites/Number of total breeding sites inspected (%)								
				Used tires	Pots	Drums	Cement tanks	Buckets	Cans	Bottles	Flower pots	Plastic containers
TOTAL		**2807**	**628 (22.4)**	**448/1268 (35.3)**	**21/122 (17.2)**	**21 /95 (22.1)**	**20/62 (32.3)**	**17/156 (10.9)**	**14/146 (9.5)**	**7/74 (9.4)**	**34/576 (5.9)**	**46/308 (14.9)**
Northern	**Total**	**617**	**228 (36.9)**	**147/ 290 (50.7)**	**14/38 (36.8)**	**6/17 (35.3)**	**10/20 (50.0)**	**13/52 (25.0)**	**9/81 (11.1)**	**6/52 (11.5)**	**8/23 (34.8)**	**15/44 (34.1)**
	Cabo Delgado	115	28 (24.3)	9/18 (50.0)	1/1 (100.0)	1/1 (100.0)	7/10 (70.0)	4/18 (22.2)	1/21 (4.8)	1/29 (3.4)	2/8 (25.0)	2/9 (22.2)
	Nampula	320	158 (49.4)	110/164 (67.1)	12/ 30 (40.0)	5/12 (41.7)	0/1 (0.0)	9/28 (32.1)	5/42 (11.9)	5/23 (21.7)	-	12/20 (60.0)
	Niassa	182	42 (23.1)	28 /108 (25.9)	1/7 (14.3)	0/4 (0.0)	3/9 (33.3)	0/6 (0.0)	3/18 (16.7)	-	6/15 (40.0)	1/15 (6.7)
Central	**Total**	**604**	**260 (43.0)**	**203/439 (46.2)**	**1/22 (4.5)**	**10/14 (71.4)**	**1/3 (33.3)**	**0/1 (0.0)**	**4/11 (36.4)**	**1/9 (11.0)**	**26/81 (32.0)**	**14/24 (58.3)**
	Manica	200	107 (53.5)	81/154 (52.6)	1/1 (100.0)	9/10 (90.0)	1/2 (50.0)	-	-	1/9 (11.0)	-	14/24 (58.3)
	Sofala	138	53 (38.4)	53/138 (38.4)		-	-	-	-	-	-	-
	Tete	52	25 (48.1)	21/43 (48.8)	-	1/4 (25.0)	0/1 (0.0)	0/1 (0.0)	1/1 (100.0)	-	2/2 (100.0)	-
	Zambézia	214	75 (35.0)	48/104 (46.2)	0/21 (0.0)	-	-	-	3/10 (30.0)	-	24/79 (30.4)	-
Southern	**Total**	**1586**	**140 (8.8)**	**98/539 (18.2)**	**6/62 (9.7)**	**5/64 (7.8)**	**9/39 (23.0)**	**4 /103 (3.9)**	**1/54 (1.9)**	**0/13 (0.0)**	**0/472 (0.0)**	**17/240 (7.1)**
	Gaza	396	52 (13.1)	38/145 (26.2)	1/13 (7.7)	1/23 (4.3)	0/5 (0.0)	1/37 (2.7)	1/40 (2.5)	-	-	10/133 (7.5)
	Inhambane	865	25 (2.9)	14/193 (7.3)	3/16 (18.8)	2/24 (8.3)	2/15 (13.3)	3/44 (6.8)	0/12 (0.0)	0/13 (0.0)	0/467 (0.0)	1/81 (1.2)
	Maputo Cidade	40	15 (37.5)	8/29 (27.6)	-	2/4 (50.0)	5/7 (71.4)	-	-	-	-	-
	Maputo Província	285	48 (16.8)	38/172 (22.1)	2/33 (6.1)	0/13 (0.0)	2/12 (16.7)	0/22 (0.0)	0/2 (0.0)	-	0/5 (0.0)	6/26 (23.1)

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(6.3%; 36/576) had a lower infestation (Table 2). The *Ae. albopictus* larvae found Moatize district, Tete Province came from a used tire.

Discussion

Arboviruses are spreading at an alarming pace across the world and a growing fraction of them have been reported in recent years in Mozambique [8, 16, 18, 43, 44]. Data on the distribution and ecology of anthropophilic *Aedes* mosquito species in the country remains limited. Previous records from the 1960's reported the presence of *Ae.* (*Stegomyia*) species in Northern to Southern regions, with highest densities in coastal areas [45]. However, the distribution may have changed.

Aedes aegypti were collected in every district sampled, which explains the transmission of DENV, CHIKV and others arbovirus in many parts of Mozambique [16–18, 43, 44, 46]. Using mathematical modeling the heterogeneity of abundance and distribution of *Ae. aegypti* shown in the present study has previously been suggested by Kraemer and others [8]. Similar findings were observed in Cameron [45] and in a prior study conducted in four cities of Mozambique in 2014 [34]. Thus, the risk of arbovirus transmission is also likely to be heterogeneous across the country, suggesting that vector control activities should prioritize the Central and Northern regions, the regions with higher *Ae. aegypti* infestation levels.

The lower abundance of *Ae. aegypti* in the South might be due to lower amount of rainfall [47, 48], relatively good environmental sanitation and a consistent water supply system, which reduces number of putative *Ae. aegypti* and *Ae. albopictus* breeding sites. In contrast, the high CI in Northern Mozambique may be due to the high annual precipitation [48], a poor water supply system (leading to an increase in water storage containers) and poor environmental sanitation, which increases the number of putative breeding sites such as, disposed cans and abandoned used tires.

Our results are in accordance with a preliminary investigation conducted in four districts in 2014 in Mozambique [34] and could explain why most of the arbovirus outbreaks reported so far occurred in the Northern region [16, 18, 46]. A similar pattern has been observed for malaria Southern regions having, lower prevalence rates than Central and North regions of the country [48, 49].

It is well known that unplanned urbanization represents an important driver of anthropophilic *Aedes spp*. expansion in sub-Saharan Africa [50]. Increasing urbanization is only likely to exacerbate the problem. According to the World Urbanization Prospect report, the urban population in Mozambique rose from 7.0% in 1970 to 32.8% in 2017 and it is predicted to be 50.0% by 2050 [51]. It therefore becomes increasingly important that control and monitoring starts soon.

Aedes albopictus was only found in Moatize district, in Tete Province, in the Central region. Our data, together with a recent report by Kampango and Abílio [15], who initially described the presence of *Ae. albopictus* in Mozambique in the south of the country, suggests that it may have already invaded and be successfully established in other areas of the country. The potential spread of *Ae. albopictus* throughout the country raises serious concerns, since it is a possible vector of at least 22 viruses affecting humans, including dengue, chikungunya, Zika, yellow fever and Japanese encephalitis virus [45, 52]. The geographical distribution of *Ae. albopictus* worldwide has expanded over the past three decades, with several countries reporting its presence for the first time [23–25, 53–56]. Climate change has been pointed out as a major determinant of *Ae. albopictus* expansion [11, 57]. Additional research is urgently needed for a better understanding of the ecological features of *Ae. albopictus* under local conditions.

The present survey showed that the preferred breeding site of *Ae. aegypti* were used tires, cement tanks and drums. This was not surprising, considering that *Ae. aegypti* is highly synanthropic. Old tires are commonly used in Mozambique for fencing in peri-urban and rural households, to weigh down the tin sheeting used for roofing material in some houses and to control soil erosion [34]. Furthermore, used tires are frequently sold along the main public highways, where they usually remain unattended and exposed to rainfall and sunlight for long periods. Cement tanks and drums are the most frequently found water-storage containers in communities with intermittent or deficient water supplying. Data from Cameroon, India and Vietnam [45, 58–60] also showed that water storages for domestic use in cement tanks and drums are among the most productive breeding sites of *Aedes* mosquitoes.

Thus, *Ae. aegypti* and *Ae. albopictus* control programs should concentrate their interventions on the education and engagement of residents in appropriate use and disposal of old tires and covering of water drums and tanks.

Since Mozambique has a well established sentinel surveillance system for malaria vectors, we recommend that *Aedes* surveillance be integrated into the existing surveillance system for malaria vectors that is being carried out in urban and rural areas of the country. The surveillance for *Aedes* should be enhanced to urban areas where *Ae.* (*Stegomyia*) mosquitoes are more frequent, in order to ensure its sustainability and optimize use of scarce resources.

Although we were only able to undertake samples from 32 out of the 152 districts of Mozambique ours remains the largest study conducted so far in the country. Our results

indicate that *Ae. Aegypti* is present in all regions of the country with, therefore, a risk of dengue, Zika and chikungunya transmission in urban areas.

In conclusion, we found that *Ae. aegypti* has heterogeneous distribution throughout Mozambique. The mosquito is likely to be present throughout the country, enhancing the risk of dengue, chikungunya and Zika transmission. *Aedes albopictus*, another potential vector of these arboviruses, may have a more limited distribution. Further systematic studies are required to determine the degree of ecological association between these two vectors, as well as their contribution in the arboviruses transmission in the country. A national surveillance system for *Aedes spp*. in Mozambique is required.

Supporting information

S1 File.
(XLSX)

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Consent for publication

Our manuscript does not present any individual person's data.

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Parasites & Vectors

SHORT REPORT Open Access

First confirmed occurrence of the yellow fever virus and dengue virus vector *Aedes* (*Stegomyia*) *luteocephalus* (Newstead, 1907) in Mozambique

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Abstract

Background: Mozambique, same as many other tropical countries, is at high risk of arthropod-borne virus (arbovirus) diseases and recently two dengue virus (DENV) outbreaks occurred in the northern part of the country. The occurrence of some important vector species, such as *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) and *Ae.* (*Stg.*) *albopictus* (Skuse), besides several other sylvatic vectors, have been reported in the country, which may indicate that the transmission of some arboviruses of public health importance may involve multiple-vector systems. Therefore, knowing the occurrence and distribution of existing and the new important vectors species, is crucial for devising systematic transmission surveillance and vector control approaches. The aim of this study was to map the occurrence and distribution of mosquito species with potential for transmitting arboviruses of human and veterinary relevance in Niassa Province, Northern Mozambique.

Methods: Field entomological surveys were undertaken in April 2016 in Lago District, Niassa Province, northern Mozambique. Breeding sites of mosquitoes were inspected and immature stages were collected and reared into adult. Mosquitoes in the adult stages were morphologically identified using taxonomic keys. Morphological identification of *Aedes* (*Stegomyia*) *luteocephalus* (Newstead) were later confirmed using dissected male genitalia and molecular based on the phylogenetic analyses of the sequenced barcode (*cox*1 mtDNA) gene.

Results: A total of 92 mosquito larvae collected developed into adults. Of these, 16 (17.39%) were morphologically identified as *Ae. luteocephalus*. The remaining specimens belonged to *Ae.* (*Stg.*) *aegypti* ($n = 4$, 4.35%), *Ae.* (*Aedimorphus*) *vittatus* ($n = 24$, 26.09%), *Anopheles garnhami* ($n = 1$, 1.09%), *Culex* (*Culiciomyia*) *nebulosus* ($n = 28$, 30.43%), *Eretmapodites subsimplicipes* ($n = 18$, 19.57%) and *Toxorhynchites brevipalpis* ($n = 1$, 1.09%), taxa already known to the country. Male genitalia and phylogenetic analyses confirmed the identity of *Ae. luteocephalus* specimens collected in this study.

Conclusions: To our knowledge, this is the first detection of *Ae. luteocephalus* in Mozambican territory, a vector species of yellow fever virus (YFV), Zika virus (ZIKV) and dengue virus (DENV) in Africa. Further studies are encouraged to investigate the role of *Ae. luteocephalus* in the transmission of arboviral diseases in Mozambique.

Keywords: New record, Arthropod-borne, Virus, Vector, Aedine, Mosquito

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Background

The occurrence and distribution of mosquito-borne arboviruses, particularly those transmitted by *Aedes* species, such as dengue (DENV), Zika (ZIKV) and yellow fever viruses (YFV) represent a serious threat to global health, particularly to African sub-Saharan countries [1–6]. It has been estimated that annually nearly 3,312,040 cases and 4,032 deaths of DENV infections occur worldwide [3, 7]. Zika virus, on the other hand, has been declared a public health emergency of international concern since 2016 [6]. Likewise, re-emergence of yellow fever cases has been lately observed in some African countries and Brazil, despite the existence of an effective vaccine that could protect populations and control the disease [8].

Mozambique is home to several mosquito species, some of which are widely known and suspected arbovirus vectors [6, 9–11]. However, the role of the country's mosquito fauna in sustaining the transmission of endemic arboviral diseases such as dengue, yellow fever and chikungunya still remains poorly understood. One possible reason for such neglect may be due to the overwhelming number of malaria transmission cases that has been recorded throughout the country [12]. Most recently, two dengue virus outbreaks were observed in the northern region of Mozambique amounting to thousands of cases of infection [13, 14]. Likewise, the presence of major arbovirus vectors such as *Ae.* (*Stg.*) *aegypti* and *Ae.* (*Stg.*) *albopictus*, besides others with a more sylvatic distribution, has been reported in the country [6, 9], implying that arbovirus transmission dynamics may likely involve multiple-vector systems. These findings underscore the need for a thorough understanding of occurrence and arbovirus transmission role, of overlooked important potential vectors of public health importance. *Aedes* (*Stg.*) *luteocephalus* (henceforth, *Ae. luteocephalus*) is a mosquito species native to Africa, reported in *circa* twenty countries, particularly in the western and central regions of the continent, as well as in southern African countries such as Botswana and Zimbabwe [15–22]. This species has varied distribution throughout different geographical landscapes comprising forests, savannah, mangrove gallery, as well as intermediate landscapes between sylvatic and urban areas, where it has been found breeding in a diversity of natural and human-made larval sites [15, 17, 20].

Aedes luteocephalus is a competent vector for YFV [23] and can be an important vector of ZIKV and DENV, as observed in competence assays elsewhere in West and Central Africa [17, 20, 24]. Therefore, an in-depth understanding of the occurrence and distribution of these important vectors of arboviruses is crucial for devising accurate and effective evidence-based transmission control measures. The aim of this study was to map the occurrence and distribution of mosquito species with potential of transmitting arboviruses of human and veterinary relevance in the region.

Therefore, this report represents, to the best of our knowledge, the first confirmed record of *Ae. luteocephalus* in Mozambique. The implication of this discovery in the design of arthropod-borne virus (arbovirus) surveillance and control measures in Mozambique is briefly discussed.

Methods

Study site and sampling strategy

Entomological field surveys were conducted in April of 2016 in Lago District, neighborhood of Maniamba (12°41.881′S, 34°48.539′E), Niassa Province in northern Mozambique. All potential types of natural and artificial mosquito breeding sites were surveyed for the presence of mosquito immature stages. Mosquito larvae and pupae were sampled following standard operating procedures [25]. Additionally, used car tyres filled with water were placed for approximately 500 m apart in a transect along the main road crossing Chapama forest and Luaui River in an effort to collect as many samples as possible at different sites in the vicinity, to better sample the area. The tyres were left in the field for 8 days, after which they were surveyed for immature mosquitoes. Each breeding place was surveyed using a Pasteur pipette. Collected specimens were sorted, placed in the 500 ml plastic bottles, filled up to 75% of water from specific breeding place and labelled accordingly. All samples collected were then transported to local insectaries for rearing to adults [25, 26]. Preliminary morphological identification was conducted on adult stages emerged, using taxonomic keys [15, 21, 27–29]. Adult specimens were preserved individually in single 1.5 ml Eppendorf tubes at − 80 °C for further morphological and molecular analysis. Whole mosquitoes, male and female, of *Ae. luteocephalus* were re-observed and male terminalia were separated from the abdomen and adsorbed in Marc André solution [27]. Genitalia were dissected under stereomicroscope and mounted in formic acid-polyvinyl alcohol (PVA) solution between a slide and a cover slip [27, 28] and photographed under Olympus stereomicroscope SZ51 (Olympus, Seoul, South Korea), Olympus microscope (BX51, Olympus, Seoul, South Korea) and an Olympus SC30 digital camera (Olympus, Tokyo, Japan), respectively.

