Modulation of Arbovirus Infection by Mosquito Saliva

Daniella Andrea Lefteri

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
Faculty of Medicine and Health

September 2019

The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

The work in this thesis would not have been possible without the teamwork, help and support of numerous people. This completed thesis, the writing of which literally broke me with my lung collapsing sometime during the writing of chapter 8, is dedicated to everyone who has supported me over the last 3 years.

Firstly I would like to give huge thanks to my supervisor Clive McKimmie. You were always hugely supportive and always had the ability to look at data in a positive light (even when it seemed impossible). This work would have been impossible without you. Also thanks for the cookies at the end of long experiments.

I would also like to thank Steven for all the help in the lab, for showing me the quickest way possible to extract RNA from tissue samples via the incorporation of numerous hacks, for cheering me up on dull work days, for the continuous provision of well needed coffee and for all the potentially dangerous adventures. Also thank you for taking me around Glasgow so many times, I expect another crazy night out at Nice n Sleazy's when I move!

I would also like to thank Marieke who helped train me at the beginning of my PhD and for always providing excellent feedback and much needed after work drinks sessions with rum weasleys. Next I would like to thank Georgi for always keeping me updated will all the latest gossip, Emma for all the interesting conversations and imaginary scenarios and Dario for all the random trivial knowledge. We were the best lunch group.

I would like to thank Emilie Pondeville and her group at the CVR for providing the majority of saliva samples and *Ae.aegypti* eggs used in this thesis, this work would not have been possible without it! Also thanks to Francesco Baldini and Dorothy Armstrong at the University of Glasgow for providing me with *An.gambiae* eggs. In Leeds I would also like to thank my co-supervisor Stephen Griffin and all of his group for all their input throughout this project and also all the people of level 5 WTBB for their support. Also a huge thanks to the technical staff at the SBS for helping will the huge amount of *in vivo* work conducted in this thesis.

Away from the lab I would like to thank my family; my wonderful parents, Annette and Dinos, for all their support and belief in me over the years. You always encouraged my curiosity as a child by providing me with an insane amount of books as well as my first "lab equipment' which allowed me to catch and study any insect I could find in our garden. Also, a huge thanks to my amazing sisters, Vanessa and Tiffanie. My life would have been incredibly boring without the madness and craziness which can only be found between three sisters. Thank you all for always being there for me despite the distance! Jag älskar er!!

Next, thank you to the person I basically consider my third sister, Marie. I am so happy we both chose the random city of Leeds to study. You were my first lab partner in crime helping me cover up several lab mishaps (mainly me clumsily spilling out precious materials). Thanks for everything!!

And of course, last but definitely not least, most thanks to the person that has had to put up with most of my madness over the past 9 years; Chris. I cannot express in words how thankful I am for everything you have done for me over these years and for helping keeping me sane. I could not have managed this without you. Also thank you for being my personal graphic designer. Σ'αγαπώ!!!

Abstract

Arboviruses constitute a major public health problem; in particular mosquito-borne arboviruses that continuously emerge and re-emerge. Arbovirus infection of mammals is enhanced by the presence of a mosquito-bite at the inoculation site, or by the co-inoculation of extracted mosquito saliva alongside virus, in comparison to virus experimentally administered by needle inoculation in the absence of bite/saliva. Host responses elicited against saliva appear to be key in facilitating this enhancement. As such, we have studied the mechanistic basis for these observations by investigating mosquito-bite factors, as well as host responses, involved in facilitating viral enhancement. Using an *in vivo* mouse model we demonstrate that salivary microbiota does not modulate virus infection. Instead proteinaceous salivary factors inside saliva is responsible for enhancing virus infection. We have studied whether saliva from different mosquito species successfully enhance virus infection. Interestingly, while saliva from *Aedes* genus enhance virus infection, *An.gambiae* saliva does not. This could partly explain why *An.gambiae* mosquitos are unsuitable vectors for transmitting most arboviruses. By comparing the effects that saliva from these different species have at the inoculation-site, we have further specified which inflammatory responses modulate arbovirus infection in the skin. Using an *in vivo* mouse-model we demonstrate that *An.gambiae* causes significantly less oedema than *Ae.aegypti* and that histamine induced oedema in the absence of salivary-factors also enhances infection. Also, measuring cytokine responses to *Aedes* and *Anopheles* saliva, showed that several key anti-viral chemokines such as CCL5 were significantly more upregulated by *Anopheles*. Hence, we're providing important insights into how mosquito saliva modulates infection. A better understanding of this will aid the development of anti-viral treatments targeting factors within the mosquito bite that are common to many distinct infections.

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Abbreviations

Ab	Antibody
Abx	Antibiotics
AgBR1	*Ae.aegypti* bacteria responsive protein 1
Ago2	Argonaut 2
BCR	B-cell Receptor
BHK	Baby Hamster Kidney Cells
Bp	Base Pairs
BSA	Bovine Serum Albumin
C	Cytosine
CARDS	Caspase activation and recruitment domains
CCL	Chemokine (C-C motif) ligand
CGRP	Calcitonin gene related peptide
CHIKF	Chikungunya fever
CHIKV	Chikungunya virus
CNS	Central nervous system
CO_2	Carbon dioxide
CoV	Coronavirus
CPV-1	Cytoplasmic vacuolar structures type I
Ct	Cycle threshold
CTD	C-terminal domain
CTP	Cytidine triphosphate
CXCL	Chemokine (C-X-C motif) ligand
C57BL/6	C57 black 6
DALY	Disabilty adjusted life years
DAMPs	Damage associated molecular patterns
Dcr2	Dicer 2
DC-SIGN	Dendritic-cell-specific ICAM-grabbing non-integrin
DDT	Dichlorodiphenyltrichloroethane
dLN	Draining lymph node
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleoside triphosphates
E1	Envelope Protein 1

EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EEEV	Eastern equine encephalitis virus
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
EU	European Union
FCS	Foetal calf serum
G	Guanine
Gluc	Gaussia Luciferase
GMEM	Glasgow Modified Essential Medium
GPCR	G-protein coupled receptor
GRP78/BiP	Glucose-regulating protein 78
Hz	Hertz
IFIT	Interferon induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNγR	Interferon gamma receptor
IHR	International Health Regulations
IL1β	Interleukin 1 beta
IRF9	Interferon regulatory factor 9
IRSE	Interferon stimulated response element
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JEV	Japanese encephalitis virus
JH	Juvenile hormone
LGP-2	Laboratory of genetics and physiology-2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LTβR	Lymphotoxin β receptor
MAPKs	Mitogen-activated protein kinases
MAVS	Mitochondrial antiviral signalling protein
MCSF	Macrophage colony-stimulating factor
MDA-5	Melanoma differentiation-associated gene 5

MEF	Mouse Embryonic Fibroblasts
MEM	Minimum Essentials Media
miRNA	Micro RNA
ml	Milliliter
mm	Millimetre
MMPs	Matrix-metalloproteinases
MOI	Multiplicity of infection
Mya	Million years ago
NeSt1	Neutrophil stimulating factor 1
NK	Natural killer
NLR	NOD-like receptor
NOD	Nucleotide oligomerization domain
NTS	Non-template control
ONNV	O'Nyong-Nyong virus
PAHO	Pan American Health Organization
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PBSA	PBS with BSA
Pen/Strep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PFU	Plaque forming unit
PRR	Pattern recognition receptor
qPCR	Quantitative Polymerase Chain Reaction
RIDL	Release of Insects carrying a Dominant Lethal
RIG-I	Retinoic acid-inducible gene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRV	Ross River Virus
RSAD2	Radical-SAM-domain-containing-2
SARS	Severe acute respiratory syndrome
SAT	Saliva assisted transmission
SFV	Semliki Forest Virus
SHM	Somatic hypermutation
SINV	Sinbis virus
siRNA	Small interfering RNA

SIT	Sterile insect technique
STAT1/2	Signal transducer and activator of transcription 1/2
TAE	Tris-acetate-EDTA
TBE	Tick borne encephalitis
TCR	T-cell receptor
Th1	T-helper 1
Th2	T-helper 2
TIR	Toll/IL-1 receptor
TLR	Toll like receptor
Tm	Melting temperature
TNF	Tumour necrosis factor
TNFSF	Tumour necrosis factor superfamily
TPB	Trypto phosphate broth
VCAG	Vector Control Advisory Group
VEEV	Venezuelan equine encephalitis virus
VEGF	Vascular endothelial growth factor
WEEV	Western equine encephalitis virus
WHO	World Health Organization
WNV	West Nile Virus
WT	Wild type
YFV	Yellow fever virus
ZIKV	Zika virus

CHAPTER 1: Introduction

Female Aedes Aegypti

1.1 Brief history of infectious diseases

Infectious diseases have plagued humanity throughout our entire existence. They have repeatedly swept across the globe devastating communities, forever changing the course of human history by causing the deaths of millions and even bringing mighty empires, such as the Han and Roman empires, to their knees. Infectious diseases have had, and are still having, a tremendous impact on our way of life.

The story of infectious disease stretches as far back as humanity's own. Historians and archaeologists have successfully unsurfaced numerous accounts of infectious diseases in literature, at archaeological sites and depicted in art. In Egyptian mummies that are over 3000 years old we can detect evidence of outbreaks of smallpox, and Egyptian papyrus reveal evidence that Egyptians of the time were plagued by infectious diseases such as poliomyelitis [1]. Also in literature, we can find accounts of previous outbreaks of disease along with humanities earliest attempts at understanding them; one notorious example being the "Cough of Perinthus" cited in the Corpus Hippocratium, thought to be referring to the first account of an influenza outbreak in human history [2].

Hippocrates, commonly referred to as the "Father of Medicine", was the first to correctly suggest various routes of transmission of disease including air, water and food whilst Thucydides was the first to draw the conclusion that infectious disease could be contagious between individuals; a conclusion he drew from his observations during the plague of Athens in 430-427 B.C.E [3]. In the 1600s, Leeuwenhoek, also known as the "Father of Microbiology" invented the microscope, enabling the visualisation of microorganisms for the first time whilst the 18th century brought us the development of the first vaccine by Edward Jenner [4] [5]. Despite these advances, it was not until the 19th century that "germ theory" became widely accepted following the work of Louis Pasteur who demonstrated that infectious disease was caused by microorganisms [6]. The existence of viruses however was not confirmed until the late 19th century by Dmitri Ivanovsky and scientific and medical advancement continued to develop rapidly from there [7].

From the mid to the end of the 20th century there was a common belief that the battle on infectious disease was largely won. In 1948, the US secretary of State, George Marshall, stated that "the conquest of all infectious diseases was imminent", a point of view that was shared by many at the time [8]. This belief had been brought on by the successful development of numerous vaccines, the discovery of antibiotics, the eradication of smallpox and rinderpest and the development of DDT (dichlorodiphenyltrichloroethane) which was believed to be the solution to the malaria problem. These scientific breakthroughs wrongly instilled a sense of hubris amongst scientists and world leaders alike whom in the end were surprised that instead of a continuation of the trend, they saw a re-emergence of old diseases previously believed to have been controlled and the emergence of completely new or resistant variants of previously known diseases.

1.2 Understanding Emerging and Re-emerging Infectious Diseases

The word disease, originating from the old French word of "desaise" (lack of ease), can refer to any condition that disrupts the normal function of the body. The aetiology of disease can however vary greatly; some diseases, such as cystic fibrosis, are caused by mutations in the genetic code whilst infectious diseases are acquired when a host has become infected with an outside agent that causes the disease, hence the term "infectious disease".

Infectious diseases can be further divided into emerging infectious disease or re-emerging infectious disease; the former referring to the appearance of novel, previously unheard of diseases whilst the latter group refers to the reappearance of infectious disease that was previously believed to have been eradicated or kept under control, an infection that re-appears with new disease symptoms or simply a disease that materializes in a completely new, previously unheard of geographical location. Whilst the possibility of emerging novel diseases was widely accepted by scientist for an extensive period of time, it was not until the appearance of a range of haemorrhagic fevers, including the Crimean-Congo haemorrhagic fever and Ebola in the mid 1970s, that emerging diseases received an increase of attention. The first documented use of the terms "emerging and re-emerging disease" was by Joshua Lederberg, Robert B. Shope, and Mary Wilson in 1987 [9].

One of the most renown examples of an emerged infectious disease is the HIV/AIDS epidemic. Acquired immunodeficiency syndrome (AIDS) which is caused by the lentivirus HIV (Human immunodeficiency virus) is thought to have originated from the closely related SIV (simian immunodeficiency virus) which primarily infects primates and only overcame the species barrier via repeated contact of humans with infected bushmeat in 1910 in Kinshasa [10]. AIDS was officially recognized as a new disease in 1981 and since then, despite the tremendous progress that has been made in combating the disease, over 25 million people have died due to AIDS related illnesses [11].

1.2.1 Factors determining emergence or re-emergence of disease

In order to understand the concept of emergence and re-emergence of disease it is important to comprehend the difference between disease and infection. Whilst infection refers to the introduction of an agent such as a virus into a host where it can then multiply, disease is a word describing the symptoms and signs the host typically exhibits by such an introduction. By making this distinction it is possible to understand that a host can be infected without actually exhibiting any symptoms and therefore the infectious agent can precede the existence of the disease. Therefore, whilst it may seem as if new diseases can just appear randomly, there is almost always a reason explaining its occurrence. In fact, most newly emerged diseases originate from infectious agents that are already present in our environment and it is estimated that the majority of human diseases have their origin in zoonoses, which are animal infections that are also transmissible to humans. [12, 13]. It is changes made to the environment of these infectious agents, usually by humans, that can cause a new disease to emerge or re-emerge.

There are 5 evolutionary stages required for a pathogen to evolve into a human specific pathogen. These are: 1) Infectious agent is present in the environment but not in humans; 2) The infectious agent can infect humans but it cannot be transmitted from one person to another; 3) Transmission between humans is possible but infections are limited to smaller outbreaks that tend to die out; 4) Transmission between humans is frequent but environmental reservoirs such as other animals are

still required to constantly enhance infection numbers; 5) The infectious agent is human specific and self-sufficient [14].

1.2.1.1 Ecological Changes

Changes in environmental conditions is one of the major drivers for emerging diseases. Agriculture appears to be one of the major culprits as it tends to place people in close proximity with environments were potential infectious agents are present. This exposure of humans to an infectious agent for a prolonged time, increases the chances of it successfully infecting and causing disease. An environmental change can also cause disruptions of the natural ecosystem potentially shifting an infectious agents host preference.

Ebola outbreaks are believed to commonly originate from human contact with infected bats either via bushmeat or via infected droppings [15]. Fruit bats were confirmed to be the reservoir host of Ebola as viral titres have been detected in three separate bat species [16]. Major deforestations of the fruit bats natural habitats could have contributed to the likelihood of the 2014 Ebola epidemic occurring, as it altered the distribution of the fruit bats as well as bringing humans into closer proximity to the virus. The presence of infected fruit bats in human habitats, feeding from the same fruit trees eliminated the need for infectious bushmeat being the source of infection [17].

In contrast to the deforestation events possibly resulting in the outbreak of Ebola, the emergence of Lyme disease in the United States is believed to have been a consequence of reforestation. The reforestation caused an increase of deer populations and with them an increase of Lyme disease carrying ticks [18]. Similarly to the emergence of novel disease, environmental changes can also be the aetiology of disease re-emergence and can have a major impact on the prevalence of arthropod borne diseases as arthropod vectors constitute a major route of infectious agent transmission. In east Asia, flooding of rice fields has become associated with an increase in the incidence of Japanese encephalitis (JE). This is due to the fact that the accumulation of water allows for a perfect breeding ground for Japanese encephalitis virus (JEV) vector *Culex tritaeniorhynchus* [19] and consequently also for

the virus [20]. These examples illustrate the impact environmental changes can have on disease frequency and occurrence and how difficult they can be to predict.

1.2.1.2 Human Populations and Demographics

Approximately 11,000 years ago, humans made a shift from predominantly hunter-gathering to systematic agriculture and with this change human societies started to take form with people living in closer proximity and larger numbers. As a consequence, numerous novel infectious diseases emerged specifically targeting mankind as diseases could adapt and spread more readily from one person to another [21]. Currently, there are over 7 billion people living in the world and it is estimated that the global human population will continue to increase to approximately 11 billion before finally levelling out (see figure 1.1) [22] [23]. Projections also estimate that in 2050, 68% of all people on earth will be residing in cities (currently it is about 55%) [24]. With more than half of the human population living in urban areas people are living in closer proximity to each other and in larger numbers than ever before and such changes have impacts on infectious disease incidences.

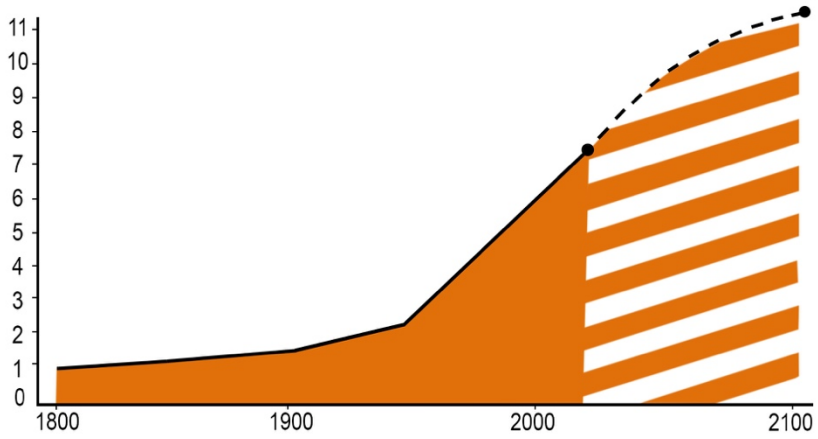


Figure 1.1 World population projection.

World population projections estimate that there are currently over 7 billion people living in the world and the global human population will continue to increase to approximately 11 billion before finally levelling out [22] [23].

Urban environments possess the enhanced risk of transmissions of disease as the density of the population means the existence of a larger number of potential hosts. If an emerging infectious disease gets into a major city, it can quickly become a hot spot for transmission. Ebola for example usually makes an appearance as an isolated

incidence in isolated rural areas resulting in a very low number of infected individuals. The outbreak in 2014 however resulted in over 10,000 peoples deaths as it managed to spread to the capital of Guinea and from there on to the capitals of Sierra Leone and Liberia [25]. Apart from environments with higher population density, major cities can also create more temperature stable environments which can indirectly expand the season when vectors are able to transmit disease [26] [27]. The evolution of dengue virus (DENV) has been closely linked with the development of urban areas as it has benefited from both the density of humans but also of the beneficial climate to its mosquito vector [28] [29].

1.2.1.3 Globalization

The last decades have seen an immense increase in aviation and goods transportation with the numbers of airline passengers exceeding 4 billion in 2017 and with 53.9 million tons of goods being shipped by air [30]. Increase in international travel and commerce has proven to be a very effective way of spreading diseases across the globe. With international travel rates being higher than ever, diseases have an unprecedented opportunity to reach far off locations, and quarantine measures and border screenings have often proven to be inadequate at preventing spread, as infectious agents can hitch a ride in travellers not yet presenting any symptoms of disease [31].

Due to international travel HIV succeeded in spreading across the entire globe, and SARS (severe acute respiratory syndrome) successfully spread to 28 countries in 5 separate continents claiming almost 800 people's lives before the epidemic was successfully controlled [32]. International shipments have also helped spread diseases indirectly by the involuntary transportation of infected animals or stagnant microbe containing water. Hantavirus has been spread across the globe by the introduction of infected rats to novel locations [33] and shipping of tires containing infected mosquito eggs has been linked to the introduction of the mosquito vector *Aedes albopictus* to parts of North America and the continent of Africa [34] [35]. Another common arthropod-borne disease that is commonly imported via air travel is malaria, and several cases of "airport malaria infections" have been reported [36] [37].

1.2.1.4 Adaptation

Infectious agents are constantly changing and adapting to their environment in response to evolutionary pressures. When a treatment is introduced to help treat an infection this can lead to selection pressure for escape mutants to occur. This is especially common in bacteria and their constant selection towards antibiotic resistant strains due to the widespread misuse of antibiotics. Similarly, fast evolving viruses can quickly become resistant to antivirals which is why drugs with differential targeting are often required to minimize the risk of resistance occurring as is true in the treatment of HIV [38]. Changes can also result in new strains that can cause variations in symptoms caused and in the case of influenza, constant genetic drift results in the need for new vaccines each year [39].

1.2.2 Socioeconomic Burden of Infectious Disease

Out of the 56.9 million deaths recorded in 2016, 3 million were caused by the deadliest infectious disease globally; lower respiratory infections. Diarrhoeal diseases contributed to 1.4 million diseases, tuberculosis caused 1.3 million deaths whilst deaths from HIV/AIDS were estimated at 1 million individuals in 2016. Whilst the impact of infectious diseases is less prevalent in higher income countries, in lower income countries infectious disease related deaths are still the most common cause of death (see figure 1.2) [40].

Measuring the impact of infectious diseases by looking at deaths alone gives a skewed and incomplete picture of the actual socioeconomic burden that infectious diseases have. A more complete estimate of impact can be gained by measuring disability-adjusted life years (DALYs). In this system, one DALY represents the loss of 1 year of full health. In the EU/EEA (European Union/European Economic Area) it has been estimated that there was a yearly loss of 275 DALYs per 100,000 individuals between 2009-2013 due to infectious disease with the highest disease burden attributed to influenza with 81.8 DALYs lost yearly [41]. DALYs have a huge societal impact as affected people are less capable of working and may need medical support and care thereby becoming a financial burden on their community.

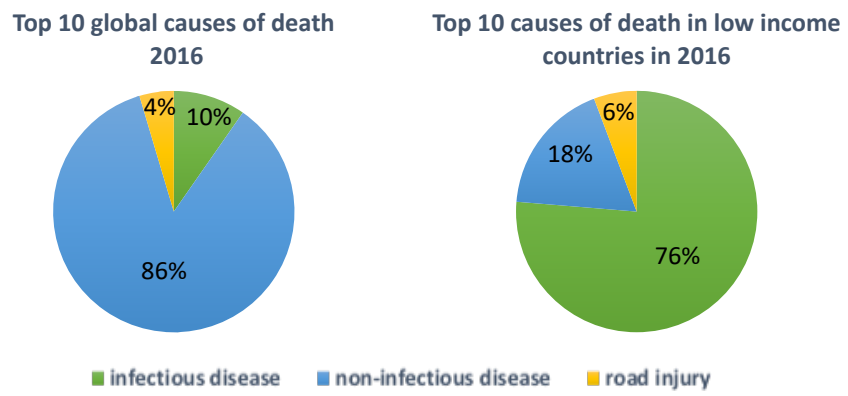


Figure 1.2. Top 10 causes of death globally and in low income countries.

Pie charts depicting the percentages of the top 10 global causes of death, or causes of death in low income countries in 2016. The top 10 causes of death have been grouped into 3 categories; infectious disease (green), non-infectious disease (blue) and road injury (yellow). Data acquired from world health organization [40].

1.2.3 Future prospects of Infectious disease

The pandemic of 1918, most commonly referred to as the "Spanish flu", ended up wiping out 3-5% of the human population of the time [42]. There is a consensus that a new pandemic will occur eventually and humanity must be prepared for the potentiality of this unknown danger. The outbreak of SARS in 2003 which led to a rapid spread of the previously unheard of coronavirus (SARS-CoV) with a death rate of approximately 1:10 infected, led to a wakeup call for the necessity of international measures to combat future outbreaks [43]. Up until the 1990s the international health regulations (IHR) only required countries to report cases of cholera, yellow fever and plague as these were the only diseases recognised for having the potential of being an international threat. Due to several outbreaks in the early 1990s, a need for a revision of the IHRs was recognized and the incorporation of more accurate and timely accounts of ongoing outbreaks were deemed necessary to aid in the combat of diseases. Whilst the SARS outbreak, which initially proved to be an immense challenge, was controlled within 5 months of its emergence due to successful global collaborations, it did also help highlight the flaws of the IHR of the time [43]. This led to a new revision that went into effect in 2007. These revisions now require countries to report to the World Health Organization (WHO) any new cases of smallpox, polio, new subtypes of human influenza, and SARS as well as other potential infections depending on the assessment of their public health impact. They have also enforced regulations to minimize spread of disease at international

ports such as airports, ports and ground crossings whilst also providing WHO with additional powers and duties including a constant global surveillance of epidemic intelligence and risk assessments and to investigate all cases of potential concern [44]. Currently, the most pressing question is, are these measures enough to control future threats?

In 2009, with the H1N1 influenza outbreak, the new IHR measures were put to the test and whilst the new modifications to the IHR helped, the world continued to be vulnerable for future pandemics. Several shortcomings were highlighted including the lack of adequate healthcare in low and mid income countries and the lack of capability of some countries to successfully monitor disease cases and disease progressions [44]. Also, since the implementation of the new IHR measures, many countries have proven incapable to meet the requirements needed for compliance. Gaps in the system have been detected following several recent outbreaks including the Ebola epidemic in Western Africa in 2014. These gaps are related to the lack of availability of timely care, limits in early detection of disease occurrences, lacking quarantine procedures and also an inefficient timely coordinated global response to these occurrences. As these limitations have had severe implications on epidemics restricted to certain geographical regions, it is possible to assume that the consequences of such gaps in our response to infectious diseases, can be quite severe in the case of a full blown global pandemic [45] [46]. It is therefore essential for all our benefit that we make a global effort to help lower income countries to meet the desired IHR measures, the implementation of a Universal Health Coverage where affordable health services are available for everyone, and the continuous monitoring and control of zoonotic pathogens in order to prevent any future outbreaks from occurring [47] [48].

The complexity between the interactions and dynamics between zoonoses, animals and potential human hosts illustrates the difficulty in predicting the occurrence of emerging and re-emerging disease. Even if humanity successfully achieves the latest goals on IHR, emerging diseases will continue to pose a genuine global threat; underlining the importance of scientific research for new treatments and medicines for the prevention and treatment of any new emerging or re-emerging pathogens. Recent outbreaks of newly emerged Zika virus (ZIKV) in Brazil [49] and re-

emergence of viruses such as Chikungunya (CHIKV) in La Réunion [50], as well as DENV have highlighted the knowledge gap for a specific group of neglected emerging and re-emerging pathogens; the arthropod borne viruses.

1.3 Introduction to Arboviruses

Arboviruses is a classification used to characterize arthropod-borne viruses which are essentially viruses that successfully replicate in, and transmitted by an arthropod vector such as mosquitoes, ticks and sand-flies. The successful maintenance of the transmission cycle of such viruses is dependent on the spatiotemporal interaction of the virus, the host and the vector. Most arboviruses retain an enzootic cycle, i.e. the virus main host is a non-human vertebrate that only occasionally will spill over and infect humans such as in the case of West Nile virus (WNV); certain arboviruses are mainly transmitted directly between humans however, such as is the case for dengue virus DENV. As a general rule, most arboviruses are named after either the disease they cause; such as in the case of yellow fever virus, or of the location it was first discovered e.g. ZIKV.

The majority of the non-segmented positive stranded RNA arboviruses belong to the *Togaviridae* and the *Flaviviridae* virus families, whilst the majority of the segmented negative stranded RNA arboviruses belong to the order of *Bunyavirales*. As an order, *Bunyavirales* contains a large number of approximately 350 genetically distinct viruses organised into 9 distinct families: *Hantaviridae, Nairoviridae, Peribunyaviridae, Phenuiviridae, Tospoviridae, Feraviridae, Fimoviridae, Jonviridae* and *Phasmaviridae*.