Molecular analyses of adult mosquito specimens

Genomic DNA was extracted from remaining the abdomen and legs of 4 males, as described in Mixão et al. [27]. Molecular analysis was targeted at the barcoding section between positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1 mtDNA). Amplification of *cox*1 mtDNA was performed using LCO1490 and HCO2198 specific primers under PCR conditions as described by Folmer et al. [30]. The nucleotide (nt) sequences obtained were deposited in GenBank [31] under the accession numbers LC536733-LC536736).

The degree of correspondence between the barcode *cox*1 mtDNA gene sequences obtained in this study were compared against those at GenBank database using BLASTn, (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Barcode of Life Data Sytems-v4 (http://www.boldsystems.org/) [32].

Phylogenetic reconstructions using *cox*1 molecular data were carried out from multiple alignments of nt sequences obtained using the iterative G-INS-I method as implemented in MAFFT v. 7 [33]. Subsequently, attained sequences were edited using both GBlocks [34] and visual inspection using BioEdit 7.0.5 [35] to ensure the correct alignment of homologous codons. Phylogenetic analysis was carried out using the Maximum Likelihood (ML) optimization criterion and GTR + Γ + I (GTR-General Time Reversal, Γ-Gamma distribution, I-proportion of invariant sites) as the dataset best-fitting evolutionary model, as suggested by jModelTest2 [36]. The ML phylogenetic tree was constructed with W-IQ-tree [37], using the bootstrap test (with 1000 random data resampling's) for assessment of the tree topological stability. The tree was edited with FigTree 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Due to the lack of a satisfactory number of examined sequences and collected specimens, we were unable to run haplotype network analysis for robust inference of the origin of *Ae. luteocephalus* collected in this study.

Results and discussion

A total of 92 adult mosquitoes emerged from collected larvae and pupae; of these, 16 were tentatively identified as *Ae. luteocephalus* (12 females and 4 males) based on morphological features. The remaining specimens were identified as *Anopheles* (*Celia*) *garnhami* ($n = 1$), *Ae.* (*Aedimorphus*) *vittatus* ($n = 24$), *Ae.* (*Stg.*) *aegypti* ($n = 4$), *Culex* (*culiciomyia*) *nebulosus* ($n = 28$), *Eretmapodites subsimplicipes* ($n = 18$) and *Toxorhynchites brevipalpis* ($n = 1$) (Table 1).

Ten females of *Ae. luteocephalus* collected in this survey were deposited in the insect depository of Instituto Nacional de Saúde (INS) in Maputo Province, Mozambique, stored in individual Eppendorf® tubes (accession numbers MZ113-a1.2, a1.4–a1.12) and 6 specimens (2 females and 4 males) deposited in the Entomoteca (Insect collection) of the Institute of Hygiene and Tropical Medicine (IHMT), Lisbon, Portugal (accession numbers MZ113-a1.1–a1.3 and MZ113-a2.1–a2.4).

All 16 larvae, which gave rise to the adults *Ae. luteocephalus* and 5 *Ae. vittatus* were found cohabiting in a rock-pool of clear water, with approximately 20 × 15 cm (Additional file 1: Figure S1), located at the Luaui riverbank and exposed to sunlight. Other species including the remaining 19 *Ae. vittatus* were obtained from other breeding sites, namely the tyres that were placed as "ovitraps", while no specimens of *Ae. luteocephalus* were obtained from any other breeding site (Table 1).

Table 1 Date of collection, mosquito species, sex, their respective niches, percentage (%) and number of mosquitoes collected from Mozambique

Date of collection	Habitat	Species	Sex (*n*)	Subtotal (%)
1 April 2016	Rock-pool of clear water	*Aedes* (*Stegomyia*) *luteocephalus*	M (4)/F (12)	16 (17.39)
		Aedes (*Aedimorphus*) *vittatus*	M (5)	5 (5.43)
8 April 2016	Tyre placed as "ovitraps"	*Anopheles* (*Celia*) *garnhani*	M (1)	1 (1.09)
		Aedes (*Stegomyia*) *aegypti*	M (4)	4 (4.35)
		Aedes (*Aedimorphus*) *vittatus*	M (3)/F (16)	19 (20.65)
		Culex (*Culiciomyia*) *nebulosus*	M (10)/F (18)	28 (30.43)
		Eretmapodites subcimplicipes	M (3)/F (15)	18 (19.57)
		Toxorhynchites brevipalpis	F (1)	1 (1.09)
Total collected				92

Abbreviations: F, female; M, male

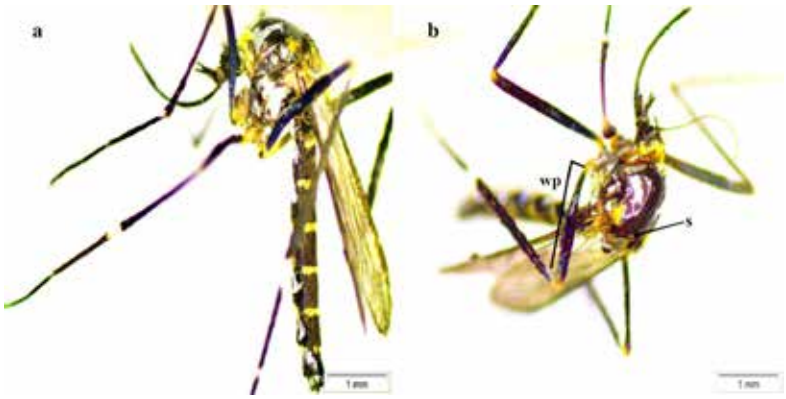


Fig. 1 **a** Adult female of *Aedes luteocephalus* specimen showing general characters. **b** Adult specimen highlighting the main diagnostic features including for the scutum (with median-longitudinal yellow stripe) (s) and hind tarsomere anteriorly with large pale band at base and two large white patches on median and apical areas (wp), both images at 20× magnification

Exhaustive studies are required to collect more information about *Ae. luteocephalus* distribution and infestation to better understand the importance of this and other arbovirus vectors throughout the country.

The habitat where they were found corresponds to its natural range of tropical forest habitats. Indeed, *Ae. luteocephalus* can be found in forests, savannah, mangrove gallery forest and also in intermediate landscapes between sylvatic and urban areas [15, 17, 20]. Bionomically the specie utilizes varied range of breeding places as rot holes, tree holes, rock holes, bamboos, bamboo stems, tree fork, plastic bottles and artificial containers in height up to 9 metres [15–20]. *Aedes luteocephalus* breeding sites with similar features have also been reported elsewhere and in association with *Ae. africanus*, of the same group, but not to our knowledge, with *Ae. vittatus*, which is not surprising, as this later species also favors rock pools [15–20].

Preliminary analysis of the nucleotide sequences of *cox*1 mtDNA obtained from the 4 males revealed completely identical sequences, which suggests that the *Ae. luteocephalus* larvae sequenced were siblings [28], possibly hatched from eggs laid by a single female, as mtDNA is maternally inherited, quite possible given the small dimensions of the breeding rock pool (Additional file 1: Figure S1).

Laboratory experiments have shown that *Ae. luteocephalus* can transmit yellow fever with an efficiency comparable to *Ae. aegypti* [23], readily bites humans and is involved in the transmission of YFV in West and Central Africa, and chikungunya virus (CHIKV), ZIKV and DENV2 have been isolated from it in West Africa [15]. Although *Ae. luteocephalus* habitat has been essentially rural and sylvatic, increasing demographical expansion and human pressure on forest resources, for logging and farming as it has been observed in the studied place. This condition might also increase the likelihood of vector-human and, therefore, the risk of rural arbovirus epidemics. Therefore, additional studies are urgently needed to investigate the effect of anthropogenic activity on arboviruses transmission risk in Lago District.

All *Ae. luteocephalus* specimens collected in this study, had a distinct middle longitudinal yellow stripe of thin scales in the scutum region; scutellum with wide white scales on lateral lobes; basal pale band on terga II-VI more yellow; and hind femur anteriorly with a huge light band at base and alongside two sizable white spots on median and apical regions (Fig. 1). These characteristics

Fig. 2 Dissected male genitalia of *Aedes luteocephalus* showing gonocoxites with gonostylus (**a**) with gonostylar claw and claspette (**b**) large, lobed with distal expanded portion, oval in dorsal view, with numerous simple setae on the apicolateral portion, and with some short setae on the apicomesal portion and aedeagus (**c**) at 100× magnification. *Scale-bar*: 100 µm

are similar to those described by Huang [15] and Jupp [21].

All four dissected male genitalia showed gonocoxites with gonostylus simple with few setae in the apical quarter and a long slender gonostylar claw, claspette large, lobed, with distal expanded portion, oval in dorsal view, with numerous simple setae on the apicolateral portion, and with some short setae on the apicomesal portion (Fig. 2). These are considered the most important distinctive features that separate the species from other members of the *africanus* group, namely, *Aedes* (*Stg.*) *africanus*, to which it belongs [15, 21].

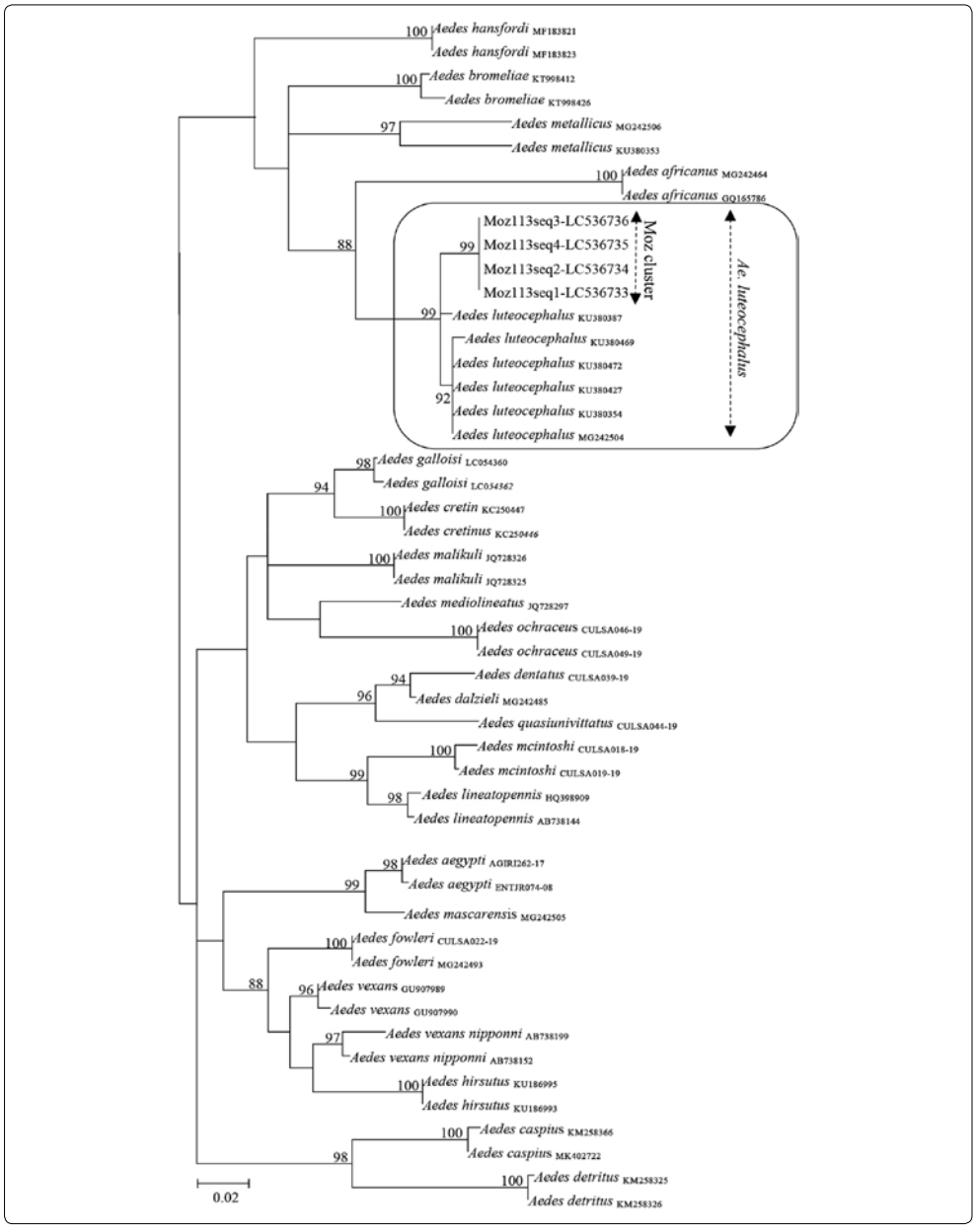
Barcode gene sequences of all specimens analyzed displayed 97.65–98.12% sequence identity with homologues using BLAST (MegaBlast option) and 97.82–98.26% identity in the BOLDSYSTEMS database with sequences of *Ae. luteocephalus* from Tanzania and Kenya, thereby confirming its taxonomic identity [19, 20, 22]. These findings represent, as far as we are aware, the first confirmed record of *Ae. luteocephalus* in Mozambique, while all other specimens collected in this survey correspond to taxa already known for the country. Additionally, phylogenetic reconstruction analysis carried out on the basis of a dataset of multiple *Aedes* species of the subgenera *Stegomyia*, *Aedimorphus*, *Neomelaniconion* and *Ochlerotatus*, clearly placed the *cox*1 sequences obtained in the course of this study in a topologically stable monophyletic cluster that only included *Ae. luteocephalus* reference sequences (Fig. 3). This further confirms the morphological, barcode and sequence similarity-based identifications presented above. Therefore, our results clearly confirm that *Ae. luteocephalus* collected in the study area are quite similar to those from the neighboring countries Tanzania and Kenya [19, 20, 22] and have now been found as part of a wide survey in the country for vectors of arboviruses Abílio et al., unpublished data). Further haplotype network analyses are recommended to ensure for robust inference of exact origin of *Ae. luteocephalus* from Mozambique.

Conclusions

Comparative morphological, molecular and phylogenetic analyses have consistently shown, for the first time, the occurrence in Mozambican territory of *Ae. luteocephalus*, a competent vector of yellow fever virus and dengue fever virus in Africa. This finding may help fill the gaps of our knowledge about the distributional ecology of this important and overlooked arbovirus vector. Further field

(See figure on next page.)

Fig. 3 Maximum likelihood phylogenetic tree based on partial *Aedes cox*1 sequences. Nodal support values ≥ 75 are shown. The reference sequences used are indicated with either their GenBank accession number or BoldSystems code. The sequences generated in this study are indicated in bold by their laboratory code and accession numbers and are grouped in a monophyletic cluster indicated as Moz cluster. The scale-bar indicates the number of nucleotide substitutions per site


Aedes hansfordi MF183821
Aedes hansfordi MF183823
Aedes bromeliae KT998412
Aedes bromeliae KT998426
Aedes metallicus MG242506
Aedes metallicus KU380353
Aedes africanus MG242464
Aedes africanus GQ165786
Moz113seq3-LC536736
Moz113seq4-LC536735
Moz113seq2-LC536734
Moz113seq1-LC536733
Moz cluster
Ae. luteocephalus
Aedes luteocephalus KU380387
Aedes luteocephalus KU380469
Aedes luteocephalus KU380472
Aedes luteocephalus KU380427
Aedes luteocephalus KU380354
Aedes luteocephalus MG242504
Aedes galloisi LC054360
Aedes galloisi LC054362
Aedes cretin KC250447
Aedes cretinus KC250446
Aedes malikuli JQ728326
Aedes malikuli JQ728325
Aedes mediolineatus JQ728297
Aedes ochraceus CULSA046-19
Aedes ochraceus CULSA049-19
Aedes dentatus CULSA039-19
Aedes dalzieli MG242485
Aedes quasiunivittatus CULSA044-19
Aedes mcintoshi CULSA018-19
Aedes mcintoshi CULSA019-19
Aedes lineatopennis HQ398909
Aedes lineatopennis AB738144
Aedes aegypti AGIRI262-17
Aedes aegypti ENTJR974-08
Aedes mascarensis MG242505
Aedes fowleri CULSA022-19
Aedes fowleri MG242493
Aedes vexans GU907989
Aedes vexans GU907990
Aedes vexans nipponni AB738199
Aedes vexans nipponni AB738152
Aedes hirsutus KU186995
Aedes hirsutus KU186993
Aedes caspius KM258366
Aedes caspius MK402722
Aedes detritus KM258325
Aedes detritus KM258326
0.02

and laboratory surveys are encouraged to investigate the role of *Ae. luteocephalus* in the transmission of arboviruses in Mozambique.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13071-020-04217-9.