Clinical disease typically caused by arbovirus infections can be divided into 4 separate categories [51];

- illnesses affecting the central nervous system (CNS)
- haemorrhagic fevers
- polyarthritis and rash
- acute fever and rash

1.3.1 History of arboviruses

Historically, most mosquito borne viruses were restricted to certain geographical locations. *Ae.aegypti* for example, one of the most prevalent mosquito vectors was originally only found in parts of Africa and only spread to the rest of the world via the slave trade between the 15th and 19th century [52]. This expansion of the geographical spread of *Ae.aegypti* led to the first recorded yellow fever outbreak of the new world in 1647 in Barbados, and later, the recording of several epidemics in in the southern parts of the US in the 18th and 19th century [53] [54].

Since then, the Americas have been plagued by numerous outbreaks of yellow fever and dengue that ranged as far north as New York. An attempt was initiated in 1947 to try to tackle the issue by the Pan American Health Organization (PAHO). This mission was based on a campaign involving emptying of potential mosquito breeding grounds and the extended use of DDT (dichlorodiphenyltrichloroethane) and often involved direct access to people's homes to conduct these control measures [55]. These measures proved to be highly successful as by 1962 *Ae.aegypti* had been eradicated in approximately 20 countries [56]. However, despite these successes, due to the lack of interest of participation in the program by the US government, the US provided an easy route of re-introduction of *Aedes* mosquitoes across the border. In addition to this, when the eradication program disintegrated in the 1970s the mosquito vector subsequently managed to spread across the continent yet again reaching similar distribution levels as before the campaign (see figure 1.3) [57].

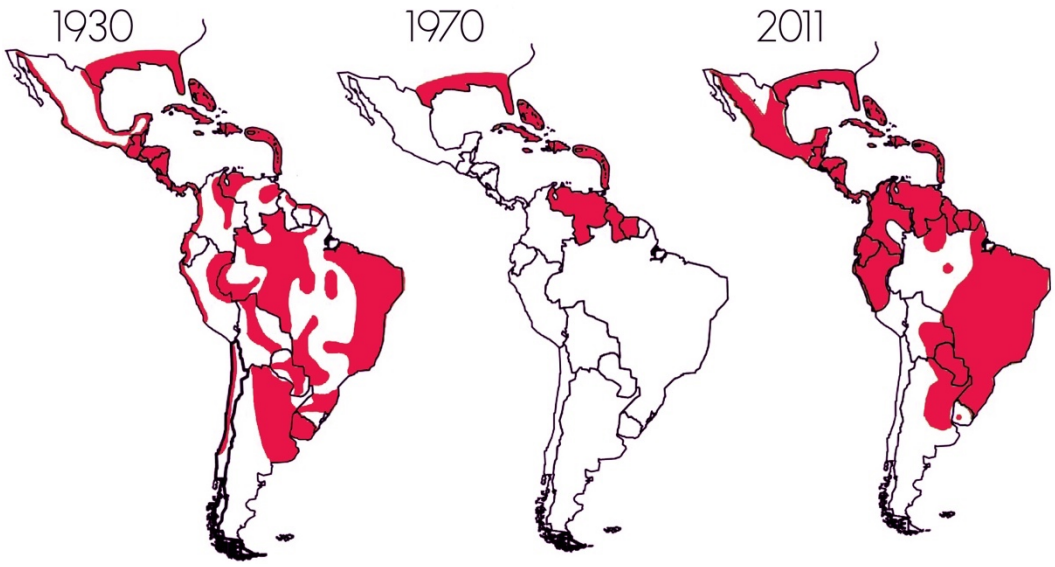


Figure 1.3. *Ae.aegypti* eradication attempt.

Map illustrating the spread of *Aedes* mosquitoes in the Americas; in the 1930s before the eradication attempt; in the 1970s after the eradication attempt; and in 2011 after abandoning the eradication attempt. Figure edited from Duane et al 2011 [57].

The ability of mosquitoes acting as transmitters of disease remained undiscovered until it was suggested as a concept by a Cuban doctor in 1881 called Carlos Finlay [58]. It took another 20 years until this was backed up by scientific evidence by Major Walter Reed [59]. During the 1930s several arboviruses were discovered via their isolation directly from blood of infected patients or from autopsy samples [60]. Via this method, the first tick borne virus was discovered in the USSR in 1937 following outbreaks of tick borne encephalitis (TBE) by a team of scientists led by Professor Lev A. Zilber [61].

1.3.2 Arboviruses today

Whilst arboviruses have been emerging and re-emerging for centuries, in recent years we have observed a far swifter global dispersal and emergence of such viruses due to globalization, adaptation of mosquito species to human habitats and human failure of controlling mosquito populations. Limitations in surveillance and health care in common endemic areas are believed to be causing an underestimation of arbovirus disease cases, but nevertheless DALYs due to infections with YFV, JEV, CHIKV and RVFV were estimated to be somewhere between 300,000 and

5,000,000 in 2005 [62]. The most prevalent alphaviruses and flaviviruses as well as the viruses implicated in this thesis will be discussed at greater length in the following sections.

1.3.3 Alphaviruses

Alphaviruses are a virus genus consisting of 31 known medically and veterinary important viruses. It has recently been suggested that the terrestrial alphaviruses originated from the oceans due to the recent discovery of aquatic alphaviruses that are most likely transmitted via aquatic lice [63]. Alphaviruses are endemic on 6 continents with certain viruses being more restricted than others in their geographical spread [64]. However as has already been discussed, geographical spread of such viruses is forever changing.

1.3.3.1 Taxonomy and distribution

Alphaviruses belong to the family of *Togaviridae* with 31 virus species having been ascribed to this family so far. Phylogenetic studies suggest that they arose in the southern oceans only to evolve terrestrial transmission where they were then capable of disseminating to the Old and New world. The non-aquatic alphaviruses are therefore commonly divided further into New world and Old world alphaviruses; two groups which arose following several introductions and re-introductions of alphaviruses from the ocean [63]. Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV) are a few examples of medically relevant New world alphaviruses whilst CHIKV, Semliki forest virus (SFV) and O'nyong 'nyong virus (ONNV) belong to the Old world alphaviruses grouping. Neuroinvasive disease is a common manifestation of infections with New world alphaviruses whilst Old world alphavirus infections are more commonly associated with febrile illness, arthralgia and myalgia.

1.3.3.2 Structure and Replication

Alphaviruses are small enveloped viruses with a size of approximately 65-70nm in diameter and with a capsid consisting of 60 asymmetric units formed by 4 types of

protein resulting in a T=4 icosahedral symmetry. The envelope is covered with 80 glycoproteins made up of 2 or 3 subunits forming a trimer made up of E1 and E2 proteins [65]. Within the nucleocapsid the virus' 12kb single stranded positive sense (ss+) RNA is located (see figure 1.4).

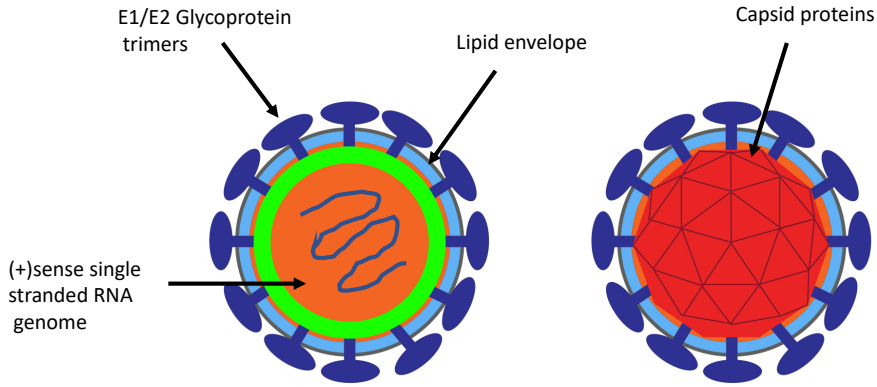


Figure 1.4. Alphavirus structure

Alphaviruses are small (700A diameter) enveloped viruses containing a 12Kb positive sense RNA genome within an icosahedral protein nucleocapsid. The envelope is host cell lipid derived and is decorated with 80 glycoprotein spikes containing E1 and E2 heterodimers.

In order for infection to occur, the virus particle must first successfully enter a susceptible cell. This occurs with the attachment of the E1/E2 glycoprotein to a specific receptor on the cell surface via protein-protein interaction. The receptors facilitating alphavirus entry into host cells remain unclear. However, due to the broad cell tropism observed by alphaviruses which is necessary for their dependency on their ability to replicate in both vertebrate and non-vertebrate cell lines, it is possible that alphaviruses use surface receptors widely expressed on a variety of cell types from a wide range of species. Another suggestion is that they potentially possess the ability to bind to multiple receptors. Receptors that have been suggested to be responsible for aiding viral entry include proteins in the histocompatibility complex such as DC-SIGN (dendritic-cell-specific ICAM-grabbing non-integrin) and L-SIGN, heparin sulphate and, for joint specific alphaviruses, the recently discovered cell adhesion molecule Mxra8 [66] [67] [68]. Following binding to cell surface receptors, the virus particle will be internalized into the cell via the process of receptor mediated endocytosis by the formation of a clathrin-coated vesicle. The virus will then fuse with the endosomal membrane in a pH dependent manner [69].

Lowering of pH triggers conformational changes on the virus particle with the E1 protein forming a fusion pore with the membrane [70]. An alternative viral entry method has recently been proposed for alphaviruses however. This alternative suggests a method of viral entry independent of endocytosis and pH-dependent membrane fusions. Instead, it is suggested that the virus particle forms a pore-like structure directly with the cell membrane via the interaction of viral and cell membrane proteins and the direct transfer of the viral RNA into the host cell via this method [71].

Alphavirus replication occurs in proximation with cellular membranes which triggers the formation of so-called "spherules". These spherules are essentially invaginations of a membrane with a diameter of approximately 50nm and they are located in the membranes of cytoplasmic vacuolar structures type I (CPV-1) where replication occurs, and also at the plasma membrane where they have potentially been directed by nsp1 [72, 73].

The ss+ RNA alphavirus genome consists of a 5' cap and a 3' poly(A) tail. There are two open reading frames (ORF) within the genome which can give rise to two polyproteins (see figure 1.5). The first ORF which is located at the N-terminal, encodes the non-structural polyprotein translated by the genomic RNA, whilst the structural polyprotein which is encoded by the C-terminal ORF is translated by subgenomic 26S RNA. Further cleavage of the polyproteins is achieved by the viral protease cysteine as well as host proteases. The non-structural polyprotein can be cleaved into 4 separate proteins; nsp1, nsp2, nsp3 and nsp4. Nsp1 is responsible for binding replication complexes to cell membranes; nsp2 possesses enzymatic properties such as helicase and protease responsible for cleaving the nsp complex; nsp3 has phosphatase and RNA-binding activity whilst nsp4 is an RNA-dependent RNA polymerase (RdRP) [74]. Initially nsp4 is cleaved from the nsp1234 complex via nsp2 to form the nsp123 and nsp4 (-)RNA strand replicase complex. The synthesis of (-) strand RNA will eventually trigger the cleavage of nsp123 complex into nsp1 and nsp23 which will lead to the formation of a complex in charge of the task of replicating the genomic and subgenomic RNA [75]. Some alphaviruses possess a leaky stop codon located between nsp3 and 4 resulting in the translation of

nsp4 10% of the time. Interestingly the substitution of this stop codon with an arginine base appears to be linked with virulence [76].

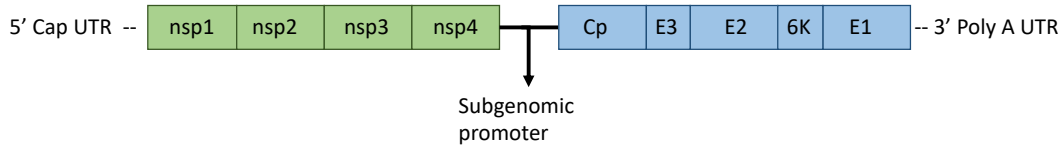


Figure 1.5. Alphavirus genome layout

Newley synthesized viral RNA is then thought to move into the cytoplasm via the pore at the neck of the spherule as it accumulates [72]. Subgenomic RNA will then be translated into structural proteins which consist of Cp, p62 (the E2 precursor, termed p62 in SFV and pE2 in SINV), 6K, E1, and transframe (see figure 1.5). Cp, which is the first structural protein to be constructed, will assemble the genomic RNA and form the nucleocapsid [77]. The structural proteins will then assemble into a newly constructed virus particle in the cytoplasm. When the virus capsid is fully assembled it will bud at the cell membrane, simultaneously gaining an envelope leading to the release of the mature virion [75, 78].

1.3.3.3 Semliki forest virus – an *in vivo* model

In 1942 in Bwamba, Uganda, 130 mosquitoes of the species *Aedes abnormalis* were collected in the Semliki forest by the Uganda Virus Research Institute, which were found to be carrying a previously undescribed alphavirus. This positive stranded RNA virus was named after the forest where it was discovered, and was therefore given the name Semliki forest virus (SFV) [79]. SFV can commonly be found in sub-Saharan Africa where it is mainly transmitted by *Aedes aegypti* and *Aedes africanus* [80]. Whilst the enzootic source of this virus remains unknown, there have been accounts of SFV infections in horses, rodents, rabbits, monkeys and even humans [81] [82]. In 1976 it proved that an outbreak of equine encephalitis in Senegal was in fact caused by SFV [83]. A decade later, samples acquired from patients suffering from headaches, fever, arthralgia and myalgia in 1987 in the Central African Republic revealed that SFV was the aetiological agent behind the outbreak [84]. Although SFV infections in humans are not considered to be fatal, historically there has been

only one death associated with encephalitis induced by SFV in a laboratory worker that was believed to have been immunocompromised [85].

Since its discovery, SFV has become the staple model for alphavirus infection and a well-used tool for understanding the molecular biology of positive sense RNA viruses in general. By mere chance, SFV has become the model alphavirus utilised in Europe, whilst in the Americas, the alphavirus Sindbis virus (SINV) is more commonly used in research [86]. There are many different strains of SFV that have been, and that are currently in use in laboratories around the globe. SFV has been determined to be neurotropic with the capability of infecting both neurons and perivascular oligodendrocytes [87]. Whilst alphavirus infection of the CNS and viral dissemination to the brain is a characteristic more commonly associated with new world alphaviruses, the neurovirulence observed with certain SFV strains has most likely arisen due to passaging of virus through mouse brains. Strains of SFV can very roughly be divided into two groups; the virulent strains and the avirulent strains. The earliest isolated strain of SFV, now known as L10, belongs to the virulent strain grouping as it was discovered to be neurovirulent and causing lethal encephalitis in mice by its ability to infect the central nervous system (CNS). Other strains in this grouping include V13, SFV4 and SFV6 [88-90]. Strains in the avirulent group cause demyelination instead of lethal encephalitis and include the strains A8, A7 and its derivative A7(74) and MRS MP 192/7 [80, 90]. As only SFV4 and SFV6 have been used for the purpose of this thesis, only these will be discussed further.

L10, which derives from the original isolate of SFV acquired in Uganda, was passaged 8 times intracerebrally in adult mice and a further 2 times in baby mice [91, 92]. In a murine *in vivo* model, infection with SFV L10 leads to high viral titres detectable in the blood as well as efficient infection of the brain resulting in lethal panencephalitis. SFV4 is significantly less virulent and rarely neuroinvasive in mice [93]. Sequence data from the different SFV strains were compared in an attempt to determine the cause for the difference in virulence and neuroinvasion between the strains. 12 nucleotide differences were detected between SFV L10 and SFV4 out of which 6 were nonsynonymous. A single amino acid change in the E2 glycoprotein at position 162 as well as 247, from glutamine in L10 to a lysine in SFV4 appeared to be directly affecting the different strains virulence and neuroinvasion efficiency.

This amino acid change results in an alteration in binding efficiency with strains containing the charged lysine residue at positions 162 and 247 demonstrating a higher affinity of binding to the glycosaminoglycan heparin sulphate. Heparin sulphate is ubiquitously expressed on most vertebrate and invertebrate cells and this higher affinity in binding likely results in an increased retention of SFV4, minimizing its presence in circulation [94].

These 6 nucleotide differences were used to engineer a new molecular SFV clone now known as SFV6. This newly generated strain proved to be highly virulent and very neuroinvasive. Unlike L10, SFV6 is genetically homogenous whilst the original isolate, L10, consists of a mixture of SFV variants with varying phenotypes and pathogenicity and so SFV6 represents the most virulent consensus sequence of L10 [94]. The SFV4 and SFV6 strains are obtainable as cDNA clones which can be transcribed into viral RNA which, when electroporated in to eukaryotic cells will result in infectious virus [86].

Several studies have been conducted characterizing the pathogenicity and viral dissemination of SFV in mice. Whilst most work has focused on the pathogenicity of SFV in neural tissue following intracerebral inoculation, recent studies have investigated the pathogenesis of virus infection following the subcutaneous inoculation of virus in the skin in the presence of a mosquito bite [95]. Subcutaneous injection of SFV4 leads to dissemination of virus 3 to 6 hours following virus infection to the draining popliteal lymph node (dLN) and a peak of viraemia 24h post infection. Systemic spread of virus can be observed 48h following infection with only sporadic episodes of neuroinvasion. If SFV4 is injected alongside a mosquito bite however there is a significant increase in amount of viral RNA detected at the site of infection and amount of infectious virus particles located in the serum at 24h post infection. Whilst certain aspects of infection remain poorly understood, such as which the key cellular targets are for viral replication and the trajectory of viral dissemination to distal tissues, it has been established that a key aspect of disease progression is the early host responses at the bite site and the associated inflammation triggered by the bite which result in a significant enhancement of viral replication and dissemination [95]. This will be discussed in greater detail in section 1.7.

Due to the extensive research conducted on SFV and given how well characterised SFV infection is in mice it makes this virus an excellent model for studying arbovirus infection *in vivo*. Another factor that makes SFV an excellent *in vivo* model system is this virus ability to successfully replicate and disseminate effectively in immune competent wild type mice (unlike many other arboviruses). Also the fact that it is categorised as a biosafety level 2 pathogen, whilst most other medically relevant arboviruses are not, allowing for work to be conducted in a lower containment setting. Therefore, in terms of the work conducted for this thesis, SFV formed a useful tool for studying the modulation of arbovirus enhancement in the skin by mosquito saliva. A further benefit of using SFV is the fact that there are numerous reporter strains available as plasmids that are easily genetically manipulated and grown in bacteria which can be used for the visualization and quantification of infection with these viruses such as the SFV6-Gluc strain which was utilised in several experiments in this thesis.

1.3.3.4 Chikungunya virus – a medically relevant alphavirus

CHIKV infection in humans leads to the development of chikungunya fever (CHIKF) in approximately 75%-95% of affected individuals [96] [97]. CHIKF is most frequently associated with high fever, myalgia, arthralgia, polyarthralgia and potentially haemorrhage occasionally accompanied by a rash [98]. Chikungunya infection was first described following an outbreak of the virus in the early 1950s in Tanzania and infectious CHIKV was first isolated in 1953 from human sera of infected patients as well as directly from infected mosquitoes of the species *Ae.aegypti* and several species from the Culex family [99] [100]. The virus is named after the arthralgia symptoms associated with the disease as chikungunya is a word from the Makonde language depicting the crocked posture of a person infected with the virus [99]. CHIKV is now classified as an Alphavirus and a member of the Semliki forest virus antigenic complex, a complex that also includes other alphaviruses including SFV, Ross River virus (RRV) and the closely related ONNV amongst others [101].

Infection with CHIKV is initiated following the deposition of the virus into the skin of the host via the help of its mosquito vector which are most commonly *Aedes* mosquitoes [102]. Whilst the progression of infection and the exact steps involved

are poorly understood, it has been established that following initial infection the virus will readily spread to distal tissues as the virus preferentially targets the joints, muscle and skin for replication and occasionally also the liver, kidneys and eye [103]. Several studies have shown that following infection, replication of CHIKV can occur to very high viral titres ranging between 10^5 to 10^9 copies of viral RNA per ml. Amount of viral load appears to correlate with severity of symptoms [104] [105].

CHIKV is not considered to be classically neurotropic in the sense that it does not disseminate readily to the brain and is not commonly associated with manifestations of encephalitic disease. Despite that, the virus can occasionally disseminate to the CNS and cause infection of the choroid plexus, where it can infect meningeal and ependymal cells [106] [107]. Also, there are several accounts of paediatric cases of CHIKV infections that have been associated with neurological complications [108]. Disease tends to manifest 2-4 days following infection, and depending on the strain responsible, in 10% - 20% of infections with CHIKV lead to long lasting polyarthralgia and/or polyarthritis that can linger for several years [109] [110] [111]. This persistence of polyarthralgia associated with infections of some alphaviruses appears to be associated with the persistence of viral antigens in the joints and the immune responses triggered by these antigens [112] [109] [111] [113]. Viral RNA and antigens can be detected in the synovial tissues of affected patients. A study utilising a macaque animal model have demonstrated that virus can infect and replicate in synovial macrophages [114].

Animal models have shown to be key in researching CHIKV immunity and pathogenesis, with mice and non-human primates making up the most commonly used model systems. Unlike the SFV mouse models where the virus replicates efficiently in WT mice, CHIKV does not. Infection of WT immunocompetent mice with high-titre CHIKV inoculum leads to a peak of viraemia at 48h post infection which is then cleared 5 days following infection. Also, whilst CHIKV is able to effectively infect the foot joint close to the inoculation site and cause arthritis and localised infection of synovial tissues, the virus is unable to disseminate efficiently to distal joints away from the site of inoculation. Therefore, this model fails to closely mimic what occurs in CHIKV infections of humans where virus causes disseminated polyarthralgia as well as failing to model vertical transmission from the mother to

the infant and enhanced disease observed in older individuals [115] [116]. In addition, CHIKV joint arthritis resolves fairly quickly mice, whilst human infections can take several weeks up to several years for virus to completely clear. Alternative mouse models have therefore been developed to better study vaccine efficacy and antivirals which encompass mice with impaired innate immune responses such as interferon receptor null mice [106]. A major drawback of utilising immune deficient mice is that it may compromise our understanding of disease progression and pathogenesis.

With its enzoonotic transmission cycle between mosquitoes and non-human primates, re-emergence is very likely, and with the spread of its mosquito vectors to the continent of Europe it is possible that the future will hold CHIKV emergence in new geographical locations [117]. The high risk of re-emergence in combination with the lack of treatments or vaccines are currently available for CHIKV, emphasizes the likelihood of future complications caused by a CHIKV outbreak.

1.3.3.5 O'nyong nyong virus – an exception

ONNV is an alphavirus belonging to the Semliki forest virus complex which is very closely related to CHIKV, and the two viruses share a very high percentage of sequence similarity [118]. The two viruses appear to have diverged from a common ancestor several thousand years ago [119]. ONNV was first isolated in Uganda in 1959 during the first recorded outbreak with this virus. The name o'nyong nyong originates from the Nilotic language of Uganda and Sudan and roughly translates to "weakening of the joints" which is associated with the symptoms triggered by the infection with this virus [120]. Three major outbreaks with ONNV have been recorded in the past, the first between 1950s and 1960s which initially started in north-western part of Uganda only to spread to Kenya, Malawi, Tanzania and partly to Senegal affecting more than 2 million people [120]. The second outbreak occurred between 1996 and 1997 in Uganda whilst the third outbreak also occurred in Chad in 2004 [121] [122] [123]. To date, ONNV has never been detected outside of sub-Saharan Africa.

Infections with ONNV in humans are associated with symptoms such as fever, arthralgia and myalgia accompanied by a rash. The symptoms tend to clear up after a few days with occasional individuals suffering from long term arthralgia [124]. Unlike CHIKV however, ONNV has never been associated with causing lethal disease [125]. As the symptoms associated with ONNV infection closely resemble those of other African tropical diseases such as dengue and chikungunya, it is believed that there is a recurring underreporting of ONNV infections which means that the true prevalence of ONNV infections is unknown. In addition to this, antibodies generated against ONNV and CHIKV are highly similar and have almost complete cross reactivity [126] [122]. This is of major concern as control measures taken against these viruses differ as they are predominantly transmitted via different mosquito species.

Dissimilar to most mosquito borne arboviruses that are transmitted predominantly by culicine mosquitoes, ONNV is the only known alphavirus that is transmitted mainly by anopheline mosquitoes with its main vectors being *Anopheles funestus* and *Anopheles gambiae* [127] [128]. The reason for this remains unknown. Due to the sequence similarity of CHIKV with ONNV, comparison studies between the two viruses have been made in an attempt to shed some light on potential differences that could help explain the difference in vector competence. Studies comparing ONNV ability to infect different cell types *in vitro* to that of CHIKV have shown that whilst ONNV was able to successfully infect the *Ae.albopictus* cell line C6/36 as well as the *An.gambiae* cell line MOS-55, CHIKV could only successfully infect C6/36 cells [129]. Another study, using the construction of a range of chimeric viruses of ONNV with CHIKV in order to compare the infection rates of *Anopheles* mosquitoes to these constructs. The study concluded that by replacing the nsp3 of CHIKV with nsp3 of ONNV they could observe a significant increase in infection rates of *An.gambiae* mosquitoes suggesting that nsp3 plays an important role in determining the vector competence of ONNV [130].

There are a few different strains of ONNV commonly used in research. These include SG650, MP30 (Gulu strain) and the Igbo Ora strain. The MP30 strain was isolated during the original outbreak in Uganda in 1959 from human serum of infected patients and has since been passaged 14 times in brains of suckling mice and

then once through Vero cells [131]. SG650 was originally isolated from human serum during the 1996 outbreak in Uganda, and was passaged once in Vero cells [132]. The Igbo Ora strain was initially isolated from an infected individual in Nigeria in 1966 and was passaged 6 times in baby mouse brains and once in Vero cells [133] [134]. Igbo Ora was originally thought to be a separate virus but sequence analysis later proved that it was just a strain of ONNV [135] [132]. Several reporter variants of these strains have also been created. For the purpose of this thesis a strain called ONNV-2SG-ZsGreen was utilised which was created from the Igbo Ora strain where the ZsGreen gene has been placed between the ns-protein and structural protein region, under the duplicated promoter.

A lot still remains unknown about ONNV which is a neglected tropical pathogen meaning there is very limited research associated with this virus. For example, the reservoir host remains undetermined and the course of infection, dissemination and pathogenesis of the virus is poorly understood. In addition to this, there are no well described animal models available for ONNV. To date there is only one study that has investigated ONNVs ability to replicate in mice which demonstrated that infection of wild-type, RAG1 KO, and IFNγR KO mice exhibited no signs of illness or viraemia. However when inoculating mice subcutaneously (s.c.) in the back with 100 µL of ONNV (10^2 to 10^4 pfu) in STAT1 KO mice and A129 (IFNα/βR KO) mice, infection caused 50-55% mortality when infected with ONNV of the strain SG650. The study also demonstrated that the SG650 strain was more virulent than MP30 [136]. More research is urgently required for this neglected pathogen.

1.3.4 Flaviviruses

Flaviviruses have affected humanity for centuries with the first cases of dengue-like disease recorded in 1779 [137] [138]. Since then these viruses have become prevalent globally as viruses such as dengue have become endemic in over 100 countries. Today flaviviruses are responsible for affecting millions of individuals annually with DENV being the most prevalent causing 20,000 deaths per year [139] [140]. Flaviviruses are a genus that belong to the family of the *Flaviviridae* and consists of more than 70 distinct viruses, many of which are of medical importance as they can cause disease in humans. Important human pathogens in this genus include

DENV, JEV, YFV and ZIKV. The genus is named after the Latin word for yellow, "*flavus*", which refers to the jaundice commonly associated with yellow fever infections [141]. The majority of flaviviruses are transmitted via an arthropod vector and infections with flaviviruses are associated with a wide variety of symptoms ranging from a mild fever and malaise to haemorrhagic fevers and lethal encephalitis.