Additional file 1: Figure S1. Corresponding author collecting larvae of *Ae. luteocephalus* in a rock-pool with clear water approximately 20 × 15 cm, located at the Luaui riverbank, Lago District, neighbourhood of Maniamba, Niassa Province, northern Mozambique.

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Authors' contributions
APA and APGA conceived the study. APA, EJA and AK performed field surveys and preliminary data analysis. APGA and RP performed molecular analysis and phylogenetic analysis. APA, AK, RP and APGA reanalysed the data and wrote the draft of the manuscript. APGA performed genitalia dissection and photographs. ESG, RP, LCBN, JMF, MS and APGA contributed with reagents, materials and equipment and reviewed the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
The study was approved by the Mozambican National Bioethics Committee (Ref #: 05/CNBS/ 2016) and the Faculty of Medicine of Eduardo Mondlane University (Ref #: CIBS FM&HCM/15/2018).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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PLOS ONE

Abundance and diversity of mosquito communities potentially associated with arbovirus transmission in two districts of Mozambique

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Corresponding Author:	Ana Paula Abilio Instituto Nacional de Saúde (INS) MOZAMBIQUE
Keywords:	Mosquito community, abundance, composition, diversity, Goba, Mopeia
Abstract:	Background: Accurate knowledge of the occurrence and distribution of mosquito community assemblages is critical for the establishment of mosquito-borne disease risk profiles and the unraveling of their epidemiology in particular settings, in order to address effective vector control measures. Therefore, this study aimed to investigate the abundance and dynamics of mosquito communities that are potentially associated with arbovirus transmission in two districts of Mozambique. Methods: Longitudinal surveys were conducted from 2014 to 2015 to investigate the structure of mosquito assemblage in Goba (Namaacha district) and Mopeia districts, located in the southern and central regions of Mozambique, respectively. The two districts had been previously affected by Rift Valley fever outbreaks. Host-seeking mosquitoes were sampled overnight, once a month using CDC light traps enhanced with CO 2 and CO 2 - baited Tent/Net traps. Collections were performed outdoors near occupied homesteads and animal shelters. Mosquito assemblages' abundance was estimated as the average number of specimens collected per site/month. Mosquito diversity within the community was estimated as the total number of species collected, and mosquito species diversity was estimated as total number of species (0 D), the effective number of equally common (1 D) and highly abundant species (2 D) in the community. The difference in abundance and mosquito community composition between sites and, as well as, possible influence of climate factors was investigated using Multivariate Abundance Linear Models (manyGLM). Results: A total of 33,201 mosquitoes were collected, 87.7% (29,109/33,201) of which in Mopeia and 12.3% (4,092/33,201) in Goba, Namaacha district. A total of thirty-one and thirty-seven mosquito species were found in Goba and Mopeia, respectively. Mansonia africana was the most dominant species in Goba, whereas Culex antennatus was the most dominant species in Mopeia. However, Chao1 species richness estimator indicated that eighteen more mosquito species are expected to occur in Mopeia, apart from those identified in this study. Results suggest also overall mosquito abundance and diversity peak during rainy season. However, diversity can remain higher for five months beyond end of rainy season in Mopeia. There was significant variability of mosquito abundance and composition between sites, season and also a significant association with rainfall and high average monthly air temperature. However, the magnitude of the effect of covariates is species-specific. Conclusion: The findings show a high diversity of vector species in mosquito communities from Goba and Mopeia. The two-mosquito communities showed significant between sites and seasonal dissimilarities are mostly driven mostly driven by variability of monthly rainfall and average maximum air temperature. The study underscores the need for further investigation on factors contributing to vector species establishment and arbovirus transmission in the studied sites.
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Abundance and diversity of mosquito communities potentially associated with arbovirus transmission in two districts of Mozambique.

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Results: A total of 33,201 mosquitoes were collected, 87.7% (29,109/33,201) of which in Mopeia and 12.3% (4,092/33,201) in Goba, Namaacha district. A total of thirty-one and thirty-seven mosquito species were found in Goba and Mopeia, respectively. *Mansonia africana* was the most dominant species in Goba, whereas *Culex antennatus* was the most dominant species in Mopeia. However, Chao1 species richness estimator indicated that eighteen more mosquito species are expected to occur in Mopeia, apart from those identified in this study. Results suggest also overall mosquito abundance and diversity peak during rainy season. However, diversity can remain higher for five months beyond end of rainy season in Mopeia. There was significant variability of mosquito abundance and composition between sites, season and also a significant association with rainfall and high average monthly air temperature. However, the magnitude of the effect of covariates is species-specific.

Conclusion: The findings show a high diversity of vector species in mosquito communities from Goba and Mopeia. The two-mosquito communities showed significant between sites and seasonal dissimilarities are mostly driven mostly driven by variability of monthly rainfall and average maximum air temperature. The study underscores the need for further investigation on factors contributing to vector species establishment and arbovirus transmission in the studied sites.

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Introduction

Mosquito-borne diseases (MBDs) remain one of the largest contributors to human and veterinary disease burden. It has been estimated that more than half of the global population is at risk of MBDs, with particular emphasis on tropical regions (WHO 2017; Wilder-Smith *et al.* 2017). Despite considerable control efforts, the prevalence of MBDs has shown a dramatic increase in endemic regions (Paixão *et al.* 2018; WHO 2020). Malaria, for instance,

caused 405,000 deaths in 2019, after years of astonishing reduction of incidence and mortality rate (Bhatt *et al.* 2015; Gething *et al.* 2016), followed by stagnation, and a recent rising, in the number of cases again (WHO 2020). On the other hand, 104,771 cases of dengue, and 4,050 deaths were also reported over the last two decades (Disease *et al.* 2018). Furthermore, the prevalence of dengue in individuals residing in Africa also increased during the same period (Simo *et al.* 2019). Concomitantly, it has been observed an unprecedented increase in frequency and severity of several other mosquito-borne arboviral disease outbreaks, including Zika, chikungunya, yellow fever, Rift Valley fever, West Nile fever (Wilder-Smith *et al.* 2017). This has been accompanied by the spread of vectors and pathogens to formerly non-endemic regions (Wilder-Smith *et al.* 2017; Kraemer *et al.* 2019). Rapid demographic expansion coupled with environmental changes due to unplanned land use, deforestation and habitat fragmentation, climate changes, and reduced susceptibility of vector populations to conventional insecticide-based control measures, have been agreed as being the main drivers of the observed surge in MDBs incidence worldwide (Norris 2004; Sutherst 2004; Ryan *et al.* 2019).

Past and recent evidence strongly suggest that Mozambique is highly susceptible for arboviral diseases epidemics (Gubler & Clark 1995; Massangaie *et al.* 2016). The country is also home to several known mosquito vectors (Worth & De Meillon 1960; Kampango & Abilio 2016; Abilio *et al.* 2020). However, despite increasing evidence on possible autochthonous transmission (Gubler & Clark 1995; Fafetine *et al.* 2013; Gudo *et al.* 2015; Fafetine *et al.* 2016; Gudo *et al.* 2016), the burden of arboviral diseases in the country and implicated vector populations still remain poorly delineated. It has been widely accepted that, in the absence of an efficient and affordable vaccine, vector control remains the most effective approach for the prevention and control of MBDs transmission (Wilson *et al.* 2020). However, the design and implementation of state-of-the-art control measures will greatly depend on a thorough understanding of the local mosquito community's ecology, spatiotemporal dynamics of vector species assemblage, and modulating factors. Therefore, this study aimed to investigate the abundance and dynamics of mosquito communities that are potentially associated with arbovirus transmission in districts of Namaacha and Mopeia in Mozambique. Recent studies had reported active circulation of Rift Valley fever virus (Fafetine *et al.* 2013; Fafetine *et al.* 2016), as well as screening for other arboviruses in these two districts.

Material and methods

Study sites

Studies were basically conducted in two districts of two provinces, *viz.*, Maputo (southern region) and Zambézia (central region) Provinces. In Maputo Province surveys were performed in Goba locality (Namaacha district), whereas in Zambézia, surveys were undertaken in Mopeia district, located along the Zambezi River. The two locations are highly endemic for malaria and there has been also an indication of arbovirus transmission in the region (Fafetine *et al.* 2016). Namaacha district is located southwest of Maputo Province, bordering the West with the Republic of South Africa and the Kingdom of Eswatini (former Swaziland), to the North with Moamba district, to the East with Boane district, and to the South with Matutuíne district. The climate is tropical dry with two seasons, the hot/rainy season between October and April, and the winter/dry season, between April to September. The average annual temperature is 21°C and the average annual precipitation is 751.1 mm (751 mm in Goba and 680 mm in the Changalane administrative post). There is a vast hydrographical network formed by the Movene, Mabenga, Calichane, Impaputo and Umbelúzi rivers, as well as, the Pequenos Libombo reservoirs (Ministério da Administração Estatal (MAE) 2005b). Mopeia district is located in the Lower Zambezi region, to the southwest of the Zambézia Province, and it is limited to the North by Morrumbala district, to the South by Chinde district, to the East by Nicoadala and Inhassunge districts, and to the West by the Zambezi River. The district has been influenced by the dry steppe climate with dry winter. Annual average temperature is 26.5 ºC, varying from 20.5 ºC to 32.5 ºC. The rainy season usually occurs from November to March, and the average annual precipitation can be over 800 mm, particularly in the coastal regions, reaching in most cases 1,200 or even 1,400 mm (Ministério da Administração Estatal (MAE) 2005a). The study settings were selected based on the recent reports of both confirmed and suspected cases of mosquito-transmitted arboviral diseases, particularly, Rift Valley fever virus in the Goba and Mopeia districts (Fafetine *et al.* 2013; Fafetine *et al.* 2016).

Mosquito sampling strategy

Series of overnight (6:00PM-6:00AM) host-seeking adult mosquito collections were

performed outdoors in each study site using conventional CO_2 – baited (dry ice) CDC light-traps and Tent/Net traps equally baited with CO_2 as dry ice. Therefore, temporary mosquito sampling sentinel sites were set up close to previously identified homesteads and mosquito traps were deployed near animal shelters (cows, goats, sheep herds) in an occupied room. Traps were rotated between sites to reduce the influence of sites on mosquito catches sizes and composition. Collections in Goba and Mopeia were performed all year round, from November 2014 to December 2015, once monthly in each site, to estimate mosquito community fluctuation trends. However, due to logistics limitations the collections were not possible all year round in Goba.

Sample processing and identification

Mosquitoes were transported to the laboratory for sorting and species identification, in dry ice. Morphological identifications were performed using the keys proposed by Edwards (Edwards 1941) Gillies and De Meillon (Gillies & De Meillon 1968), Service (Service 1990), Jupp (Jupp 1986), and Ribeiro and Ramos (Ribeiro *et al.* 1980). Immature mosquitoes were reared to adults in holding cages at insectary standard conditions of temperature (27 ± 2 ºC) and humidity (87 ± 10%). Newly emerged adults were removed from the cage, killed by freezing in the camping freezer for morphological identification. Female mosquitoes were pooled according to species, locality, and time of collection, for arboviral screening (Abilio et al., 2020). Male mosquitoes and specimens of either sex that for some reason (*e.g.*, loss of body parts, rubbed scales) were not possible to be identified morphologically were submitted to identification by genitalia dissection and mounting, for later and broad systematic study along with male genitalia dissection and mounting (ongoing study), and are hereby referred to as "genera sp". Pools submitted for virus screening that were morphologically identified only to the genera level were confirmed by molecular approaches, which consisted of sequencing of the barcoding section between positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome oxidase gene subunit I (*cox*1 mtDNA) in order to identify to the species level, as mentioned in (Abilio *et al.* 2020).

Statistical analysis

Overall mosquito community abundance was estimated as the average number of specimen's sampled/Trap/night. Observed mosquito community richness was determined as the total number of species observed by location/month. Diversity of mosquito community in each

location was estimated as mosquito species richness (SR), representing the total number of all mosquito species expected to occur in the studied and the effective number of species (ENS), expressed by transforming of the Shannon-Weaver and Simpson diversity indices into Hill's numbers (^{q}D), where q is the Hill´s number order which controls the weights of common and rare species; q = 0 for species richness, q=1 for a number of equally common species, obtained by the exponential of the Shannon–Wiener Index [exp(H')], and q = 2 for highly abundant species resulting from the transformation of Gini-Simpson (D) (Jost 2007; Chao *et al.* 2014). The true SR was estimated by the Chao1 bias-corrected (Chao1 bc) species estimator (Chao *et al.* 2014). The completeness of overall mosquito samples for estimation of global community ecology metrics was assessed by mean of species-accumulation curves (Hsieh *et al.* 2016). The degree of variability in mosquito abundance and composition between sites, season, and, as well as, the association with climate variables, namely temperature, humidity, and rainfalls was determined using multivariate abundance generalized linear models (manyGLM) proposed by Wang and colleagues (Wang *et al.* 2012). The manyGLM model assumed negative binomial distribution of mosquito abundance with log-link function to predictors. Possible non-linear effect of continuous predictors (temperature, humidity, and precipitation) was modeled using Penalized spline function (Eilers & Marx 1996). All the data processing tasks and statistical analyses were undertaken using R software version 4.0.2 (R Core Team 2020).

Results

Mosquito abundance and species diversity

A total of 33,201 mosquitoes were collected during the study period, of which 87.7% (29,109/34,539) were collected in Mopeia district, and 12.3% (4,092/33,201) in Namaacha district (Fig. 1). In Mopeia district, *Culex (Cux) antennatus*, *Mansonia (Mnd) africana, Anopheles (Cel) funestus* and *Ma. (Mnd) uniformis* were the four most abundant (65.8%) mosquito species found accounting for 27.5% (8,005/29,109), 21% (6,113/29,109), 17.7% (5,140/29,109) and 15.6% (4,534/29,109), respectively. Similarly, in Goba, *Ma (Mnd) africana*, *An (Cel) funestus* and *Ma (Mnd) uniformis,* were the most frequent, accounting for 34.8% (1,423/4,092), 32.9% (1,346/4,092) and 8.3% (339/4,092), respectively (amounting to 76%) (Fig. 1). Due to logistic hindrances, collections were not feasible in Goba from February to May 2015.

A total of forty-three (n=43) mosquito species were identified during the study period, 31 of which were found in Goba and 37 in Mopeia, districts. Four species and two genera (*Ae. mcintoshi*, *Ae. vittatus*, *Cx. zombaensis*, *Cx. annulioris*, *Ficalbia sp* and *Uranotaenia sp*) were exclusively found in Goba, whereas nine species and one genus (*An. pretoriensis*, *Ad. africana*, *Ad. furfurea*, *Ae. fowleri*, *Ae sudanensis*, *Coquillettidea metallica*, *Cq. microannulata*, *An. marshallii*, *Cx. univitattus*, *Eritemapodites sp*.) where only found in Mopeia district. Chao1 bias corrected species richness estimator indicates that of the 31 species identified in Goba, 14 rare species and 17 were dominant species. Similarly, of the 37 species identified in Mopeia district, 15 are rare species and 22 are dominant (Fig. 1). Overall mosquito abundance and species richness peak in December and January (Fig. 1). In both regions, the number of equally abundant (^{1}D) and most abundant (^{2}D) species remains high from January to July after which these types of diversity reduce sharply till reach the minimum number in October. Chao1 species estimator also indicated that nearly 31.8 (31.1 - 39.4) mosquito species occur in Goba and 55 (41 – 118.3) species in Mopeia district. Species-accumulation curve (Fig. 2) indicates that sampling strategy was able of detecting nearly all mosquito taxa expected to be found in Goba but failed to estimate the true number of species occurring in the Mopeia district (Fig. 2). The overall mosquito abundance, richness, and diversity found in Goba and Mopeia is summarized in Tab. 1.