1.3.4.1 Flavivirus structure and replication

Flaviviruses are icosahedral, enveloped viruses approximately 50nm in diameter. The flavivirus single stranded positive sense RNA genome which encodes three structural proteins, E (envelope) protein, C (capsid) protein and M (membrane) protein, as well as seven non-structural proteins (see figure 1.6) [142]. Unlike the alphavirus genome, the flavivirus genome has only one open reading frame which encodes for a single polyprotein that is subsequently cleaved to give rise to the structural and non-structural proteins.

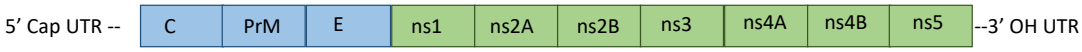


Figure 1.6 Flavivirus genome structure.

Flavivirus cell entry occurs via receptor mediated endocytosis. For DENV the proposed receptors for mediating entry into host cells are DC-SIGN, GRP78/BiP (glucose-regulating protein 78), and CD14-associated molecules [141]. Assembly of the viral particle occurs on the surface of the endoplasmic reticulum with virions maturing in the trans golgi network. Mature virions will then exit the cell via budding through the cell membrane whilst simultaneously acquiring their viral envelope [143].

1.3.4.2 Zika virus

The flavivirus ZIKV was originally isolated in 1947 from a rhesus monkey in Uganda [144]. Whilst infections in humans were known to occur in the 1960s – 1980s, it remained a largely neglected tropical virus with very few researchers studying it. It

was not until 2007 that the first larger ZIKV outbreak would occur, at the island of Yap [145]. ZIKV would then grab the attention of the world following its re-emergence in French Polynesia in 2013-2014 and later in Brazil which suffered from major outbreaks in 2015 [146] [147]. Although ZIKV causes a self-limiting infection, infection with ZIKV is commonly associated with febrile illness, rash, potentially Guillain-Barré syndrome and abnormal neurodevelopment in foetuses of infected mothers resulting in incidences of microcephaly in infants [148].

ZIKV is predominantly transmitted via *Aedes* mosquito species [149]. Unlike most other flaviviruses ZIKV can be transmitted via the perinatal route by its ability to cross the placenta and can also be sexually transmitted as ZIKV can be detected in semen up to 6 months following initial infection with the virus [148] [150].

The first isolated ZIKV strain MR766 which was isolated in 1947 in Uganda and has been passaged in mouse brains over 100 times [144]. Following the more recent outbreaks of ZIKV several new viral strains have been isolated due to concerns of the existence of potential differences between the old and newly emerged variants. Such strains include the Asian lineage isolate H/PF/2013, the American lineage isolate FB-GWUH-2016, as well as isolates from French Polynesia, Brazil and Puerto Rico [151] [152] [153]. In this study the Brazilian strain of ZIKV, ZIKV PE243164, was used, which was first isolated in 2015 by the Fundação Oswaldo Cruz, Recife in Brazil [154].

ZIKV is unable to replicate efficiently in immunocompetent mice as WT mouse strains such as C57BL/6, CD-1 and BALB/c demonstrated no signs of disease and detection of viral RNA or infectious virus in tissues was negligible [152] [153]. In humans there is evidence that ZIKV NS5 protein can promote the degradation of STAT2, thereby inhibiting the type I IFN response in humans [155] [156]. Studies carried out in mice however demonstrate that NS5 fails to degrade mouse STAT2; a phenomenon which could explain why immunocompetent mice are resistant to ZIKV infection [155]. This has led to the development of ZIKV mouse models that are reliant on interferon or STAT knockouts [157]. Such models are unreliable for the accurate description of *in vivo* infection kinetics of the virus as well as for outlining immune responses. However, wild type macaque animal models using rhesus

macaques and Mauritian cynomolgus macaques have deduced that viraemia peaks 2-3 days post infection with viral clearance occurring approximately 10 days following infection [158]. Interestingly, some studies have demonstrated that 30-40 days post infection a "viral rebound" where a significant increase in viraemia can be observed in the blood [159]. An alternative, immunocompetent mouse model modelling ZIKV infection has recently been developed as an alternative. In this model STAT2 "knockin" mice where, mouse STAT2, replaced with human STAT2 where used. When infected with a mouse adapted strain of ZIKV, virus is able to replicate efficiently with interferon responses remaining intact [160].

1.3.4.3 West Nile Virus

West Nile virus (WNV) is a flavivirus that is sustained in a zoonotic cycle between mosquitoes and avian hosts. It was first discovered in Uganda in 1937 and was later involved in several epidemics in the 50s and 60s in the Mediterranean [161] [162]. Also, New York saw the emergence of fatal encephalitis in 1999 which was then attributed to West Nile virus. Infected migratory birds enabled WNV to spread, in less than 5 years, to the southern parts of Canada, South and North America as well as the Caribbean [163]. Infection with West Nile virus has since been associated with West Nile fever in 1 in 4 infected individuals out of which approximately 1% will go on to develop neurological symptoms such as encephalitis, acute flaccid paralysis and meningitis which will prove to be lethal in 1 in 10 of affected individuals [164]. As there are no current treatments or vaccines available for WNV, future outbreaks are likely to cause a problem.

1.3.4.4 Dengue virus

DENV is one of the most globally widespread arboviruses and considered a major global health challenge in tropical and subtropical regions. It is estimated to infect 50-400 million people per year [165]. Dengue fever is known to cause fever, rash, myalgia and arthralgia but it can also cause dengue haemorrhagic fever which is associated with vascular leakage and thrombocytopenia. There are 4 separate serotypes of DENV; DENV-1, DENV-2, DENV-3 and DENV-4 [166]. Secondary infection with a different DENV serotype has been associated with causing "severe

dengue" out of which 5-20% of cases end up being lethal. Between 1990 and 2015, 262 outbreaks of DENV have been recorded worldwide with the majority of those transpiring in India, China and Brazil [167]. In Europe, the continent least affected by DENV, 4 outbreaks took place in France and 2 in Portugal [168]. Lack of treatments available leads to the hospitalization of approximately 500,000 individuals per year, most of which are children which is having a tremendous economic burden on affected countries.

1.4 Introduction to vectors and epidemiology

Arthropoda constitutes the most species rich phylum, containing over 80% of all living species. Estimations indicate that there are approximately 5-10 million distinct species of arthropods on the planet with a small fraction of these making up the hematophagous arthropods with more than 14,000 species [169, 170]. Hematophagy, which refers to the act of blood feeding, has evolved independently 1400 times, an adaptation that has had enormous impacts on the co-evolution of hematophagous insects with their vertebrate hosts [169]. Blood feeding has allowed for the evolution of a range of pathogens that hijack this mechanism of feeding and utilise it as an efficient method of their own transmission. These pathogens have a profound impact on human health as there are hundreds of diseases of viruses, bacteria and parasites that are transmissible to humans via arthropod vectors including dengue, malaria and Lyme disease [171]. The maintenance of the pathogen transmission cycles is heavily dependent on the spatiotemporal interactions of the pathogen, the vector species and the host species. Vector competence and host susceptibility to infection adds further complexity. In humans, the majority of arthropod borne diseases are transmitted via arthropods such as mosquitoes, ticks, sandflies and midges [172].

1.4.1 Mosquitoes

Mosquitoes are classified as arthropods under the order of Diptera belonging to the family *Culicidae*. 3,490 species of mosquitoes have currently been identified and they are spread across the globe, with species residing in the tropical regions stretching to above the arctic circle. Out of these less than 1 in 10 are known vectors of viral

disease as there are approximately 300 different mosquito species that can transmit arboviruses. Nevertheless, mosquitoes are the most prevalent arthropod transmitters of viral disease, with *Aedes* and *Culex* mosquitoes being the most common vector-competent species; with their ability to transmit 115 and 105 arbovirus strains respectively [173].

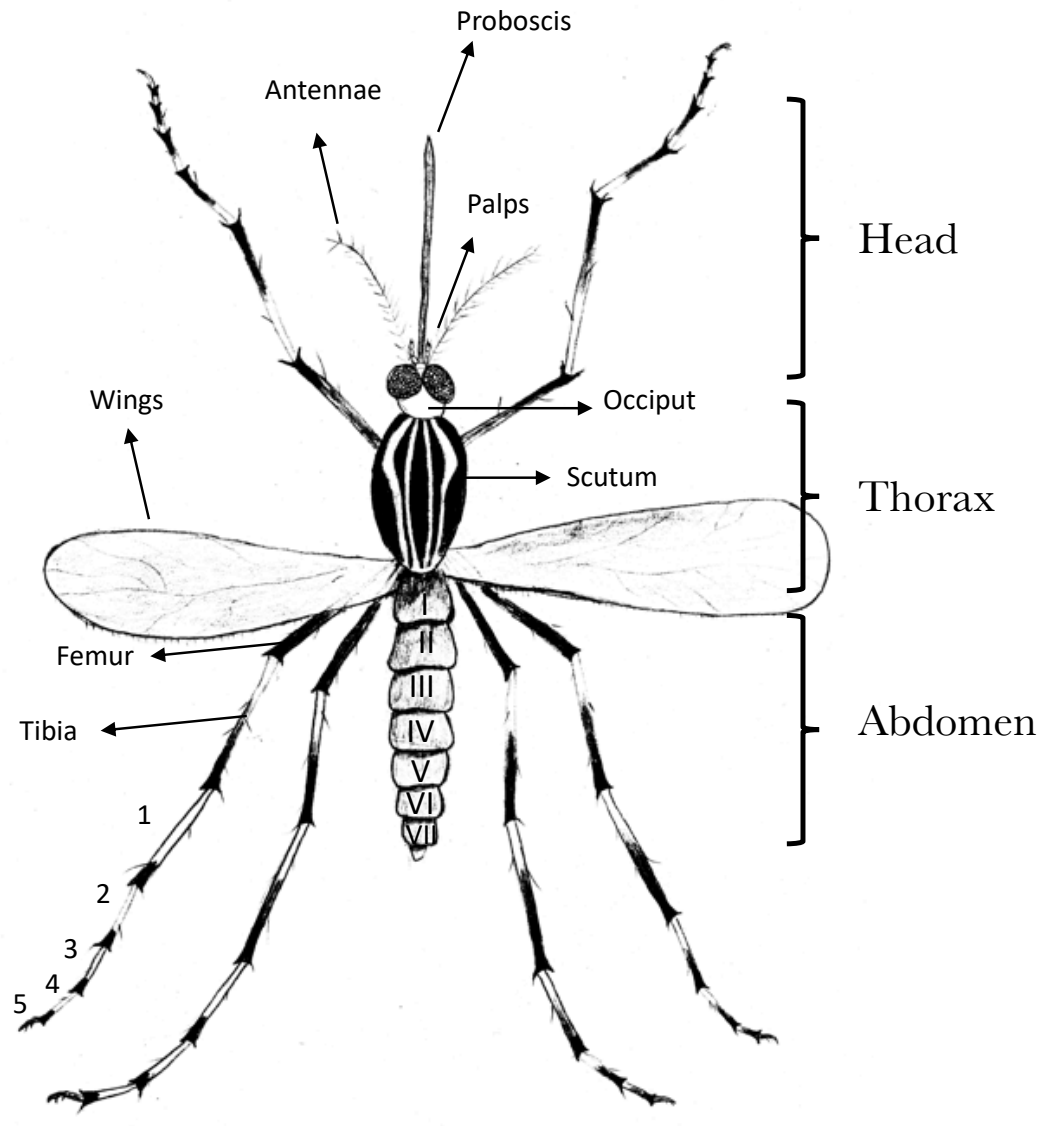


Figure 1.7. Mosquito diagram.

Diagram of a female *Ae.aegypti* mosquito. *Ae.aegypti* mosquitoes are easily identified by the lyre-shaped pattern on their scutum with two lines down the centre of their thorax. The abdomen is made up of 7 sections.

1.4.1.1 Mosquito Life cycle

Whilst the majority of mosquitoes reside in close proximity to ground water, the life cycle of mosquitoes can vary greatly between species, with certain species specialised to occupy anything from holes in trees, bromeliads and snail shells. Whilst this section will only focus on the life cycle and aspects of relevant mosquito vector species, some aspects are universal to all mosquito species.

All mosquito species lay their eggs in, or in close proximity of, water. The eggs, less than a millimetre in length, whilst initially white in colour darken quickly during the course of a day into black or brown. Eggs laid directly in water tend to hatch within a couple of days whilst eggs of other species, such as the *Aedes*, which are typically laid just above the water line of stagnant water, will typically hatch when they are eventually submerged in water. This delay in hatching prevents the eggs from hatching before conditions are right as *Aedes* eggs are more resilient to fluctuating temperatures than hatched larvae and emerged adults, enabling them to potentially survive throughout winter [174]. Rain, rising of water levels or even human intervention will eventually submerge the eggs enabling them to hatch into mosquito larvae.

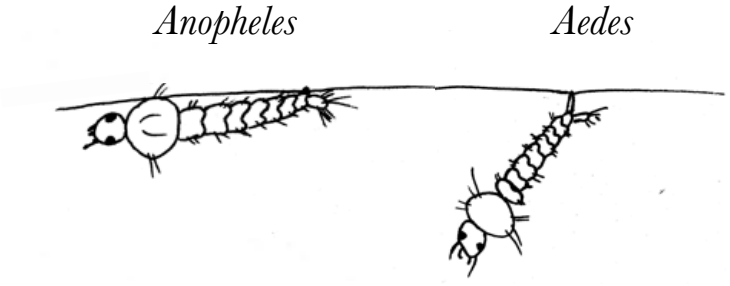


Figure 1.8. Mosquito larvae.

Mosquito larvae of different species can be observed lying flat at the water surface, such as *Anopheles* larvae or "hanging" from the surface such as *Aedes* larvae.

Mosquito larvae reside in water but are entirely dependent on breathing air via a breathing tube and must therefore regularly return to the surface. Depending on the species, resting larvae can be observed "hanging" from the surface or lying flat at the water surface (see figure 1.8). Larvae typically feed on aquatic microorganisms via two separate methods of feeding, filter-feeding at the surface or bottom scraping and the prevalence of the two methods varies between species [175] [176]. Feeding allows

the larvae to grow and undergo three separate stages of shedding, commonly known as instar phases. These transitions and the eventual transformation of the larvae in to pupae are heavily regulated by the release of hormones such as ecdysteroids into the water [177]. Larval molts are triggered by the presence of ecdysteroid and the sesquiterpenoid juvenile hormone (JH) whilst the final transformation into pupae is initiated by the absence of JH with pupal stages lasting a couple of days depending on species [178].

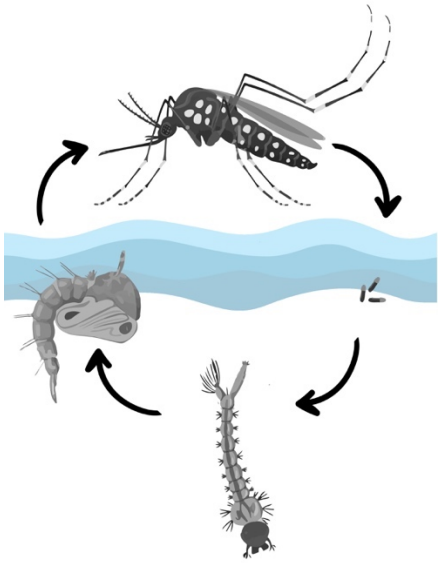


Figure 1.9. *Ae.aegypti* life cycle.

Diagram depicting the life cycle of *Ae.aegypti* from an egg to a hatched larvae, to a pupae to a fully emerged adult mosquito.

Adult mosquitoes have sexually matured by 3-5 days post emergence. Whilst the majority of mosquitoes feed exclusively on plant nectar, the females of certain species are hematophagous as the acquisition of a blood meal plays a key role in their gonotrophic cycle of egg maturation and oviposition [179]. Even within the hematophagous mosquito group feeding patterns vary greatly between the different mosquito species, which exhibit dissimilarities in feeding locations, host preferences as well as time of feeding. Certain species of mosquitoes are preferentially nocturnal (active at night); such as is the case for the majority of *Anopheles* mosquitoes [180]; while other mosquito species are diurnal (active during the day) or crepuscular (active at twilight), and occasionally both diurnal and crepuscular as is the case of *Ae.aegypti* [181] [182]. A better understanding of the ecology and life-cycle of important vector species is, and has been vital in the development of specific and more targeted mechanisms for vector control [183].

1.4.1.2 Arbovirus replication in mosquito vector

Mosquito-borne arboviruses are transmitted via infected mosquitoes and this mechanism of transmission relies on the successful infection and viral replication within the mosquito [184]. These viruses have therefore acquired specific mechanisms in order to successfully replicate within the arthropod vector whilst simultaneously overcoming a range of physical and immunological barriers within the mosquito (see figure 1.10) [185] [186].

After the ingestion of infected blood following the feeding upon an infected host, the infected blood moves into the posterior section of the mosquitoes midgut thereby circumventing the diverticula of the gut which are reserved for carbohydrate storage [187]. It is of huge importance that the mosquito ingests virus of high enough titre in order to override the threshold required for a successful infection. When inside the lumen of the midgut virus particles have to successfully infect mesenteronal cells via gut microvilli before the formation of the peritrophic matrix which is secreted by the midgut epithelium following the ingestion of a blood meal, which once formed prevents infection of mesenteronal cells [188]. Whilst it remains unknown exactly how viruses manage to overcome the midgut barrier, it has been observed that the virus will undergo a genetic bottleneck in the process as only a select few particles will succeed [189]. Successful virus will then replicate in the cells of the mesenteron until it reaches peak titres when it can successfully spread to the haemocoel by crossing the basal lamina [188]. The haemocoel is the primary, haemolymph containing cavity of invertebrates and the escape of virus in to the haemocoel cavity allows for the spread of viral infection to a multitude of susceptible tissues such as muscle, pericardium, fat bodies and ganglia [187].

Finally, for virus to successfully complete its life cycle, it has to infect salivary glands which are located in the thorax of the mosquito. This stage of the infection is associated with a second genetic bottleneck event as only a few viral genotypes are able to successfully infect the salivary glands [189]. Infection of the glands is believed to occur via dissemination of virus via the haemolymph, prior infection of adjacent organs or via a combination of both [190]. When inside the salivary glands the virus has to replicate to high enough titres that will enable a successful transmission to a

vertebrate host during the next blood meal. The virus can require several days, depending on the strain, before it reaches optimal numbers. Apart from the salivary glands, infection of mosquito ovaries have also been observed providing arboviruses with a potential alternative route of transmission; vertical transmission from mother to offspring [191].

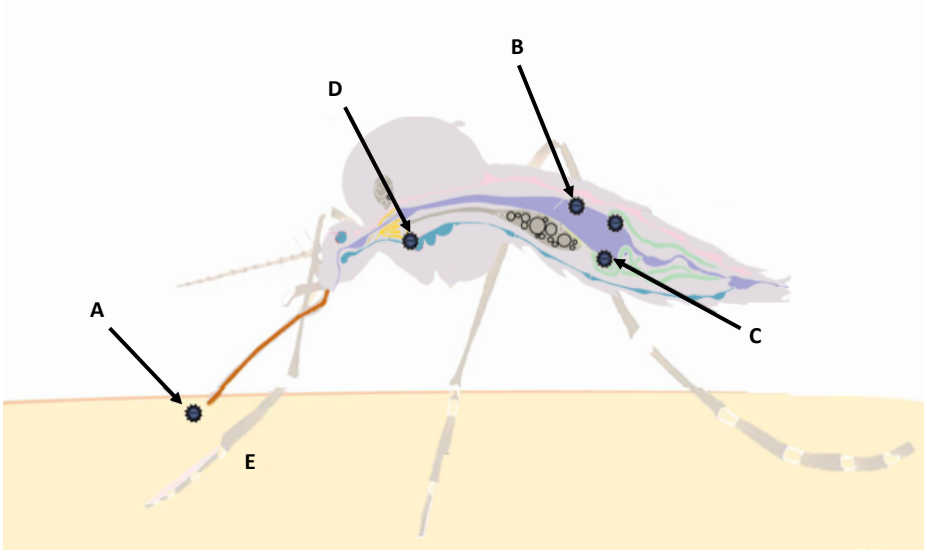


Figure 1.10. Arbovirus replication inside vector.

Figure depicting the steps of the infectious cycle of arboviruses inside the mosquito vector. A. Mosquito ingests virus infected blood from infected mammal. B. Virus travels to the midgut. C. Virus enters circulatory system. D. Virus reaches the salivary glands. E. Virus injected into new host alongside mosquito saliva following secondary blood feed.

1.4.1.3 Mosquito immune response to arbovirus infection

Mosquito borne viruses are transmitted to vertebrate hosts via bite of an infected mosquito. This mechanisms of viral transmission relies on the successful replication of the virus inside the arthropod vector. In order for this to occur the virus must overcome the obstacle that is the mosquitoes innate immune response. Such responses include interfering RNA pathways (RNAi), toll pathway, the immune deficiency (IMD) pathway, the Janus kinase - signal transducers and activators of transcription (JAK-STAT) pathway as well as apoptosis [192]. Arthropods lack an adaptive immune response and are therefore dependent solely on mounting a successful innate immune response against an invading pathogen .

The majority of research investigating the innate immune responses of mosquitoes to viral infection have been conducted utilising *Drosophila melangoster* as a model system as drosophila is susceptible to experimental infection with arboviruses. It was using this model system that the Toll pathway of arthropods was first described. Toll pathway was discovered to play an important role in the protection against fungi, viruses and gram positive bacteria [193] [194] [195]. This pathway shares certain similarities with the vertebrate NF-kB signalling pathway as it relies on pattern recognition receptors (PRRs) to recognise and bind pathogen specific ligands. Recognition by PRRs result in the cleavage of Späetzle which is a cytokine released by the presence of pathogens. Späetzle will bind to Toll triggering a signalling cascade that results in the subsequent transcription of antimicrobial peptides [196]. The Toll pathway that is conserved in mosquito species, has a key role in mediating responses against DENV infection. Studies infecting *Ae.aegypti* with DENV have shown significant upregulation of Toll post infection is associated with the reduction of viral titres in the mosquito midgut [197] [198]. Similarly, knockdown of the IMD, which, like Toll upregulates antimicrobial genes in response to PRRs, leads to an increase of DENV in the midgut suggesting a role for IMD pathway in the innate immune response of mosquitoes against arboviruses [199] [200]. The involvement of the JAK/STAT pathway in insect innate immunity was initially observed in *An.gambiae* mosquitoes in response to bacterial infection [201]. Whilst the role of JAK/STAT against arbovirus infection remains poorly understood, JAK/STAT does play a role in regulating DENV infection in *Ae.aegypti* mosquitoes [202].

RNAi is another mechanism that plays a key role in mosquito antiviral immunity. The RNAi mechanism can be subdivided into three distinct pathways, namely the small interfering (si)RNA, the micro (mi)RNA, and the Piwi-interacting (pi)RNA [203] [204]. The RNAi driven immune response is activated via the recognition of pathogen associated molecular patterns (PAMPs) such as dsRNA by Dicer 2 (Dcr2) which is an RNase III which recognizes and cleaves dsRNA into siRNAs which ultimately leads to the initiation of the RNAi pathway [205]. The siRNAs, Dcr2 and the dsRNA binding protein R2D2 interact with the RNA-induced silencing complex (RISC), the key component of which is Argonaut 2 (Ago2 which functions as the catalytic component of RISC) [206] [207] [208]. After degrading one of the siRNA strands and by using the second strand as a template for detecting viral mRNA,

Argo2 will degrade it utilising its endonuclease activity which ultimately will inhibit viral replication without causing apoptosis [209]. piRNAs are small RNAs that protect the genome by regulating the transcription and transposition of transposable elements and studies have suggested that they play a role in the defence of mosquitoes against arbovirus infections. Although the specific mechanisms remain unclear, piRNAs have been shown to be implicated in *An.gambiae* mosquitoes infected with ONNV as well as *Ae.aegypti* mosquitoes infected with DENV [210] [211].

Whilst these immune responses are broadly similar in most mosquito species there are some species-specific variations. Studies of *Anopheles gambiae* mosquitoes which are known to be inefficient vectors of most arboviruses with the exception of ONNV, have illustrated key differences in the immune responses to arboviruses to that of the main arbovirus vectors, *Aedes* and *Culex*. Whilst there is debate about whether JAK/STAT and IMD play a role in *Anopheles* antiviral immunity, studies have shown that Toll does not impact infection of *Anopheles* with ONNV, and that ONNV has the ability to actively inhibit the Toll pathway [212] [213] . This is in contrast to *Ae.aegypti* where Toll and JAK/STAT are the key pathways of the immune response against DENV [197, 200] [202]. Such differences between mosquito species may help explain the differences in vector competence of the different species.

1.4.1.4 Mosquito species

The *Culicidae* stretch as far back in history as the Jurassic following their split from their sister group Chaoboridae [214] [215]. Due to the limited amounts of mosquitoes in the fossil records categorization of mosquito species within the *Culicidae* has historically proven difficult. More recently, with the help of genome sequencing several advances have been made in this area with mosquito species being split into two subfamilies; the *Anophelinae* and the *Culicinae*. These can then be subdivided further into 11 tribes containing at least 44 separate genera [216] [217]. Studies comparing mitochondrial DNA of mosquito species have suggested that the split between the *Culicinae* and the *Anophelinae* occurred sometime between 145-200 million years ago (mya) whilst comparisons of specific protein coding nuclear gene sequences estimate that the split occurred approximately 226 mya [218] [219].

The *Anopheles* family underwent radiation during the early Cretaceous and the rapid speciation of mosquito species has been linked to the increased speciation of mammals as these would have been the main blood source of blood feeding mosquitoes [220]. The long evolutionary history of mosquitoes, the huge numbers of mosquito species and their co-evolution with arboviruses have resulted in numerous differences between the species, including vector competence and transmission cycles. In the following section a summary will be made of the main mosquito vector families including the *Aedes, Culex* and the *Anopheles*.

1.4.1.5 *Aedes* mosquitoes

The *Aedes* mosquito genus was named after the Greek word ἀηδής, meaning sickening or disgusting, by the German entomologist Johanh Wilhelm Meigen in 1818 [221]. Since their emergence, the *Aedes* mosquitoes, with their recent spread to Europe and North America, can now be found on all continents across the globe with the exception of Antarctica [222]. *Aedes* are predominantly known for their impact on global health as several mosquito species of this genus spread clinically important human afflicting diseases such as Zika, dengue, yellow fever and Ross river arthritis. Historically, the species *Aedes aegypti* has been regarded as the most problematic arbovirus vector as it is the main vector of DENV and CHIKV. With the recent geographical expansion of *Ae.albopictus*, which while originally native to South East Asia can now be found on five continents, along with the mosquitoes co-evolution with arboviruses has resulted in a global threat matching that imposed by *Ae.aegypti* [222]. *Aedes* mosquitoes can be easily recognized by their distinctive black and white stripes on their legs and torso.

Aedes aegypti was originally named *Culex aegypti* by Carl von Linnaeus protégé, Fredrik Hasselqvist who discovered it in 1757 in Egypt [223]. It is believed to have evolved from its forest dwelling ancestor *Aedes aegypti formosus* and that its domestic behaviour originated in West Africa. The change in its behaviour provided the mosquitoes with benefits such as shelter when resting inside human habitats as well as suitable sites for egg laying in stagnant water supplies created by humans [224]. Several strains of *Ae.aegypti* have been used for research purposes including the "Liverpool" strain used in this study. Whilst this strain was originally acquired by the Liverpool school of

tropical medicine sometime between 1935 and 1938, the exact origin remains unclear. However it is believed that it was originally acquired from Sierra Leone [225].