Table 1. A total number of specimens, richness, and estimated species diversity of mosquito found in Goba and Mopeia. Shannon-Wiever and Simpson diversity were, respectively, transformed into the effective number of equally abundant species (^{1}D) and an effective number of highly abundant species in the community (^{2}D).

Location	Total	Observed richness	Estimated richness (± 95% CI)[¥]	^{1}D ± 95% CI**	^{2}D ± 95% CI**
Goba	4,090	31	31.8 (31.1 - 39.4)	6.67 (6.63 - 6.92)	4.14 (4.13 - 4.28)
Mopeia	29,109	37	55 (41.0 - 118.3)	7.16 (7.16 - 7.24)	5.51 (5.51 - 5.57)

[¥]Calculated with Chao1 estimator
**Converted to the effective number of species (Hill´s number)

Variation in mosquito composition, abundance and correlates

There was significant variation of mosquito community abundance and composition according to season, location, and changes of climate variables (Tab. 2). Season-to-season variations were mostly due to changes in *Culex bitaeniorhynchus*, *Cx. pipiens*, *Cx. antennatus*, *Cx. poicilipes*, *Ae. metallicus*, *Ae. sudanensis* and *Anopheles sp* abundances, as indicated by their contribution to overall deviances of season effect (Tab. 2). Similarly, location-to-location variability was largely caused by variations in *Aedes mcintoshi*; *Ma. uniformis*, *An. pretoriensis*, *Ma. africana*; *Ae. sudanensis* and *Anopheles sp* abundance. Regarding the effect of climate variables, rainfalls and average maximum temperature were the two climate factors that significantly influenced mosquito community composition. In general, overall mosquito community abundance and species richness increased nonlinearly with an increase in temperature and rainfalls (Fig. 3). However, manyGLM test indicates that variation of rainfalls only affected significantly *Aedes sudanensis*, *Ae. mcintoshi* and *Cx. simpsoni* abundance compared to other species in the communities, as indicated by those species' contribution to total deviance (Table 2). Similarly, effect of temperature was only significantly observed in *Ae. fowleri*, *Ae. metallicus*, *Anopheles sp*, *Cq. metallica*, *Cx. annulioris*, *Cx. zombaensis*.

Table 2. Model-based community similarity analysis showing the effect of season, location (municipality), and climate variables on mosquito community abundance and composition. Only significant variables and species that contributed significantly (at 5% level) for total variability of abundance are shown. Percentages of mosquito species contribution to total model parameters deviance are shown in brackets.

Parameters	Deviance	Residual df	*p-values*	Species with significant contribution to total deviance
Season	106.2	22	0.001	*Culex bitaeniorhynchus* (13.5%) *Culex pipiens* (10.6%) *Culex antennatus* (9.8%) *Ae. metallicus* (8.4%) *Culex poicilipes* (7.8%) *Aedes sudanensis* (5.8%) *Anopheles sp* (5.7%)
Municipality	86.2	21	0.009	*Anopheles pretoriensis* (10.2%) *Mansonia uniformis* (9.4%) *Aedes mcintoshi* (8.5%) *Mansonia africana* (5.4%) *Aedes sudanensis* (5.2%) *Anopheles sp* (5.0%).

Precipitation	77.8	20	0.033	*Aedes mcintoshi* (10.4%) *Aedes sudanensis* (8.6%) *Culex simpsoni* (5.3%)
Temperature (maximum)	86.6	19	0.006	*Anopheles sp* (12.1%) *Aedes fowleri* (11.0%) *Culex zombaensis* (8.7%) *Coquillettidea metallica* (7.6%) *Aedes metallicus* (5.4%) *Culex annulioris* (4.6%)

Table 3. Mosquito species with known or suspected public health importance and their abundance in the two studied locations of Mozambique.

Mosquito species	Know or suspected vector for	Nº*	
		Goba	Mopeia
Ad (Lep) africana	Avian malaria, VEEV	0	31
Ad (Lep) furfurea	Avian malaria, VEEV	0	1
Ae (Adm) fowleri	PGA, RVF and Spondweni arbovirus	0	13
Ae (Adm) vittatus	BBK, NRI, PGA and SF arbovirus	4	0
Ae (Muc) sudanensis	RVFV	0	8
Ae (Neo) circumluteolus	BUN, LEB, PGAV, RVF, SPON, WSL arbovirus	101	47
Ae (Neo) mcintoshi	BBK, Bunyamwera, MID, Ndumu, NRI, Pongola, and RVF arbovirus	55	0
Ae (Ste) aegypti	CHIK, Chaoyang, Dengue (1-4), RVF, YF and ZIK, WNV, VEEV arbovirus	22	1
Ae (Ste) metallicus	YF Virus	3	2
Aedes vexans	Banna, Chaoyang, Potos, TAH, TVT, WN arbovirus	1	1
Anopheles coustani	Malaria, arbovirus (Bwamba PGA; Uganda S; RVF; SFV &WSLV)	158	103
An (Ano) tenebrosus	RVF, WN arbovirus	12	277
An (Ano) ziemani	Malaria	18	22
An (Cel) funestus s.l	Malaria, arbovirus (Bwamba; O'Nyong-Nyong; SFV)	1346	5140
An (Cel) gambiae s.l.	Malaria, arbovirus (Bwamba; O'Nyong-Nyong; Ilesha; Tataguine)	99	99
An (Cel) pharoensis	Malaria, arbovirus (SIN, RVF)	2	1566
Anopheles pretoriensis	Malaria	0	312
An (Cel) rufipes	RVFV	4	4
An (Cel) squamosus/cydipes	RVFV	3	25
Cq (Coq) metallica	Avian malaria,	0	6
Cq (Coq) microannulata	EEE and RVF arbovirus	0	1
Cx (Cux) annulioris	SINV	2	0
Cx (Cux) antennatus	RVF, SIN and WN arbovirus	159	8005
Cx (Cux) bitaeniorhynchus	Bancroftian filariasis and MVEV	38	17
Cx (Cux) neavei	Bagaza, Mossuril, SIN, Spondweni, USU and WN arbovirus	8	100
Cx (Cux) pipiens s.l.	OLI, RVF, SF, SIN, Uganda S, USU and WN arbovirus	13	607
Cx (Cux) poicilipes	RVF, Bagaza, BBK and WN arbovirus	4	21
Cx (Cux) simpsoni	RVFV	119	343
Culex tritaeniorhynchus	JE, CHIK, WN, ZIK Viruses	1	1
Culex univittatus	Bagaza, SIN, USU and WN arbovirus	0	1
Culex zombaensis	RVFV	3	0
Culex tigripes	BBK, Mossuril and SIN arbovirus	2	6
Eretmapodites sp	YF, CHIK, RVF, SF, Spondwene, Bunyamwera arbovirus	0	1
Ma (Mnd) africana	Bancroftian filariasis, arbovirus (Bunyamwera, MID, Ndumu, RVF, Shokwe, SIN, Spondweni, USU)	1423	6113
Ma (Mnd) uniformis	Bancroftian filariasis, arbovirus (Bwama, Ndumu, O'Nyong-Nyong, Spondweni, ZIK)	339	4534

*Total number of specimens collected per taxa in each studied site. Virus names and abbreviations: BBKV- Babanki; BUNV-Bunyamwera; CHIKV- Chikungunya; DENV-Dengue; EEEV-Eastern Equine Encephalitis; BWAV- Bwamba virus; MID-Middelburg; MVEV-Murray Valley River; NRIV-Ngari; OLI- Olifantsvlei; PGAV-Pongola; RVFV-Rift Valley Fever; SFV-Semliki Forest; SINV-Sindbis; SLEV-St Louis Encephalitis; SPOV-Spondweri; TAHV-Tahyna; TVTV-Trivittatus; UGSV-Uganda S; USUV- Usutu; VEEV-Venezuelan Equine Encephalitis; WNV-West Nile; WSLV-Wesselbron; YFV-Yellow Fever; ZIKAV-Zika.

Discussion

Strategies for surveillance and control of mosquito-borne diseases (MBDs) depend on the accurate knowledge of mosquito community composition, seasonal dynamics, ecology and determining factors of a spatiotemporal assemblage of local vector species. This study investigated the occurrence, structure and dynamics of mosquito communities from two different eco-geographical regions of Mozambique, namely from Mopeia and Goba in the locality from central and southern regions of the country. Overall, we have identified 31 mosquito species in Goba and 37 species in the Mopeia district. Despite an apparent difference in the number of species, the core group of the ten most frequent mosquitoes occurred in both Goba and Mopeia. The number of dominant taxa comprised those that were common to both regions. Species-rarefaction analysis showed that no information regarding new taxa could be added to the data after we had reached a sample size of 4,000 individuals in Goba, indicating that the sampling routine was able to detect all possible mosquito species occurring in the studied settings, even despite the lesser number of months of collections in this site. Differently, species-rarefaction curve analyses on Mopeia data have indicated an estimate of 55 mosquito taxa that may occur in the studied settings, contrarily to the 37 observed, thus, about 18 species remained undetected. Host-seeking mosquitoes were mostly sampled by CO_2-baited light-traps. Several factors may affect the likelihood of mosquitoes detection by traps, such as differential response to type and source of the odor and trap visual components, prevailing wind speed during trapping time, moonlight, and landscape features (Bidlingmayer 1967; Snow 1970a; Snow 1970b; Service 1980). Regarding olfactory stimuli, although CO_2 is a powerful long-range generalist attractant (Gibson & Torr 1999), and it has been showing that some CO_2 outputs can either attract or repel certain mosquito species (Gibson & Torr 1999). Accordingly, wind speed above 3 m/s can inhibit flight activity of several mosquito species and, therefore, reduce the likelihood of detection by traps (Bidlingmayer *et al.* 1995). Moreover, it has long been known that moonlight can strongly influence the size and composition of mosquito catches by light traps (Ribbands 1946; Birley & Charlwood 1989; Kampango *et al.* 2011). Landscape features can also influence the probability of mosquitoes finding the trap. Bidlingmayer (Bidlingmayer 1974) reported that traps deployed in the forest collected more mosquitoes than those deployed in the open land. Another factor that may have contributed to the failure in detecting all mosquito species may be the limited number of sampling days performed per month, as well as the atmospheric conditions in those particular days the collections took place.

Both Goba and Mopeia mosquito communities contain known vectors of important pathogens, including malaria vectors (e.g., *An. gambiae s.l, An. funestus s.l, An. coustani, An. pharoensis*) and several known arboviruses vectors of medical and veterinary relevance as Rift valley fever, dengue, chikungunya, yellow fever, O'Nyong nyong, West Nile and Zika (e.g., *Ae. aegypti, Ae. mcintoshi, Ae. simpsoni, Ae. vittatus, Cx. tritaeniorhynchus, Cx. antennatus, Ma. africana, Ma. uniformis*). Assembled mosquito communities included taxa of the vector of arboviral diseases of epidemiological concerns are found in the Tab. 3. A part of that, it is known that *Ae. aegypti* one of the main vectors of arbovirus, might occur in the whole country (Abilio *et al.* 2018). While, *Ae. albopictus,* another important vector for at least 26 arbovirus (Paupy et al 2009) tends to expand (Kampango & Abilio 2016; Abilio *et al.* 2018), *Ae. luteocephalus* an important vector of dengue, yellow fever and Zika in Africa were recently confirmed to occur in Mozambique territory (Abílio *et al.* 2020). Additionally, the two existing reports of screening for different groups of RNA viruses in mosquitoes from Mozambique, one of which as mentioned, as part of this study, discloses the presence of several insect-specific flaviviruses and phenuiviruses in several widely known vector species from genera of *Anopheles spp, Culex spp, Coquillettidia spp,* and *Mansonia spp*. Hence, the presence of the most important arbovirus vector and the circulation of different RNA viruses present in mosquitoes from Mozambique coupled with the high density of the most abundant mosquito species vectors obtained in this study, clearly suggests a high risk for pathogenic arbovirus transmission.

Results also indicate that most of the vector's population usually occur all-year-round in the studied setting (Fig. 4). Further investigations are encouraged to determine the role of the aforementioned arbovirus vectors in maintaining local transmission of mosquito-transmitted arboviral agents. The overall mosquito population abundance has usually peaked in the middle of the summer/rainy season. However, for Goba results also indicated that high mosquito abundance erupts in June. Contrarily, the peak of mosquito richness, effective number of equally common species, and highly abundant species remain nearly constant for nearly six months (from January to June). A similar pattern of temporal dynamics of abundance and diversities has been reported elsewhere (Franklin & Whelan 2009). Results also suggest that climate factors, particularly, maximum temperature and average rainfalls are the main drivers of mosquito abundance and diversity at studied sites. Overall mosquito abundance and community richness increased with the increase of maximum temperature and amount of rainfall. Conversely, Shannon and Simpson diversities tend to respond negatively

to higher temperatures. These findings suggest that in general, the mosquito community from Goba and Mopeia might successfully thrive under extreme climate events, such as, rise in temperatures as a consequence of climate changes. It has been argued that climate change may turn the African continent much more suitable for arboviruses rather than malaria (Mordecai *et al.* 2020). Our results also suggest that the higher risk of exposure to pathogens potentially transmitted by vectors populations identified, concerning their densities, might extend for at least eight months a year, that is, from November to June in Mopeia, impossibility of collections all year round in Goba, prevent us from estimating the riskiest season (Fig. 1, Fig. 4). However, it is very likely that the trajectory abundance curve, depicted in Fig. 1, maybe mostly due to variations of dominant species abundance, particularly *Ma. africana*, *Ma. uniformis*, *Cx antennatus*, *An. funestus* that breed in permanent larval habitats and, therefore, not showing dramatic seasonal variations across the range of their occurrence (De Meillon 1956; Laurence 2009). Interestingly, the peak of mosquito richness and an effective number of species (equally abundant and rare) last for nearly four to five months, suggesting great stability of larval habitats even during the dry season (e.g., April – June). We observed significant variation of community composition between sites and seasons. Similarly, mosquito composition was significantly affected by fluctuation of temperature (maximum) and rainfall. However, the effect covariates on community composition were species-specific. The heterogeneity in the effect of the covariate on mosquito composition may reflect some degree of overlap in ecological niche requirements between groups of species. With exception to *Ma. africana* and *Ma. uniformis,* species that were significantly affected by variation of rainfall inputs and temperature are essentially container exploiting mosquito species and species that usually breed in marshes, swamps or another type of periodically flooded environments, namely, *Ae. sudanensis*, *Ae mcintoshi*, *Aedes fowleri*, *Ae. metallicus*, *Cx. annulioris, Ae. pretoriensis* (Edwards 1941; Lambrecht & Peterson 1977). It is also well known, that the higher risk for arbovirus transmission occurs at the end of the rainy season, as mosquito populations get older and likely more prone to higher infection rates. Therefore, future studies should aim at understanding the real risk these vector species impose on residents and, as well as, to determine the extent to which variations of local environmental and climate factors regulate the spatiotemporal vector´s assemblages and pathogens transmission exposure in Goba and Mopeia districts.

Conclusion

This study updates the information on the ecology of mosquito populations' potential vectors of arbovirus and malaria in the Goba and Mopeia district. It also provides the basis for future studies on ecological processes underlining mosquito assemblage and vector species persistence in the regions considering risk estimation and monitoring, to disease prevention and control. Mosquito communities have a predictable annual cycle and some vector species found are important vectors of pathogens of medical and veterinary health impact. Therefore, this study highlights the need for coordinated multidisciplinary strategies for the design of impacting mosquito control interventions.