1.4.1.6 *Culex* mosquitoes

Culex mosquitoes were given their name by Carl von Linnaeus meaning midge or gnat in Latin. These mosquitoes are known transmitters of several human pathogens such as WNV, JEV and St. Louis encephalitis virus [226] [227]. Whilst originally an African species, Culex mosquitoes spread to Norther Europe following the latest glaciation event [228]. Today, *Cx. pipiens* and *Cx. quinquefasciatus* can be found in the majority of human inhabited areas globally. *Culex* mosquitoes will feed on a variety of different hosts, with species ranging from birds to terrestrial mammals such as humans. This wide range of host species specificity exhibited by *Culex* mosquitoes is believed to have enabled the role of *Culex* as a bridging vector between viruses that primarily replicate in birds which, with the help of *Culex,* can cause occasional spillovers into human populations such as is the case for WNV. It is believed that a hybridization event between two groups of *Cx. pipiens*, one with a stronger preference towards bird biting with one preferentially mammal biting, may have led to the *Cx. pipiens* species we observe today [229] [230]. In contrast to the single domestication event of *Ae.aegypti* mosquitoes, *Cx. pipiens* was domesticated twice in two separate locations; once in North Africa resulting in the evolution of *Cx. pipiens* form molestus, and once in South East Asia resulting in the domesticated *Cx. quinquefasciatus* [231] [232].

1.4.1.7 *Anopheles* mosquitoes

Anopheles, like the *Aedes* genus, were named by the entomologist Johann Wilhelm Meigen in 1818. Like *Aedes*, Johann gave the genus a name with Greek origin, ἀνωφελής, meaning "useless" [233]. Since then, *Anopheles* mosquitoes have gained the reputation of being "the deadliest animal on the planet" due to their ability to transmit malaria. In terms of arbovirus transmission though, they are known to be inefficient vectors of most arboviruses, with the exception of one; the Alphavirus O'nyong 'nyong virus which is predominantly spread by *Anopheles gambiae*.

Interestingly, research investigating the virome of *Anopheles* mosquitoes, have discovered that these mosquitoes can harbour a large number of different medically relevant arboviruses such as the alphaviruses VEEV, WEEV, SINV and SFV as well as the medically relevant flaviviruses WNV and JEV [234]. To date, no studies have shown successful transmission of any of these viruses by *Anopheles* mosquitoes making it clear that *Anopheles* species cannot consistently transmit and maintain the circulation of arbovirus in the wild, with the exception of ONNV. This suggests that *Anopheles* mosquitoes are less competent arbovirus vectors than the culicinae mosquitoes. The reason behind this difference in vector competence remains a mystery however, especially in the species that are highly anthropophilic as in the case of *An.gambiae*. Several mechanisms to explain these differences in vector competence have been hypothesised including; differences in mosquito species tissue barriers; differences in the mosquito microflora; or molecular differences in the determinants required for viral replication [235]. There is some evidential support for the latter suggestion, as differences which have been observed in the sequence of nsP3 gene in ONNV is sufficient to allow for infection of *An.gambiae* under laboratory conditions [130]. However, this fact on its own is insufficient in explaining the reason behind the incapability of *Anopheles* in transmitting genetically-distinct arboviruses, suggesting another fundamental reason must exist preventing these mosquitoes from efficiently transmitting virus to the mammalian host. For the purpose of this thesis the *An.gambiae* strain of Kisumu was used. This strain was originally acquired from Kenya [236].

1.4.2 Ticks

Ticks are ectoparasites that are obligatory hematophagous. There are over 900 species of ticks documented which can roughly be divided into two separate families; the hard-bodied ticks *Ixodidae* or the soft bodied ticks *Argasidae*. Due to their hematophagous nature, tick bites have been associated with the transmission of numerous pathogens including several species of Rickettsia bacteria as well as a range of arboviruses such as Crimean-Congo haemorrhagic fever virus (CCHFV), tick borne encephalitis virus (TBEV) and African swine fever virus (ASFV). Originally however, tick borne diseases were discovered due to their impact on

livestock, such as the Louping ill virus which was associated with encephalitic disease in livestock such as sheep [237].

As an example of a tick-borne human inflicting arbovirus pathogen, TBEV is the cause of tick borne encephalitis disease (TBE) in humans. TBEV is a flavivirus that is primarily spread via *Ixodes ricinus* and *Ixodes persulcatus* [238]. Due to the virus dependency on its arthropod vector, its current geographical spread is restricted to Europe and Asia [239]. TBEV transmission cycle relies on the infection of small rodents where nymphs and larvae feed, whilst adult ticks will mainly feed on larger mammals such as roe deer with occasional spillover into humans via bites of infected ticks. In northern Europe high numbers of roe deer populations have enabled the maintenance of very high numbers of ticks. Declines in deer numbers since the 1990s have ultimately forced ticks to rely more heavily on rodents which has caused a significant increase in numbers of infected ticks [240].

Highly effective vaccines are available for TBEV with a success rate of approximately 95-99% [241]. However due to the need of repeated annual injections in combination with the relatively high cost of the vaccines, the success rates have been variable [242].

1.4.3 Culicoides Midges

Culicoides are biting midges in the family of Ceratopogonidae. Culicoides are 1-3 mm in size, and unlike mosquitoes they lack a proboscis which prevents them from biting through clothing efficiently. They are widely considered the most important vectors of arbovirus diseases in livestock with their ability to transmit viruses such as bluetongue virus (BTV) and Schmallenberg virus [243]. They can also transmit human pathogens such as Oropouche virus. Similar to mosquitoes, the Culicoides life cycle consists of instar stages, a pupae stage and the emerged adult. Adult midges typically live for 2-3 days and during their life span will only travel a maximum of a few kilometres.

BTV is an Orbivirus commonly spread by *Culicoides sonorensis* and *Culicoides imicola* which mainly affects sheep and occasionally also cattle, goats and other ruminants

[244]. Infection is commonly associated with acute disease accompanies by high mortality. Signs of disease include febrile illness, face swelling and tongue cyanosis. BTV was historically restricted to Africa and the tropics until its spread to Cyprus in the 1940s [245]. However, Europe has remained largely unaffected by BTV until very recently [246]. Since 1998, 8 out of the 24 existing BTV serotypes have spread across Europe causing severe economic damages in its path [247]. A recent outbreak in the Netherlands is estimated to have cost €175 million [248]. Effective vaccines are currently available which have the potential to help limit the spread of the disease if high enough numbers are vaccinated to create sufficient herd immunity [249].

1.4.4 Phlebotomine sand flies

The Phlebotominae are a subfamily of the Psychodidae, and they include several genera of hematophagous flies that are known transmitters of human pathogens such as leishmaniasis, bacteria and arboviruses. The majority of arboviruses spread by sandflies are Phleboviruses. Phleboviruses, which belong to the family of *Bunyaviridae*, spread by these sandflies include sandfly fever Sicilian virus, sandfly fever Naples virus, Toscana virus and Punta Toro virus [250]. The Phlebotomine sandflies are spread throughout the tropics and temperate zones with the Mediterranean being the most affected region in Europe [251]. Their geographical range has however increased in the last few years most likely due to climate change as they are restricted to areas with temperatures above 15.6^{o}C for a minimum of three months annually [252] [253]. Due to warming temperatures it is estimated that the geographical spread of the phlebotomine sandflies will increase further in the coming years spreading into northern Europe [254].

1.4.5 Vector control

Despite the medical importance of arthropod borne diseases there are very limited treatments and effective vaccines available as their development has proven to be difficult. The majority of these diseases can also be considered neglected tropical disease which means that a lack of resources have historically been set aside for the purpose of research and drug development. Therefore, in an attempt to curb disease, focus has inevitably been placed on methods of vector control. Numerous strategies

have been used collectively in an attempt to regulate vector numbers including the use of insecticides, genetic modifications of vector species, release of sterile male mosquitoes and the release of mosquitoes with reduced vector competence.

1.4.5.1 Insecticides

The use of insecticides for insect control is not a new concept with insecticide use dating back to ancient china. In Europe, over 200 years ago Armenian merchants were trading "Persian powder" which essentially contained Dalmatian pyrethrum used in the extermination of insects such as cockroaches, bedbugs, flies, and mosquitoes [255]. Insecticides used specifically in the purpose of disease prevention did not start until the discovery of DDT in the 1940s. Insecticide use has since proven its efficiency by helping in the eradication of malaria from both Europe and the US, controlling the spread of CHIKV in southern Europe as well as for combating WNV outbreaks in the US [256] [257]. Despite of the successes achieved by the use of insecticides, widespread emergence of insecticide resistances, as well as concern regarding bioaccumulation and potential negative impacts on human health, has led to a shift in the public's attitude towards the wide spread use of insecticides [258]. This led to a ban of the use of DDT in the US in the 1970s [259].

The use of insecticides remains the key method of combating arbovirus diseases today with mainly insecticides of pyrethroid class being used that ultimately leads to the emergence of pyrethroid resistant variants [260]. To combat this issue, WHO recommends rotation of the types of insecticides used to minimise risk of emerging resistance [261]. Another issue is the high cost associated with insecticide use [262]. Despite the benefits obtained by the use of insecticides in the fight against vector disease, the problems associated with their widespread use highlights the need for the development of alternative measures in combating this global issue [261].

1.4.5.2 Alternative methods of vector control

Due to the limitations of traditional insecticide use the development of alternative methods for vector control is underway; many of which are under review by the World Health Organization Vector Control Advisory Group (WHO VCAG). Such

alternatives include variations in application of insecticides such as the use of spatial repellents which are designed to deter mosquitoes from certain areas, thereby limiting human/mosquito contact; mosquito traps and insecticide treated surfaces and or sugar sources. In addition, more unconventional methods of vector control have been developed that do not rely on insecticide use.

Genetic modifications of mosquito species to confer virus resistance, lethal mutations or mosquito sterility are being assessed for efficacy of vector control. The use of sterile mosquitoes, commonly referred to as the SIT (Sterile Insect Technique), involves the breeding and irradiation of male mosquitoes with chemosterilants that ultimately leads to the generation of dominant lethal mutations in the sperm. The concept of SIT assumes that during release of these sterilized males in affected areas will result in a reduction in the ration of fertile wild type males with that of the released infertile males leading to a reduction, and eventually the elimination, of new offspring as the new males mate with wild females [263]. SIT has proven very successful in the eradication of insect pests in the past including the eradication of the tsetse fly *Glossina austeni* from Zanzibar[264]. Despite these successes, the use of sterilised males in mosquito vector control remains limited due to several limitations of the SIT including the high costs and effort associated with rearing the vast amounts of mosquitoes required for release as well as the infrastructure required to implement this technique in endemic countries [265] [266]. Additional problems associated with SIT is that current methods used are unable to successfully prevent somatic damages during the sterilization process which can lead to a reduction in the overall fitness of these mosquitoes, reduction in longevity and sexual activity making them less capable at outcompeting their wild counterparts [267].

More recently, an alternative technique has been developed, the Release of Insects carrying a Dominant Lethal (RIDL). RIDL utilizes genetic engineering for the introduction of a dominant lethal mutation which can only be supressed by an antidote. In the absence of the antidote, any larvae born to mosquitoes carrying the allele will die [268].

Finally, attempts have been made utilising the bacterial intracellular symbiont *Wolbachia* as a vector control technique [269]. *Wolbachia* which can be found in

several insect species is known to reduce the fertility of the infected insect whilst also interfering with viral replication in vector species. Indeed, studies have demonstrated resistance to infection by DENV and CHIKV of mosquitoes infected with *Wolbachia* [270] [271]. The first attempt at mosquito vector control utilising *Wolbachia* was initiated in Australia in 2011. Following 10 weekly releases of infected *Ae.aegypti*, infection rates of wild populations remained high [272]. It remains unclear however how *Wolbachia* inhibits virus infection and it remains to be seen whether viruses can/will evolve to circumvent this resistance [273].

1.5 Immunology

Whilst the scientific field of immunology can be considered a relatively new area of research, humanities earliest grasps of the concepts of immunity dates back to ancient Greece, where, during the plague of Athens, Thucydides noted that previous exposure to disease conferred immunity to the contraction of the same disease; or has he worded it, became "exempt" [274]. Active attempts to confer immunity to infectious diseases was common practice in China in the 10th century were the act of "variolation" was used to expose individuals to material from infected lesions of smallpox. This practice was also customary in the Ottoman Empire before it was picked up by the English [275]. The first vaccine was later developed by Edward Jenner in 1798 and mankind have been capitalizing on the concept of conferred immunity ever since with the continuous development of modern vaccines [276]. With the establishment of "germ theory" by Louis Pasteur and Robert Koch in the late 19th century, and with the uncovering of cellular immune responses and the existence of "humoral" immunity by Metchnikoff, Von Behring and Kitasato, the basic concepts of Immunology were defined by the start of the 20th century [277] [278].

Immune responses can be defined as defence mechanisms employed by the host in response to a potential invading pathogen, potentially harmful substances or in response to tissue damage. This is achieved by the highly specialised recognition system that is in place to aid in the detection of potential threats followed by the wide range of cellular and soluble factors responsible for preventing pathogen spread. The

system has to be carefully regulated to prevent excessive damage on the host [279]. The immune response can roughly be divided into two categories; the innate immune response and the adaptive immune response [280] [281].

1.5.1 Organs and tissues of the immune system

The immune system consists of a complex arrangement of organs and tissues interconnected by a large network of lymphatic vessels that make up the lymphatic system. These organs can roughly be separated into three categories; the primary, secondary and the tertiary lymphoid organs. The two main organs classified as primary include the bone marrow and the thymus. Bone marrow is a tissue of a sponge-like structure that resides within bones where the majority of immune cells will originate from haematopoietic stem cells and where the generation of B and T lymphocytes occurs, whilst the thymus is a gland located above the heart [282]. The secondary lymphoid organs include the lymph nodes, spleen, Peyer's patches, as well as mucosal tissues such as the nasal-associated lymphoid tissue, adenoids, and tonsils. Secondary lymphoid organs are mainly responsible for lymphocyte maturation and the collection of antigens for exposure to naive B and T cells which reside within segregated zones of secondary lymphoid organs. Thousands of lymph nodes are distributed around the body lining the lymphatic network with higher concentrations around the groin, neck, knees and armpits. Lymph will drain through the nodes carrying with it potential antigens that will activate lymphocytes. Activated lymphocytes will then exit the nodes to eventually end up in the blood stream. Unlike the lymph nodes, the spleen does not filter lymph but instead it filters blood. Similarly to the lymph nodes however exposure of lymphocytes to antigens in the spleen will lead to activation of said lymphocytes. Macrophages also reside within the spleen which are responsible for the removal of pathogens and other debris from the circulation [283]. Finally, the tertiary lymphoid organs consist of abnormal immune structures which are created at sites of chronic inflammation following the accumulation of immune cells at the site. Such structures can be observed in patients suffering from autoimmune diseases such as rheumatoid arthritis or atherosclerosis [284].