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List of figures

Figure 1. Structure and dynamics of mosquito communities of Goba and Mopeia districts. Bar charts show the abundance and composition of the respective mosquito communities highlighting the dominant (grey background area) and rare (brown background area), species. Line plots depict temporal dynamics of overall mosquito abundance, net species richness (^0D) and the effective number of equally common species (^1D) and the most abundant species (^2D) in Goba and Mopeia district.

Figure 2. Species-accumulation curves of mosquito samples from Goba (a) and Mopeia (b). The plateau in Goba curve indicates that the sample size was able to detect all possible mosquito species occurring in the region. Mopeia curve shows an increasing trend indicating that more samples needed to be taken in order to estimate the true species richness (total number of species occurring in the region). The shaded grey area represents the 95% confidence intervals obtained by bootstrap method based on 200 replicates.

Figure 3. Association between variation of average maximum temperature and precipitation on overall mosquito community abundance (a and b) and richness (c).

Figure 4. Spatio-Temporal occurrence and abundance of mosquito assemblages in Goba and Mopeia districts.

Figure

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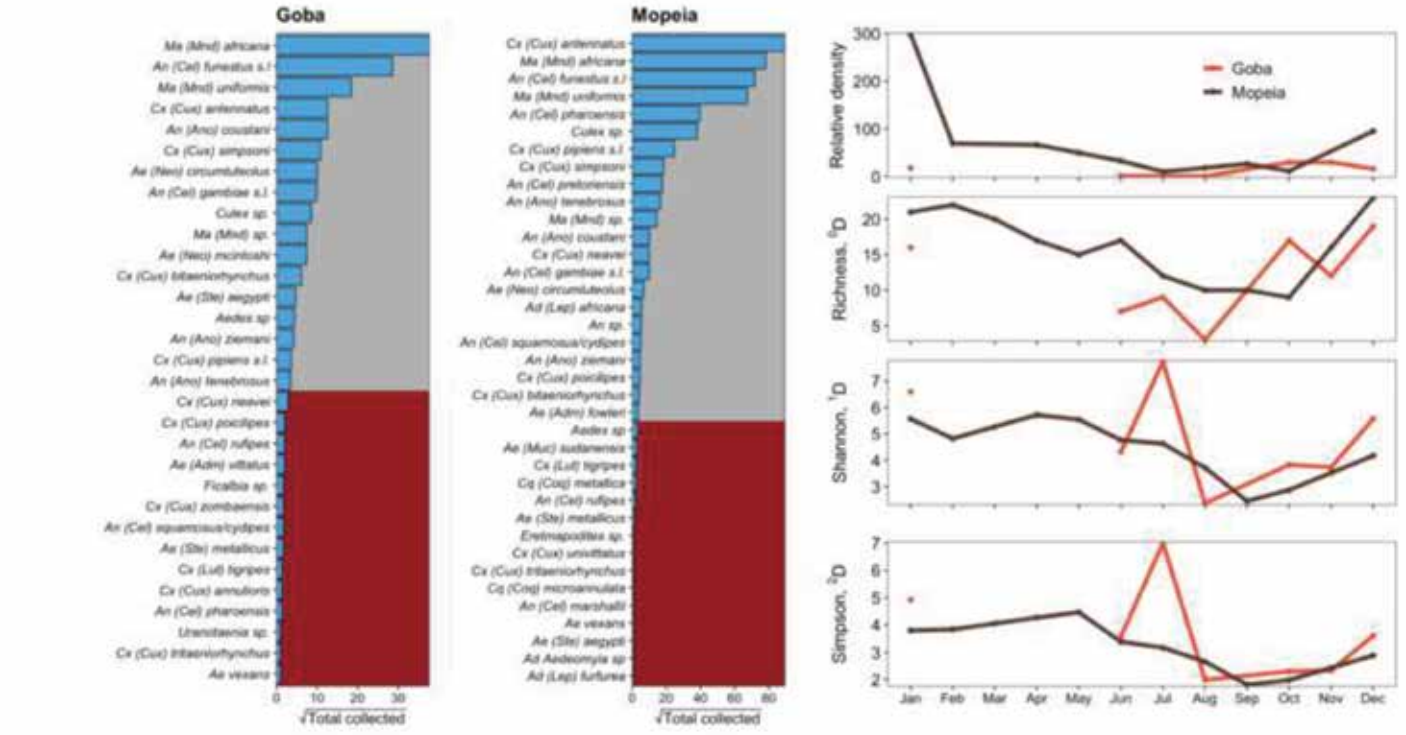

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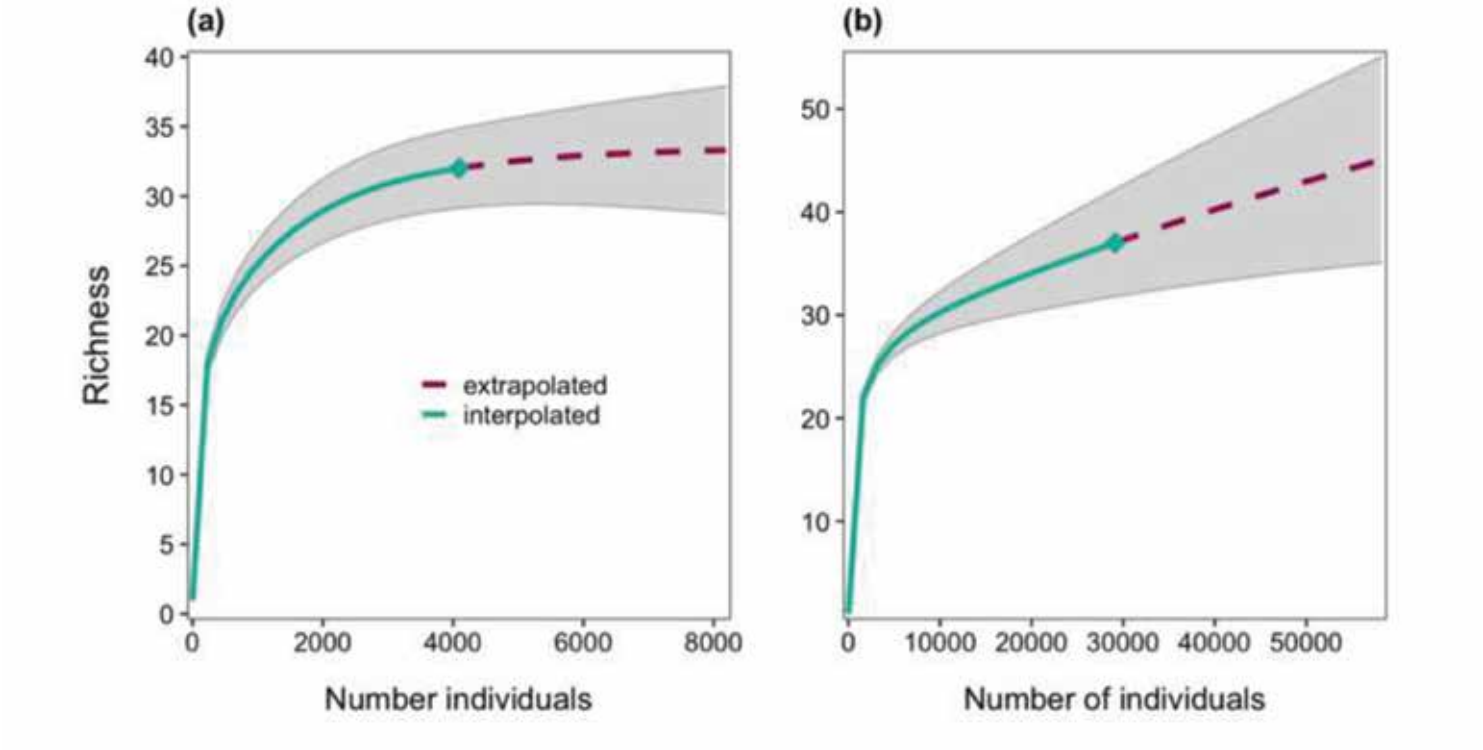

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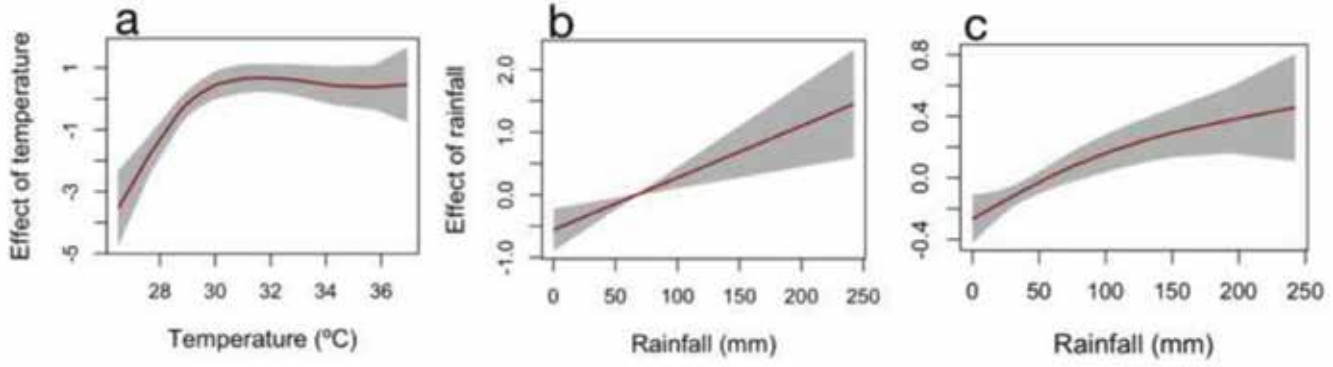

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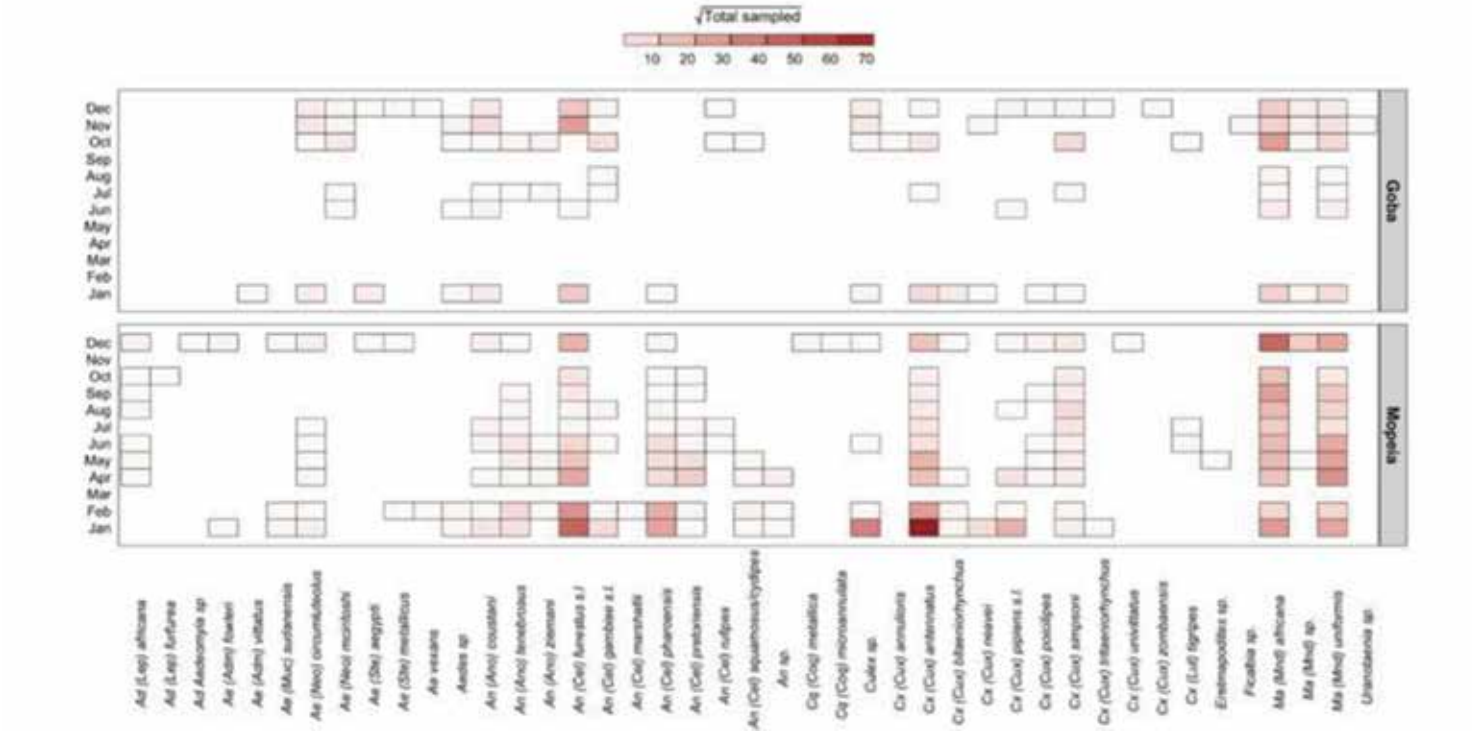

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BMC Microbiology

RESEARCH ARTICLE Open Access

A survey of RNA viruses in mosquitoes from Mozambique reveals novel genetic lineages of flaviviruses and phenuiviruses, as well as frequent flavivirus-like viral DNA forms in *Mansonia*

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Abstract

Background: Mosquito-borne diseases involving arboviruses represent expanding threats to sub-Saharan Africa imposing as considerable burden to human and veterinary public health. In Mozambique over one hundred species of potential arbovirus mosquito vectors have been identified, although their precise role in maintaining such viruses in circulation in the country remains to be elucidated. The aim of this study was to screen for the presence of flaviviruses, alphaviruses and bunyaviruses in mosquitoes from different regions of Mozambique.

Results: Our survey analyzed 14,519 mosquitoes, and the results obtained revealed genetically distinct insect-specific flaviviruses, detected in multiple species of mosquitoes from different genera. In addition, smaller flavivirus-like *NS5* sequences, frequently detected in *Mansonia* seemed to correspond to defective viral sequences, present as viral DNA forms. Furthermore, three lineages of putative members of the *Phenuiviridae* family were also detected, two of which apparently corresponding to novel viral genetic lineages.

Conclusion: This study reports for the first-time novel insect-specific flaviviruses and novel phenuiviruses, as well as frequent flavivirus-like viral DNA forms in several widely known vector species. This unique work represents recent investigation of virus screening conducted in mosquitoes from Mozambique and an important contribution to inform the establishment of a vector control program for arbovirus in the country and in the region.

Keywords: Flaviviruses, Bunyaviruses, Mosquitoes, Viral DNA forms, Phylogenetic analysis, Mozambique

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Background

Vector-borne diseases caused by arboviruses such as the Rift Valley fever, dengue, chikungunya, Zika, or West Nile viruses (RVFV, CHIKV, DENV, ZIKV and WNV, respectively), represent emerging and expanding threats in sub-Saharan Africa, and remain a major burden to global health, despite increasing funding allocated for their control and eradication [1]. Every year, more than one billion humans are infected, many of who die from vector-borne viral diseases, and more than half of the world's population may currently be at risk of infection, particularly in low-income countries [2, 3].

Our knowledge of the diversity of the viral world has significantly expanded over the last decade. During this period, a large number of studies have shown that viruses are the most abundant biological entities on the planet and display a remarkable degree of genetic diversity and genomic plasticity [4, 5], and have also allowed us to bridge apparent phylogenetic gaps in the virosphere. This is especially true when viral surveys focus on rarely sampled *taxa* or infrequently visited biotopes, and revealing novel or divergent viral groups [6–10].

Invertebrates are among the animals most frequently sampled in recent viral surveys, and their viromes seem to include a large number of genetically diverse viruses [9]. Mosquitoes (Diptera: Culicidae) are clearly the invertebrates most commonly studied due to their role as vectors of pathogenic viruses to humans and other animals [11]. However, the viromes of mosquitoes have been shown not to be limited to the latter, many of which (e.g. dengue, yellow fever or Zika viruses) have become household names in recent times. In fact, mosquitoes also host a profusion of viruses that only infect invertebrate cells and are, therefore, regarded as insect-restricted [12–14]. On the other hand, viral surveys are still frequently carried out in association with disease outbreaks, or when identifiable factors increase the probability for an arbovirus to (re)emerge and/or rapidly disperse [11]. Moreover, since there is limited knowledge on the genetic diversity, and ecology, of viruses in their natural enzootic maintenance cycles, little is also known regarding the adaptive constraints ruling the evolutionary steps that determine arbovirus emergence from their sylvatic niches [15].

Mozambique is located in a region suitable to arbovirus outbreaks, and in recent times the country was affected by two dengue virus outbreaks, which occurred in the northern regions [16, 17]. Increasing evidence also suggest that the country may be endemic to other debilitating and life-threatening arboviral threats including RVFV [18–20], DENV [2, 16, 21] and CHIKV [22, 23]. Moreover, historical and global risk projection have suggested that the country may also be suitable for the establishment of ZIKV [24–26], a virus recently linked to cases of microcephaly as well as many other neurological abnormalities in newly born infants [27]. Despite increasing evidence indicating the circulation of public heath-relevant arboviruses in Mozambique, the burden of the diseases they cause remains unknown. In addition, more than a hundred potentials arbovirus vectors have been identified in Mozambique, and these include *Aedes spp*, *Culex spp*, *Mansonia spp* and *Anopheles spp* [28–31], of which their role in maintaining arboviruses in nature remain to be elucidated.

The focus of this study was the detection, and analysis, of selected *taxa* of RNA viruses in different geographic regions in Mozambique. These regions display rich mosquito and wildlife faunas, as well as bioecological features that allow mosquitoes, wildlife, domestic animals and humans to coexist in close proximity. The viruses targeted in this viral survey included alphaviruses, flaviviruses, and different bunyaviruses. While our initial interest as far as bunyaviruses were concerned involved the detection of RVFV, in a subset of samples the viral screening also included detection of phlebovirus-like and orthobunyavirus genomes. The results obtained did not reveal the circulation of recognizable pathogenic viruses in wild-caught mosquitoes, but uncovered divergent phenuiviruses, as well as different lineages of insect-specific flaviviruses.

Results

The results presented in this work were based on the analysis of a total of 14,519 mosquitoes, collected in 3 regions of Mozambique (Fig. 1) during 12 successive collection campaigns, carried out between November/2014 and December/2015. The majority 45.55% (n = 6614/14519) of the screened mosquitoes were classified as *Culex spp.*, followed by *Anopheles spp*. 27.16% (n = 2943/14519) and *Mansonia spp* 25.22% (n = 3662/14519). Mosquitoes were grouped into 351 pools, ranging from 1 to a maximum of 128 specimens, with the average of (approximately) 41 mosquitoes each. These were subsequently processed by RT-PCR for the detection of specific viral agents (such as RVFV), or groups of viruses (such as alphaviruses and flaviviruses).

Analysis of flavivirus sequences

The genomes of flaviviruses were targeted using the primers previously described by Vázquez et al., (2012), which reveal an amplicon with the expected mass of ≈1 kbp [that on the Culex flavivirus strain CxFV-Mex07 reference sequence (EU879060) would define a section of the viral genome from coordinates 9800 to 9901] in the cDNA extracts prepared from 45/351 pools (12.8%). These results indicated the presence of flavivirus genome in 9 different species of mosquitoes from possibly 4 genera (*Anopheles*, *Culex Coquillettidia,* and *Mansonia*). A sample (n = 20) of these amplicons was sequenced, and BLASTn/x similarity searches unambiguously confirmed they had a flavivirus origin. Similarly, the mosquito

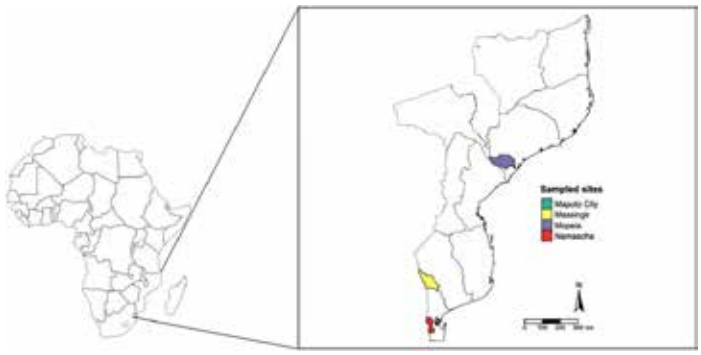

Fig. 1 Geographic coverage in Mozambique (west southern Africa) of the mosquito collections described in this report. The provinces indicated and the different municipalities where mosquito collections were carried out are color-coded

species of the pool of origin was confirmed by analysis of *COI* sequences in all but 5 pools, for lack of a PCR product. These corresponded to three of *Ma. (Mnd) africana*, and two of *Ma. (Mnd) uniformis*, all of which are very distinctive and clearly identifiable *taxa*. Table 1 lists all the viral sequences obtained in this study, as well as the date and location of collection of respective mosquito pools, their species, and respective accession numbers.

To further extend the characterization of the viral sequences obtained, a phylogenetic analysis was carried out using different methods. In all cases, the obtained phylogenetic trees indicated that none of the analyzed sequences had been amplified from bona fide arboviruses. Indeed, this is clearly revealed by their exclusion from the monophyletic cluster that assembles mosquito-borne and tick-borne flaviviruses in phylogenetic trees (cluster A in Fig. 2), the composition of which is shown in detail in the dotted box (indicated by the arrow). Conversely, all the sequences obtained in this study segregated within the large monophyletic group that assembles the so-called classical insect-specific flaviviruses, or cISF [12]. Furthermore, the analysis of the tree topologies obtained clearly suggested they did not group together in a single cluster, but rather segregated (i) either with other known viral sequences or (ii) formed independent genetic lineages. One of these lineages includes only sequences amplified from *Anopheles* spp. mosquitoes, while two others, also sharing a common ancestry, were mostly found in *Mansonia spp.* Unexpectedly, one of these sequences (LC462017) was obtained from a pool of mosquitoes identified as *Culex (Cux) antennatus* (pool Moz 182). However, the association of an apparently *Culex*-derived viral sequence with this group was considered debatable given its high similarity with the viral sequences amplified from *Mansonia* (see discussion). The above mentioned lineages of cISF include the Cuacua virus, previously identified in *Mansonia* sp. [29]. In this work, NS5-coding sequences 98–100% identical to those of the Cuacua virus were described both in *Ma. africana* and *Ma. uniformis*.

One of the other lineages of cISF identified is represented by a viral sequence obtained from *Cq. metallica* which clustered with that of Nienokoue virus (NC_024299) from *Culex* sp. However, these sequences share only 76.1% of sequence identity (as defined by Blast2 sequence comparison), clearly below the 84% limit defined by Kuno and others [32] and, therefore, indicating that they represent distinct viral species. The remainder flavivirus lineages were detected in pools of *Anopheles* mosquitoes, four of which could be classified to the species level as *An. (Cel) pretoriensis* and *An. (Ano) coustani*.

Curiously, the PCR amplification profiles of the flavivirus RT-PCR reactions frequently revealed (in agarose gels) the presence of an amplicon with approximately 0.5 kbp. This amplicon was observed in association with 31/351 (8.8%) of the pools analyzed, by itself in 9/351 (2.6%) or in combination with the expected 1 kbp DNA fragment in 22/351 (6.3%). However, given its size, it would correspond to a deleted form of the NS5 coding gene suggesting (i) that it might have been amplified from defective viral genomes and/or (ii) rearranged forms of retro-transcribed viral DNA, possibly integrated in mosquito genomes as previously observed [33–35], and/or their resulting transcripts. The association of these smaller sequences with a flavivirus origin was clearly confirmed both by sequence homology searches (using BLASTn) and the reconstruction of phylogenies (Fig. 3a). All six 0.5 kbp amplicons (indicated exclusively by NS5Δ in Fig. 3a) that had been apparently obtained

Table 1 Date of collection and location of positive *flavivirus* and *phenuivirus*-like sequence detection in mosquitoes from Mozambique, and their respective accession numbers

Date of collection	Locality/District	Province	Geo - references	Pool lab code (size)	Mosquito species[a]	Flavivirus sequence	Phenuivirus sequence	COX1 mosquito sequence
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 3 (*n* = 50)	*Mansonia africana*	LC462008	–	LC517270
04/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 9 (*n* = 55)	*Mansonia africana*	LC462001	–	LC517271
27/04/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 19 (*n* = 50)	*Mansonia uniformis*	LC462246	–	n.a.
04/06/15	Limpopo park/ Massingir	Gaza	23° 52′ 04.4399″ S; 032° 08′ 43.0852″ E	Moz 38 (*n* = 20)	*Anopheles* sp.	LC462010	–	LC517272
26/11/14	Limpopo park/ Massingir	Gaza	23° 52′ 04.4399″ S; 032° 08′ 43.0852″ E	Moz 39 (*n* = 14)	*Anopheles* sp.	LC462009	–	LC517273
18/11/14	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 47 (*n* = 48)	*Anopheles coustani*	–	LC461999	LC517274
19/12/14	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 54 (*n* = 39)	*Culex tritaeniorhynchus*	–	LC461994 LC461995	LC517275
28/01/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 76 (*n* = 40)	*Mansonia uniformis*	LC462253	–	LC517276
28/01/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 77 (*n* = 64)	*Mansonia africana*	LC462249	–	LC517277
27/04/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 89 (*n* = 50)	*Mansonia africana*	LC462005	–	LC517278
27/04/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 90 (n = 50)	*Mansonia uniformis*	LC462251	–	LC517279
01/10/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 96 (n = 50)	*Mansonia africana*	LC462016	–	LC517280
21/07/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 97 (*n* = 12)	*Anopheles coustani*	–	LC462000	LC517281
21/07/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 98 (*n* = 18)	*Anopheles coustani*	LC462013	–	LC517282
28/01/15	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 104 (*n* = 53)	*Mansonia uniformis*	LC462007	–	LC517283
27/04/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 160 (n = 50)	*Anopheles pretoriensis*	LC462011	LC461996 LC461997	LC517284
27/04/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 161 (*n* = 49)	*Anopheles pretoriensis*	LC462012	–	LC517285
09/01/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 166 (*n* = 26)	*Anopheles pretoriensis*	LC462015	LC461998	LC517286
09/01/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 182 (n = 50)	*Culex antennatus*	LC462017	–	LC517287
09/01/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 212 (n = 50)	*Culex antennatus*	LC462254 LC462255	–	LC517288
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 258 (n = 50)	*Mansonia africana*	LC462003	–	LC517289
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 259 (*n* = 54)	*Mansonia africana*	LC462002	–	n.a.
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 263 (*n* = 75)	*Mansonia africana*	LC462004	–	n.a.
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 269 (*n* = 51)	*Mansonia africana*	LC462006	–	n.a.
09/01/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 299 (*n* = 45)	*Mansonia uniformis*	LC462250 LC462256	–	LC517290
20/12/14	Goba/Namaacha	Maputo	26° 3′ 59.73″ S; 032° 10′ 23.36″ E	Moz 309 (*n* = 8)	*Mansonia uniformis*	LC462252	–	n.a.
30/07/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 313 (n = 50)	*Mansonia africana*	LC462247 LC462257	–	LC517291

Table 1 Date of collection and location of positive *flavivirus* and *phenuivirus*-like sequence detection in mosquitoes from Mozambique, and their respective accession numbers *(Continued)*

Date of collection	Locality/District	Province	Geo - references	Pool lab code (size)	Mosquito species[a]	Flavivirus sequence	Phenuivirus sequence	COX1 mosquito sequence
30/07/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 319 (n = 50)	*Mansonia africana*	LC462014	–	LC517292
26/06/15	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 324 (n = 1)	*Mansonia uniformis*	LC462019	–	LC517293
03/12/14	Chinuara/ Mopeia	Zambézia	17° 79′ 04. 748″ S; 035° 4′ 1442″ E	Moz 341 (n = 5)	*Coquillettidia metallica*	LC462018	–	LC536568

[a]Identification to the species level was confirmed based on COX1 barcoding using sequences generated by Sanger (population) sequencing of PCR products, except where marked with "n.a." in COX1 mosquito sequence accession numbers column

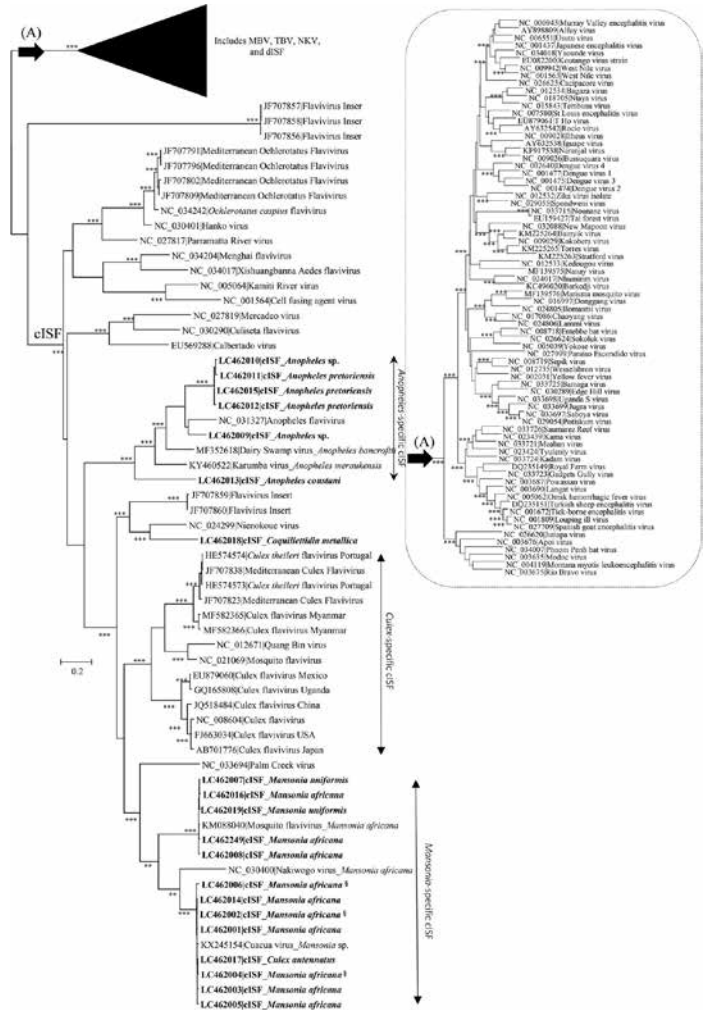

Fig. 2 a Phylogenetic analysis of flavivirus *NS5* nucleotide sequences (≈1 kbp per sequence). At specific branches, the number of "*" indicates the branch-support as revealed by the different phylogenetic reconstructions methods used, and assuming as relevant bootstrap values ≥75% (using 1000 resamplings of the sequence data in maximum likelihood analysis) and posterior probability values ≥0.80 (when Bayesian approaches were used). One, two or three "*" would indicate that a given branch had been supported by one, two, or all the phylogenetic reconstruction approaches used in the amalysis (ML and Bayesian analysis using two sets of demographic priors). At the top of the tree, the collapsed monophyletic group including reference sequences from mosquito-borne viruses (MBV), tick-borne viruses (TBV), no known vector viruses (NKV), and dual-host associated insect-specific viruses (dISF), while the branches shown comprise the so-called classical insect-specific flaviviruses (cISF), is expanded at the right (**b**). The sequences described in this work are indicated in bold-face. All the sequences used are designated by their respective accession numbers|virus name. The size bar indicates the number of nucleotide substitutions per site. §-Mosquito species could not be confirmed by *COI* sequence

Fig. 3 a Phylogenetic analysis of partial flavivirus *NS5* nucleotide sequences (0.5 kbp DNA amplicon) from insect-specific flaviviruses. cISF and dISF indicate classical and dual-host associated insect-specific flaviviruses, respectively. At specific branches, the number of "*" indicates the support revealed by the different phylogenetic reconstructions methods used (bootstrap values ≥75% and posterior probability values ≥0.80 were assumed as significant). The sequences described in this work are indicated in bold-face, and their origin (mosquito species) is also indicated. All the sequences used are designated by their respective accession numbers|virus name. The size bar indicates the number of nucleotide substitutions per site. **b** amplification of flavivirus-like from RNA and mosquito genomic DNA using a combination of treatments that included the use/or not (+/) of DNase I followed/or not (+/–) by reverse transcription (RT). The NZYtech ladder VI was used as a molecular mass marker. **c** Structure of the flavivirus wild-type and the NS5 fragments analyzed. The 3′-end of the viral genome is schematically shown at the top. §-Mosquito species could not be confirmed by COI sequence

after amplification by RT-PCR from total RNA extracted from mosquito pools were not only clearly part of the cISF radiation but also clustered together in a single, and highly stable monophyletic cluster that subdivides into two subclusters (indicated by *Mansonia*-specific cISF/NS5Δ in Fig. 3a). Moreover, these same 0.5 kbp amplicons could also be obtained when total DNA was used as a template for PCR amplification, and no reverse-transcription had been performed, but when a DNase I treatment preceded reverse-transcription, no 0.5 kbp amplification product was obtained (Fig. 3b). These results show that the origin of the frequently observed 0.5 kbp fragment was not cDNA, but rather corresponded to viral DNA forms (vDNA) contaminating the RNA extracts. Three of these amplicons (indicated by the arrows in Fig. 3a), amplified from mosquito DNA pools of *Ma. africana*, *Ma. uniformis* and *Cx. antennatus*, were cloned and sequenced. Once again, the obtained sequences fell within the same monophyletic cluster. Moreover, when the structure of these DNA fragments was investigated, all of them revealed a similar architecture (Fig. 3c), combining both different sized deletions (down to 1 nt; indicated by Δ) and point mutations.

Screening of alphaviruses and bunyaviruses, and analysis of phenuivirus L-sequences

Very different results were obtained when either *Alphavirus*-specific primers [36] or those targeting conserved sequences in the RVFV NSs coding-region [37] were used. In fact, neither of these sets of primers revealed the presence of the genomes of these viruses in any of the 351 pools of mosquitoes analyzed. Frequently, the use of the RVFV primers did result in the non-specific amplification of different sized PCR products, many of which were cloned and sequenced. In all cases (results not shown), the obtained sequences confirmed the non-viral origin of these amplicons.

On the other hand, given the overwhelming diversity of the viruses that compose the recently proposed Order *Bunyavirales*, a decision was made not to restrict the screening of bunyaviruses to RVFV, but to extend it, in a smaller subset (43/351) of the pools of mosquitoes collected in different geographic areas of Mozambique, using *Phlebovirus* and *Orthobunyavirus* primers [38, 39]. This subset of 43 pools included the species *Ae. (Adm) fowleri* (*n* = 1), *Ae. (Dic) adersi* (n = 1), *Ae. (Muc) sudanensis* (*n* = 1), *Ae. (Neo) circunluteolus* (*n* = 4), *Ae. (Neo) mcintoshi* (*n* = 2), *Ae. (Ste) aegypti* (*n* = 1), *Ae. (Ste) metallicus* (*n* = 1), *An.(Ano) coustani* (n = 2), *An. (Ano) tenebrosus* (n = 1), *An. (Cel) funestus* (n = 1), *An. (Ano) ziemani* (n = 1), *An. (Cel) pharoensis* (n = 1), *An. (Cel) pretoriensis* (n = 2), *Cq. (Coq) metallica* (n = 1), *Cx. (Cux) antennatus* (*n* = 5), *Cx. Cux) tritaeniorhynchus* (n = 2), *Cx. (Cux) neavei* (n = 2), *Cx. (Cux) pipiens* s.l. (n = 2), *Cx. (Cux) poicilipes* (n = 1), *Cx. (Cux) zombaensis* (n = 1), *Cx.* sp. (1), *Ma. (Mnd) africana* (*n* = 3), and *Ma. (Mnd) uniformis* (*n* = 4), *Mimomyia (Mim) mimomyiaformis* (n = 1), and one pool of *Ae.* (Neo) sp.

Whereas the results that were obtained failed to reveal the presence of *Orthobunyavirus* genomes, in 5 pools, two of *An. coustani* (sequences LC461999, LC462000), two of *An. pretoriensis* (sequences LC461996, LC461997, and LC461998) and one of *Cx. tritaeniorhynchus* (sequences LC 461994 and LC46195) mosquitoes, a DNA fragment with the expected size was, indeed, amplified. All these amplicons were sequenced, but while BLASTn/x sequence searches did indicate a viral origin, unexpectedly they did not seem to have derived from bona fide *Phlebovirus* genomes, and this was confirmed by phylogenetic analysis using an assemblage of *Phlebovirus*, *Bandavirus*, *Banyangvirus,* and *Goukovirus* reference sequences. Regardless of the fact that the *Phlebovirus* group was paraphyletic, the sequences obtained from the analyzed mosquitoes from Mozambique did not cluster in any of the viral *taxa* in the tree, but rather formed 3 independent genetic lineages, as indicated by the arrows in Supplementary Fig. 1. The origin of these viral sequences was investigated using phylogenetic analysis of aligned amino acid sequences of the viral L protein from viruses classified within the different families in the Order *Bunyavirales*. The obtained results (Fig. 4) showed that, while all these sequences were clearly placed within the family *Phenuiviridae*, only two of them clustered with previously known viral references [8, 40, 41], yet in a cluster with no assigned designation. The other five sequences, two amplified from a pool of *Cx. tritaeniorhynchus*, and three others from pools of *An. pretoriensis* and *An. coustani* formed isolated genetic lineages, probably representing new unassigned genera.

Isolation of viruses using C6/36 cells

In an attempt to isolate some of the identified viruses, six filter-sterilized aliquots of macerates (Moz 39/*Anopheles sp.*, Moz 54/*Cx. tritaeniorhynchus*, Moz 89/*Ma. africana*, Moz 97/*An. coustani*, Moz 98/*An. coustani*, and Moz 160/*An. pretoriensis*) were put in contact with subconfluent monolayers of C6/36 cells for virus isolation. Viral replication was allowed for 14 days (one blind passage was carried out at the end of the first week), after which the presence of viral genomes was verified by RT-PCR using the same primers used for viral screening. At day three after the first blind-passage, three of these supernatants revealed evident CPE that included an apparent cell growth arrest accompanied by cell rounding, detachment from the flask surface, and sometimes clumping (Supplementary Fig. 2. Surprisingly, none of the primers used revealed the presence of any of the viruses detected during the viral screening. Moreover, as previously the isolation of Negev-like viruses had been associated, in our lab, to CPE similar to the one described above, RT-PCR with a series of primers designed for detection of several subsets of Nelorpiviruses (Carapeta et al., 2015), was performed. Once again, the presence of these viruses was not confirmed. Therefore, to the present day, the identity of the viruses isolated remains to be elucidated, which will be carried out using a metagenomic approach.

Discussion

In this report, a screening for different groups of RNA viruses targeting the detection of some of those previously shown (genome detection) or suggested (seroprevalence studies) to circulate in Mozambique [23, 29, 42, 43]. This analysis was carried out based on a one-year sampling effort, that amounted to the screening (for viral genomes) of 14,519 mosquitoes from 3 regions of the country. As only female mosquitoes may serve as vectors of viruses to vertebrates, male mosquitoes were excluded from this viral screening. Although the detection of viral agents is facilitated when their presence is associated with visible clinical signs/symptoms in vertebrates, their screening in their natural hosts/vectors may have the advantage of signaling their circulation before any cases of clinical disease, or seroprevalence, are detected. Moreover, a viral screening effort based on the identification of *foci* of disease cases only discloses the circulation of pathogenic viruses, and these have been shown to represent only a part of the virome of mosquitoes [11–14].

The molecular screening that was carried out did not reveal the presence of RVFV or any recognizable pathogenic alphaviruses, bunyaviruses or flaviviruses. These include viruses such as DENV, ZIKV, CHIKV, o'nyong nyong, Sindbis or Middelburg [36]. While the absence of alphaviruses in this viral screening may be intriguing, we

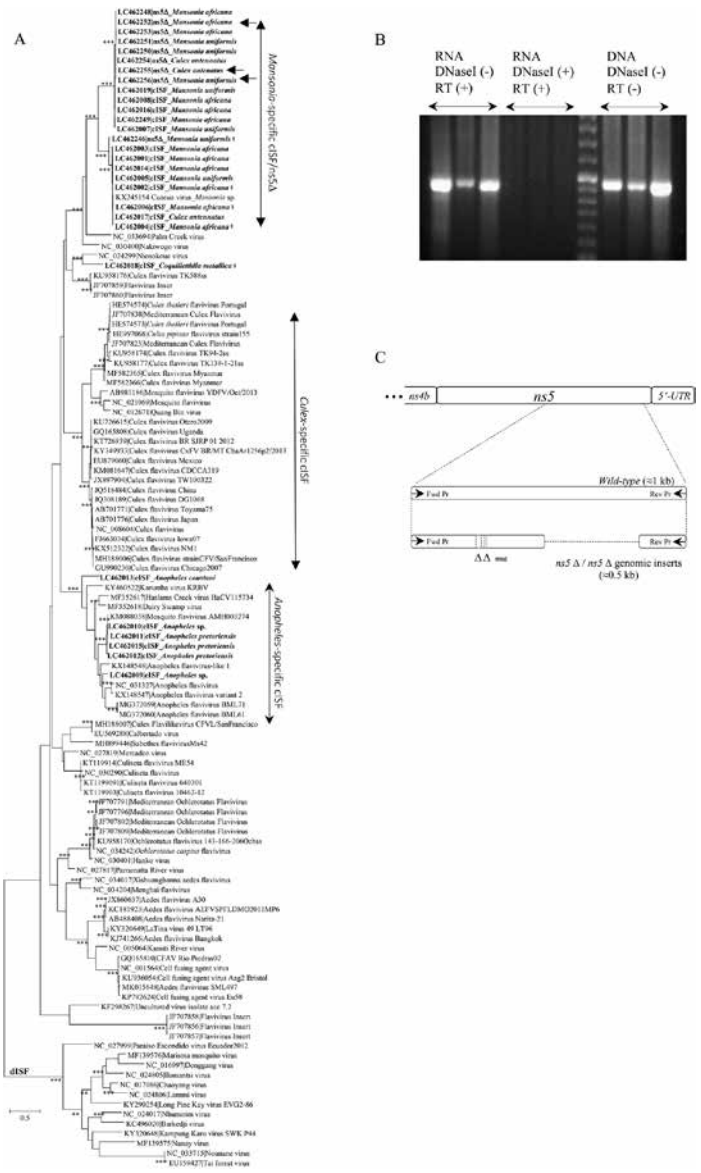

Fig. 4 Phylogenetic analysis of partial amino acid sequences of the viral-encoded RNA polymerase of viruses within the Order *Bunyavirales*. At specific branches the number of "*" indicates the support revealed by the different phylogenetic reconstructions methods used, assuming as relevant bootstrap values ≥75% and posterior probability values ≥0.80. The reference sequences used are indicated by their accession number|virus name. The sequences described in this work are indicated by their accession numbers, by the horizontal arrows and in bold-face. The size bar indicates the number of amino acid substitutions per site

must bear in mind that unlike other virus groups (e.g. flaviviruses), alphavirus ISVs have, with exceptions [44, 45], been less frequently reported in viral surveys, while pathogenic alphaviruses such as CHIKV are usually associated with *Aedes* mosquitoes which were clearly underrepresented in our screening. Furthermore, no *Orthobunyavirus* sequences were ever detected in a small sample of the pools analyzed ($n = 43$; the same subset of pools where a survey for *Phlebovirus* genomes was also carried out).

On the contrary, the use of a highly degenerate flavivirus-specific primer set [46] confirmed the presence of multiple genetic lineages of flaviviruses in a large number of pools of mosquitoes. Despite the fact that not all of the obtained amplicons were sequenced, those for which a sequence was obtained were found to segregate in the cISF radiation.

The different genetic lineages of viral *NS5* sequences were apparently associated with multiple species from 4 genera, supporting the perception that cISF are widespread in the natural populations of mosquitoes. Some of these *NS5* sequences seemed to segregate away from previously described viral assemblages and formed isolated branches in phylogenetic trees. Others were joined in clusters with multiple operational taxonomic units that were never exclusively associated with a single species of mosquitoes, adding to the possibility that cISF may not host species-restricted [47, 48]. However, while phylogenetic analysis did suggest a *Culex* origin for one of the sequences (LC462017), given the fact that it was almost identical to many others amplified from *Mansonia* mosquitoes, its association with *Culex* mosquitoes is disputable. Furthermore, although a molecular confirmation of the identity of these mosquitoes was obtained by *COI*-sequence analysis, the sequencing strategy used (Sanger) is a population-approach that only reveals the sequence of the most abundant molecular form in a PCR-product, while minor variants fail to be detected. Therefore, we cannot formally exclude the possibility that sequence LC462017 may have been derived from one/a small number or even body-parts of non-*Culex* mosquitoes (possibly *Mansonia*) originally present in the pool in question.

Surprisingly, in a high number of pools of *Mansonia* spp. ($n = 29$) in one pool of *Anopheles* sp. and another of *Culex* sp. mosquitoes, the flavivirus-specific primers used generated a smaller than expected PCR product, with approximately half the size (≈0.5 kbp). The analysis of some of these smaller amplicons showed that they corresponded to defective versions of the RdRp coding sequence and their origin was found to be DNA (vDNA), rather than RNA. For all those cases where a nucleotide sequence could be obtained, a shared ancestry between the latter and bona fide viral *NS5* sequences (obtained by RT-PCR) was also revealed.

While we cannot ascertain, at this stage, whether the flavivirus vDNA forms are present as part of the host genome (endogenized), or whether they exist in the form of a stable extra-chromosomal DNA element, flavivirus-like sequences have been known to occur in the genome of mosquitoes for over a decade, especially in association with *Aedes* mosquitoes [33, 34]. While the sequence of a vDNA amplicon amplified from *Anopheles* could not be obtained due to technical difficulties, the fact that virtually identical vDNA sequences could be amplified from DNA extracts of *Mansonia* and *Culex* mosquitoes is hard to explain given the evolutionary divergence of these *taxa*. Moreover, while these vDNA forms could result from exposure of these mosquitoes to a common source of viruses, the possibility of a contamination of pools of *Culex* mosquitoes with even a limited amount of the highly abundant *Mansonia* specimens, cannot be discarded.

Whereas the presence of bacterial symbionts of mosquitoes can alter the competence of mosquitoes for transmission of pathogenic viruses [49], to what extent the same applies to the persistent presence of insect-specific viruses in insect cells is still open to discussion. However, the highly rearranged *NS5* sequences found in this study seem to exclude the possibility that translation of an RNA transcribed from them might result in an active protein. In any case, they could participate in the establishment of persistence viral infections by controlling the siRNA response, as previously suggested [50].

Given the a priori specificity of the primers used for the screening of *Phlebovirus* sequences, the observation of a specific amplicon in association with 5 pools of 2 different species of *Anopheles* (*An. coustani* and *An. pretoriensis*) and one species of *Culex* (*Cx. tritaeniorhynchus*) mosquitoes suggested that these viruses might have been detected. However, the different phylogenetic analyses were congruent in showing (i) their inclusion in the *Phenuiviridae* family, (ii) but their exclusion from the *Phlebovirus* genus, (iii) and their separation into three genetic lineages. Two of these sequences did segregate in a stable monophyletic cluster defining a genetic lineage with no assigned designation, but that included sequences previously detected in other studies [8, 40, 41], while the other five formed two genetic lineages with no associated references.

Although no recognizable pathogenic viruses were identified in the course of this work, this may result from a combination of multiple factors that include sampling bias. In fact, collections did not focus on settings where DENV/ZIKV/CHIKV were previously known to circulate in Mozambique [23, 42, 43], but rather on areas where RVFV had been detected [18, 19]. On the contrary, *Mansonia* and *Culex* mosquitoes clearly dominate the collections in the 3 provinces of Mozambique that

were the focus of this study. However, pathogenic flavivirus such as the Spondweni virus (the closest known relative to ZIKV), have indeed been isolated from *Ma. africana* and *Ma. uniformis* [11], as well as from *Culex quinquefasciatus* mosquitoes in Haiti [51]. Association of other pathogenic flaviviruses with *Mansonia* sp. mosquitoes include the S. Louis encephalitis and West-Nile viruses (which also use *Culex* sp. for their natural maintenance), alphaviruses (including Venezuelan equine encephalitis virus), orthobunyaviruses [52], and phleboviruses, including RVFV [11].

While sampling bias may partially explain the absence of some of the arboviruses that have been previously shown to circulate in Mozambique, other factors may also explain the results obtained. These include a low natural incidence of arboviruses in the areas where mosquitoes were collected, or the concurrent absence of recorded cases of human/animal disease cases associated to the circulation of viruses such as RVFV during the mosquito collection periods. Furthermore, a technical limitation of this study is associated with the use of a less technologically advanced virus detection approach based on convectional RT-PCR, as opposed to addressing viral screening with a bona fide metagenomic experimental design combined with the use of NGS sequencing methods. To the best of our knowledge, this study and the previous detection of ISF in *Mansonia spp* [29], are the only recent virus surveys using mosquitoes from Mozambique, and clearly demonstrates the dire need for such surveys that might clarify their epidemiology.

The attempted isolation of some of the viruses identified in this work in insect cells was not successful. This fact may probably result from a combination of factors that include the use of only one blind passage and a single cell-line. Indeed, while C6/36 cells have been extensively used for the isolation of ISVs they may not be susceptible and well as permissive to all insect viruses. In this regard, it should be added that some ISF seem to be restricted to theirs hosts [53], and this may indicate that use of C6/36 cells, although convenient, may not have been ideal. For more clarification further analysis involving cell culture attempts using a larger number of cell lines originating from different species of mosquitoes is recommended. While a very short blind-passage history may have compromised the production of a high titer viral suspension, in truth the exact same RT-PCR protocols were used to screen the presence of viral genomes in mosquito macerates and culture supernatants. Moreover, only after a single blind-passage, 50% of the cultures did evidence unambiguous CPE. Taking into account the protocol used, this CPE most probably was due to viral replication.

The nature of these viruses is currently under investigation. Furthermore, bioinformatics investigations for producing the unidentified CPE observed in inoculated cells is encouraged for better understanding the prevalence of insect-specific viruses in many genera of mosquitoes [54, 55]. While these efforts should be ideally addressed using unbiased and high-throughput experimental approaches (metagenomics/NGS), the direct screening of other frequently found ISVs, including alphamesoniviruses [56] could also be pursued using a direct targeting strategy with *taxon*-specific primers.

Conclusion

This study reports for the first-time novel insect-specific flaviviruses and phenuiviruses, as well as frequent flavivirus-like viral DNA forms in several widely known vector species. While a large diversity of ISVs have been found on a global scale [57, 58] in association with a plethora of insect hosts, this work extends the results of the sole study that had, up to the present day, revealed their presence in Mozambique [29]. Although this survey did not disclose the circulation of pathogenic arboviruses, it confirmed the circulation of different RNA viruses that are present in mosquitoes from Mozambique. This article represents our professional endeavor to help to elucidate and provide higher resolution information on arboviruses vectors hotspot, transmission dynamics and routes in Mozambique and is of utmost importance to inform the establishment of a vector control program for arbovirus in the country and other region sharing the same pattern.

Methods

Study area and mosquito collection

A total of 14,519 mosquitoes were collected in rural settings in Mozambique (located in west southern Africa) between November 2014 and December 2015 as part of the work of Abílio, AP (In Preparation) at Massingir (in the province of Gaza), Namaacha (in the province of Maputo), and Mopeia (in the province of Zambézia) (Fig. 1). The general biotypes for Goba were savanna with medium grassland located around 10 to 500 m from a water stream. Collection sites in Massingir and Mopeia corresponded to forest environments located closed to the Lipompo and Zambezi rivers, respectively. The mosquitoes were collected using a combination of sampling methods that included indoor resting, tent collections and those carried out using CO_2-baited miniature CDC-light traps. These mosquitoes were stored in dry ice, and then transported to the laboratory for sorting and taxonomic identification using keys proposed by Gillies and Coetzee [59] and Jupp [60]. The manipulations of specimens for identification were carried out at temperatures close approximate to 0 °C under a stereomicroscope equipped with an ice block. Male and blood-fed specimens were

excluded from this study. All samples were then stored at − 80 °C until viral screening was carried out.

Preparation of mosquito homogenates, and nucleic acid extraction

The preparation of mosquito homogenates was based on a preliminary grouping of the collected and identified specimens in pools according to their species, sex, geographic origin, and blood-fed status. These mosquitoes were mechanically disrupted in 15 ml Falcon tubes by vortexing using glass-beads and aluminum oxide in 1 ml of phosphate buffer saline (PBS) buffer. After 3 pulses of 1 min (with 30 s breaks on ice), the mosquito macerates were clarified by centrifugation, as previously described (Carapeta et al., 2015). RNA, as well as DNA, were extracted from 200 μl of clarified mosquito homogenate using NZYol® (NZYTech, Portugal), as indicated by the supplier. The extracted RNA was dissolved in 30 μl nuclease-free water, while the obtained DNA sediments were dissolved in 40–100 μl using a 1:1 mixture of 8 mM NaOH and TE buffer (Tris 100 mM, EDTA 1 mM, pH = 7).

Viral genome detection

The extracts of total RNA served as a template for the synthesis of cDNA, that was carried out with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) using random hexamers, and a thermal profile including 10 min at 25 °C, 45 min at 52 °C and 10 min at 80 °C (for enzyme inactivation), followed by treatment with RNaseH (20 min at 37 °C).

Detection of flavivirus *NS5* sequences (encoding the viral RNA-dependent RNA polymerase, or RdRp) was carried out using previously described primers and reaction conditions [46]. A generic PCR method using degenerate primers targeting the *nsP4* gene (also encoding the viral RdRp) was used to detect the presence of the genomes of alphaviruses [36], while RVFV genomic NSs coding sequences were tentatively detected as previously described [61]. Finally, the presence of phleboviruses and orthobunyaviruses L sequences (also encoding an RdRp) was investigated using the ppL1/ppL2 sets of primers/reaction conditions previously described by Matsuno and others [38] and the technical modifications suggested by Pereira and others [62], or as defined elsewhere [39]. Nelorpivirus detection was carried out as previously defined [63]. All the PCR primers and thermal profiles used are listed in Supplementary Table 1. PCR amplifications were carried out using NZYTaq 2X Green Master Mix (NZYTech, Portugal). The obtained amplicons were purified and directly sequenced or cloned in either pGEMT-easy® (Promega, USA) or pNZY28-A using the NZY-A PCR cloning kit (NZYTech, Portugal), followed by DNA sequencing of individually purified plasmid recombinant-DNA molecules.

Cell culture and virus isolation

Aedes (Ste) albopictus C6/36 cell line was used for virus isolation. Cells were maintained at 28 °C (in the absence of CO_2) in L-15 Leibovitz Medium (Lonza, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Lonza, USA), 2 mM L-glutamine (Gibco BRL, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL, USA) and 1 × tryptose phosphate broth (AppliChem GmbH, Germany). Approximately 500 μl of filter-sterilized mosquito homogenate were diluted in the same volume of phosphate buffered saline (PBS), and inoculated onto semi-confluent layers of C6/36 cells grown in T25 culture flasks (Nunc, Denmark). After 1 h at room temperature (for viral adsorption), the viral inoculum was removed, 5 ml of L-15 Leibovitz Medium (2% FBS) was added to each flask, and the cell cultures were incubated at 28 °C for a week. Culture supernatants collected after a single blind-passage were used as viral stocks and stored at − 80 °C. Cytopathic effect (CPE) was determined by microscopic observation of the inoculated cell cultures.

DNA sequencing and genetic analyses

Multiple alignments of nucleotide (nt) or amino acid (aa) sequences were performed using the iterative G-INS-I and E-INS-I methods as implemented in MAFFT vs. 7 [64] followed by editing using both GBlocks [65], and visual inspection. The multiple sequence alignments of nucleotide sequences were systematically verified to ensure the correct alignment of homologous codons using BioEdit 7.0.5 [66].

Phylogenetic trees were constructed using both Maximum Likelihood (ML) and Bayesian approaches. The best-fitting evolutionary models used were those suggested by JModeltest2 (Darriba et al., 2012) and W-IQ-tree (Trifinopoulos et al., 2016) for the analysis of nt (GTR + Γ + I: GTR-General Time Reversal, Γ-Gamma distribution, I-proportion of invariant sites) or aa alignments (LG + Γ: Le-Gascuel, Γ-Gamma distribution). Phylogenetic analyses based on the ML optimization criterion were carried out using the Mega 6.0 software [67], and the stability of the obtained tree topologies assessed by bootstrapping with different re-samplings of the original aligned positions (1000 for nt alignments, 100 for aa sequence data). Phylogenetic reconstructions following a Bayesian approach were carried out by running two independent Markov chain Monte-Carlo (MCMC) analyses using BEASTv1.7.5 [68], assuming a relaxed uncorrelated lognormal molecular clock model [69] as suggested by the ML Clock Test implemented in Mega 6.0. The MCMC chains were run until 100,000,000 states were sampled using both logistic population growth and Gaussian Markov random field/GMRF skygrid demographic priors. The Tracer software (http://beast.bio.ed.ac.uk/tracer) was used to diagnose stationarity and adequate (> 300) effective sample size

(ESS). The trees were logged on every 5000th MCMC step, and the tree sample was summarized using TreeAnnotator v1.8.3 as maximum clade credibility (MCC) trees, with median heights used as the node heights in the tree, after discarding 10% of them as burn-in. The FigTree v1.4.2 software was used to visualize the phylogenetic trees (http://tree.bio.ed.ac.uk/software/figtree/).

The molecular confirmation of the morphological identifications of mosquitoes was carried out on the basis of the analysis of the barcoding section (from positions 58 to 705 encoding the N-terminal section of the mitochondrial cytochrome oxidase subunit I - COI) essentially using Bold Systems-v4 (available at http://www.boldsystems.org/).

The nt sequences obtained in the course of this study were deposited in the GenBank/ENA/DDBJ databases under accession numbers LC461994-LC462019, and LC462246-LC462257, and LC517270-LC517293. The reference sequences used for analyses presented in this manuscript where directly downloaded from the public sequence databases. Whenever necessary, nt sequence similarity searches were carried out using BLASTn, and BLASTx (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12866-020-01905-5.

Additional file 1: Supplementary Figure 1. Microscopic observation of C6/36 cells mock-infected cells (A; 300x), or after infection (day 3) with viruses present in three independent pools of *Ma. uniformis*, *An. ziemani*, and *An. pretoriensis* mosquitoes collected in Mozambique.

Additional file 2.

Additional file 3: Supplementary Table 1. PCR primers and thermal profiles used in this work.

Abbreviations

DNA: Deoxyribonucleic acid; RNA: Ribonucleic acid; cDNA: complementary DNA; PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase-PCR

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Authors' contributions

APA, JMF, LCBN, MS2 and APGA - conceived the investigations; APA, MS2, ESG, APGA and LCBN - Funding acquisition for the project; APA, RP and APGA - Methodology of the study; APA, JMF, LCBN and APGA - collected and performed pre-identified of the specimens; APA, AK and APGA - Confirmation of specie identifications; APA, MS1, IN, APGA, RP - Molecular and Phylogenetic Analysis; ESG, JMF, RP - Reagent and Materials. APA, APGA and RP - Data analysis. APGA and RP - Data curation and Validations. APA, APGA and RP - Writing original draft. APA, MS1, AK, ESG, MS2, LCBN, JMF, APGA and RP - Writing, revised and approved the final manuscript. The authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from corresponding author on reasonable request.

Ethics approval and consent to participate

The Ethics Committee from the Faculty of Medicine of Eduardo Mondlane University approved the study (Ref #: CIBS FM&HCM/15/2018). All head of the household or properties owners provided written informed consent prior to participation.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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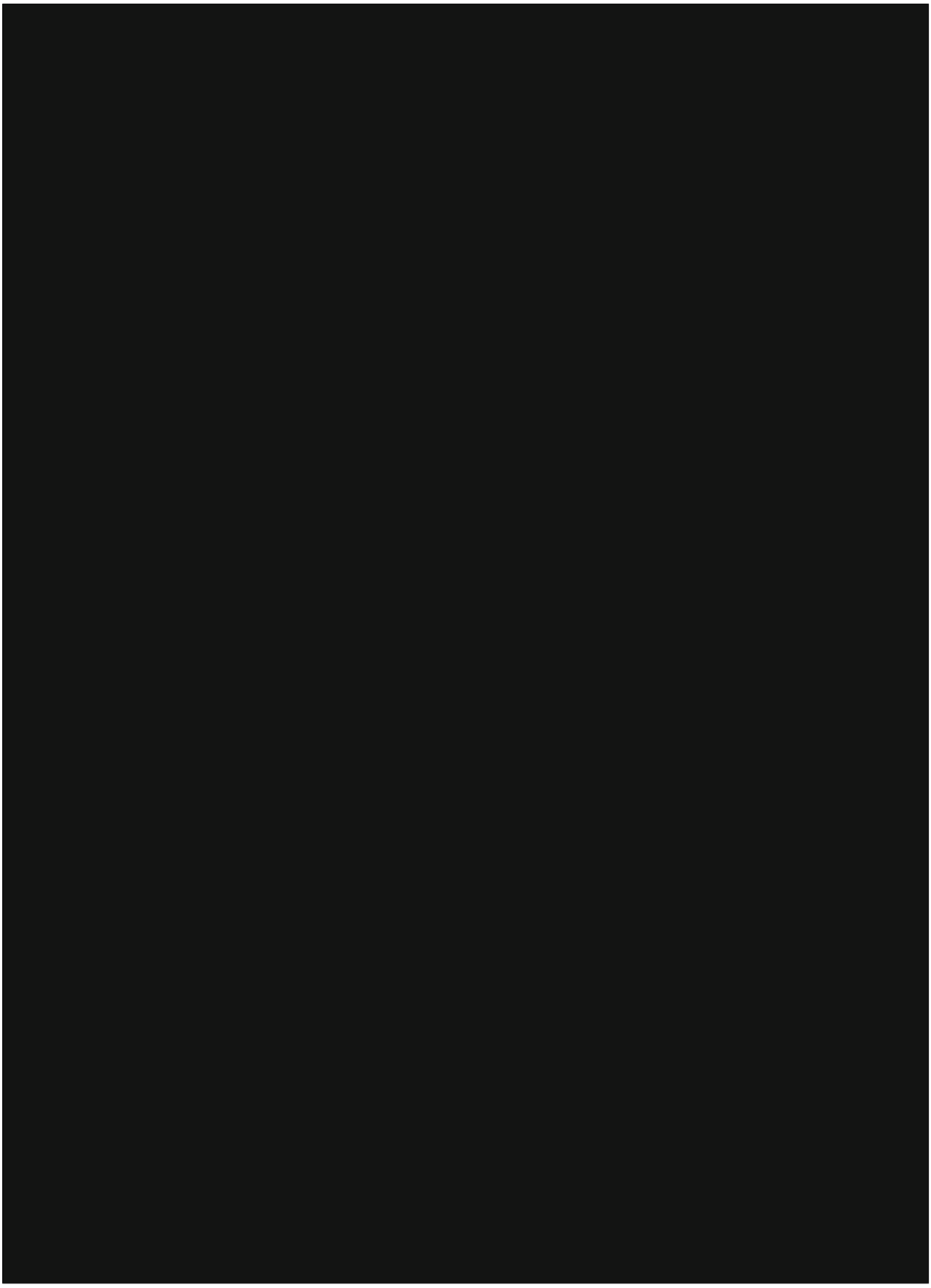

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