1.5.2 Innate immunity

The innate immune response is generally thought of as fast acting and activated by common pathogen associated molecular patterns. It occurs minutes following infection and makes up the hosts first line of defence against invading pathogens. In arbovirus infections the innate immune response is crucial for the suppression of viral replication and for the containment of viral dissemination across the body of the host. Via the use of knock out mouse models where important aspects of the innate immune response have been knocked out, such as the type I interferon response, studies have shown that the absence of type I interferon greatly affects severity and clinical outcome of infection. Susceptibility to arbovirus infections, such as SFV and WNV, was significantly increased in type I interferon knockout mice with all mice succumbing to disease [285] [286]. Similarly, lack of γδT-cells (innate immune lymphoid cell) resulted in an increase in severity of disease following WNV infection, suggesting a key role of innate cellular immunity in tackling infection [287]. This, in combination with the knowledge that many arboviruses have evolved to circumvent the mammalian innate immune response, stresses the significant impact the innate immune response has on arbovirus infections.

1.5.2.1 Acute innate immunity

In order for a host to become infected the pathogen has to successfully surpass the physical and chemical barriers that are in place to minimize infection risks at the epithelial and mucosal surfaces. These epithelial surfaces are stratified with tight junctions which help prevent anything from coming through whilst simultaneously possessing the ability to produce antimicrobial enzymes and peptides such as lysozymes, defensins and histatin which have the ability to digest bacterial cell walls [288] [289]. Another acute innate immune response is the complement system which consists of three separate complement pathways; the classical, the alternative and the lectin pathway. While the three pathways are initiated via different mechanisms they will eventually result in the lysis or phagocytosis of the invading pathogen as well as initiation of other inflammatory responses [290].

Interestingly mosquito borne viruses are able to completely surpass the epithelial host defences via the mechanism involved in their deposition into the skin. By hitchhiking on the mosquito vectors mechanism of saliva injection during blood feeding, the virus is injected directly in to the dermis alongside the mosquito saliva [291]. On the other hand, complement activation does appear to play a role in innate immunity against certain arboviruses such as in the case of WNV where inhibition of complement pathways resulted in earlier viral dissemination to the CNS in mice [292].

Following the successful bypassing of the earliest innate immune barriers, pathogens then encounter a range of immune cells and cytokines that are responsible for the initiation of the induced innate immune response. This induction is reliant on the recognition of pathogens or pathogen associated molecules by specific receptors.

1.5.2.2 Pattern recognition receptors

In order for the host system to mount a successful immune response to reciprocate against an invading pathogen, it has to be able to recognise it. In 1989, Janeway refined and proved a long-postulated theory that this recognition is achieved via the use of highly specialized receptors now commonly referred to as pattern recognition receptors (PRRs). These receptors recognise specific molecules commonly associated with foreign pathogens or damage including specific glycans, proteins and nucleic acids. Molecules recognized by PRRs are known as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs).

Toll like receptors

Toll like receptors were the first PRRs to be identified, and to date, 10 different TLRs have been found in humans whilst mice have 12. Humans have lost TLR11, TLR12 and TLR13 whilst TLR10 is non-functional in mice due to a retroviral gene insertion event. Some of these receptors are located on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10) whilst the remaining (TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13) are located intracellularly [293]. The localization of TLR receptors are important as it tends to affect the patterns they recognize, with membrane bound receptors mainly recognizing surface components of invading pathogens whilst intercellular TLRs recognize foreign nucleic acids. TLRs all share

a common structure with leucine rich repeats forming a horseshoe resembling ectodomain responsible for PAMP recognition, a transmembrane domain as well as a Toll/IL-1 receptor (TIR) domain which is involved in downstream signalling. Whilst sharing similar architecture, each TLR has evolved to specifically recognise conserved structures expressed by different pathogens such as: TLR4 recognizes bacterial lipopolysaccharide (LPS) [294], TLR5 recognizes bacterial flagellin [295], TLR7 recognizes single stranded viral RNA, TLR9 recognizes viral CpG RNA whilst TLR2 recognizes a wide range of virus, bacteria, fungal and damage associated PAMPs [296] [297].

Following recognition of PAMPs or DAMPs the TLRs recruit TIR domain-containing adaptor proteins such as MyD88, TIRAP, TRIF and TRAM that activate signalling pathways that trigger the initiation of specific immune responses. These signalling pathways can broadly be divided into two separate groups; MyD88 dependent and TRIF dependent pathways. MyD88, which was the first adaptor protein to be discovered, is used by all TLRs with the exception of TLR3. Recruitment of MyD88 results in the transcription of pro-inflammatory cytokines via the activation of NF-κB and mitogen-activated protein kinases (MAPKs). TRIF is recruited by TLR3 and 4 for the activation of the transcription factor NF-κB and IRF3 which ultimately upregulates pro-inflammatory cytokines as well as type I interferon. In contrast, the function of adaptor proteins TIRAP and TRAM is to aid in the recruitment on MyD88 or TRIF to their respective receptors [298]. Alphavirus and flavivirus recognition has been suggested to occur via TLR3, TLR7 and TLR8 [299] [296].

RIG-I like receptor family

The RIG-I like receptor family is another PRR associated with viral detection which consists of the retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and laboratory of genetics and physiology-2 (LGP-2). These receptors which are located within the cytoplasm, specialize in the detection of double stranded RNAs most commonly associated with viral genomes or genomic intermediates during viral replication. All three contain an RNA helicase core, responsible for the recognition of dsRNA and attached to the core is a zinc-binding C-terminal domain (CTD). Located at the N-terminus of RIG-I and MDA-5 are

caspase activation and recruitment domains (CARDs) which are used in signalling [300]. However LGP-2, which lacks CARDs but retains its ability to recognise dsRNAs, appears to play a key role in acting as an accessory protein responsible for the regulation of RIG-I and MDA-5 and the aiding of their association with PAMPs [301].

RIG-I distinguishes between host RNA and viral ssRNA by recognizing 5′ triphosphorylated uncapped ssRNAs commonly found in viral genomes in contrast to the capped ends commonly found in the host, as well as by recognizing the short dsRNA bi products of viral replication (typically smaller than 1kb). In contrast MDA-5 does not appear to recognize uncapped ends but instead distinguishes viral RNA by its size by binding to larger strands (over 2kb), as long dsRNA is not normally found in the host [301]. Following binding to the receptors, the receptors are activated by undergoing a conformational change which will allow for the interaction with the mitochondrial antiviral signalling protein (MAVS) which will eventually lead to the activation of IRF3/IRF7 and the subsequent transcription of type I interferon. MAVS will also activate NFkB via the recruitment of TRADD, RADD and caspase-8 and 10.

Studies have shown that MDA-5 and RIG-I are able to detect different viruses with MDA-5 recognizing viruses such as encephalomyocarditis virus (EMCV) whilst RIG-I can recognize viruses such as JEV and RSV. Both MDA-5 and RIG-I have been found to be implicated in the recognition of DENV and WNV [302].

Nucleotide oligomerization domain (NOD) like receptors

Nucleotide-binding oligomerization domain (NOD) like receptors (NLRs) are mammalian PRRs located in the cytoplasm of epithelial and immune cells which are able to detect a range of bacterial motifs including flagellum, LPS as well as iE-DAP. Humans have 22 different NLRs whilst mice possess 34. NLRs are made up of a central NOD domain, an N-terminal homotypic protein-protein interaction domain as well as leucine-rich repeats (LRRs) located at the C-terminus which are involved in PAMP recognition and binding [303].

Additional cytosolic pattern recognition receptors

Lastly there are mammalian PRRs specialised in the detection of intracellular pathogen associated DNA, known as cytosolic PRRs. These PRRs, which were discovered very recently via the observation that TLR9 deficient cells retained their ability of producing type I IFN in response to dsDNA [304]. These will not be discussed in any further detail in this thesis.

1.5.2.3 Cells of the innate immune response

Innate immune cells play a key role in facilitating the detection of PAMPs, carrying out effector functions such as phagocytosis as well as helping prime and guide adaptive immune responses. Immune cells are made up of leukocytes, which are white blood cells, which derive predominantly from pluripotent hematopoietic stem cells in the bone marrow [279]. There are exceptions of certain subset cell types such as some tissue-resident macrophages and Langherans cells that instead derive from the embryonic yolk sac and the foetal liver [305]. Hematopoietic stem cells derived from the bone marrow can differentiate into two separate differentiation pathways including the common lymphoid progenitor cells that give rise to either lymphocytes such as T-cells and B-cells, lymphoid derived dendritic cells and natural killer cells (NK cells) or the common myeloid progenitor cells that can give rise to the granulocytes, monocytes, myeloid derived dendritic cells, mast cells and macrophages [306]. A brief descriptions of key innate immune cells will be outlined here with cited examples illustrating their individual roles in arbovirus infections.

Granulocytes

Granulocytes are a group of immune cells originating from the bone marrow derived from myeloid progenitor cells characterizes by the dense granules located in their cytoplasm. There are three different types of granulocytes; neutrophils, basophils, eosinophils which can be distinguished from each other using haematoxylin and eosin staining where neutrophils are pink, basophils are blue and eosinophils are red. Eosinophils are involved in the host protection against parasitic infections via the release of toxic granule proteins, reactive oxygen species (ROS) and pro-inflammatory cytokines. Basophils, which are the only histamine containing circulating leukocyte, will release leukotrienes, histamine and cytokines in response

to pathogens whilst also being able to directly kill parasites that have been tagged by antibodies [307].

Neutrophils, which are the most abundant leukocyte, can be considered one of the most important cells of the innate immune response. Originating from the bone marrow where they are produced in large numbers of approximately 10^{11} cells a day in humans, these cells will eventually be released into the blood stream following their maturation via the increase of the expression of the chemotactic cytokine receptor CXCR2 (further discussed in section 1.5.4.7) [308]. Neutrophils can leave the blood and migrate into inflamed tissues following chemotactic cues. They are considered the "first respondents" to sites of inflammation. Following activation, neutrophils can perform several antipathogenic activities including phagocytosis, degranulation, and the release of a range of chemotactic and inflammatory cytokines which in turn can attract more neutrophils as well as monocytes, dendritic cells and T-cells [309].

In terms of the innate immune response to arbovirus infections, neutrophils play different roles in the outcome of infections depending on the virus. During infection with mosquito borne viruses, studies have shown that the act of mosquito biting accompanied with the simultaneous injection of saliva by the mosquito into the skin, induces a rapid CXCL2 driven recruitment of neutrophils to the bite site as early as 90min post bite with a peak at 180min post bite. In turn, recruited neutrophils release a range of inflammatory and chemotactic factors that facilitates the migration of other immune cells to the site of inflammation. Some of these newly recruited immune cells have been shown to be susceptible to infection by a number of mosquito-borne viruses including SFV, DENV and BUNV suggesting that this immune response to mosquito biting/mosquito saliva indirectly aids arbovirus replication [95] [310].

Mast Cells

Mast cells are hematopoietic myeloid cells originating from the bone marrow. Progenitor mast cells migrate to mucosal and epithelial tissues spread across the body where cytokines and specific stem cell factors cause them to differentiate into mast

cells. Mast cells are involved in the regulation of a range of functions including vasodilation, angiogenesis, vascular homeostasis as well as regulating the action of a range of different cells such as dendritic cells, macrophages, T and B cells, endothelial and epithelial cells, eosinophils as well as fibroblasts. Activation of mast cells can occur in response to a variety of stimuli which can have different outcomes. Most frequently, activation leads to mast cell degranulation most notably in response to antigen-bound IgE crosslinking of IgE molecules bound to FcεRI located on the cell surface of the mast cell. Degranulation can also occur in response to activation of complement, certain toxins as well as neuropeptides [311] [312]. Degranulation leads to the release of bioactive amines, proteoglycans, proteases and importantly histamine. In addition to degranulation, activation of mast cells leads to the synthesis of compounds such as leukotriene C4, prostaglandin D2 and platelet-activating factor in addition to specific cytokines and chemokines. Mast cells utilize a number of different PRRs that aid them in the detection of pathogens including TLR1-7, TLR9, RLR's and NLR's. Upregulation in the transcription of inflammatory cytokines occurs following binding of PAMPs to the PRRs including TNF-α, IL1β, and IL-6 [312] [311].

Whilst the response of mast cells to parasitic and bacterial infections is well characterized, the role of mast cells in antiviral responses is much less explored. However, studies have shown that mast cell activation and subsequent degranulation occurs in response to the detection of DENV in humans, mice and non-human primates [313]. Furthermore, activation of mast cells via the detection of DENV, results in the upregulation of antiviral immune pathways such as the interferon response [314]. It has also been suggested that host responses against mosquito saliva can include activation of mast cells [315].

Monocytes

Monocytes belong to a family of myeloid cells called the mononuclear phagocyte system which consists of monocytes, macrophages and dendritic cells (discussed in more detail in the following sections). Monocytes derive from myeloid progenitor cells in the bone marrow under the influence of transcription factors such as PU.1 [316]. Chemokine receptor CCR2 direct their release into the blood stream where

the majority of monocytes will remain throughout their lifespan, with only a few migrating into tissues where they will differentiate into macrophages to replenish resident cell populations. Maintenance of monocytes in the blood occurs via the aid of several cytokines, the most important being macrophage colony stimulating factor (M-CSF). Monocytes can be divided into two separate groups; the migratory monocytes and the patrolling monocytes. In mice, these subsets of monocytes can be distinguished by the expression of Ly6c which is expressed on the surface of migrating monocytes but not on the patrolling monocytes. Ly6c^{hi} cells can be recruited to sites of inflammation where they rapidly differentiate into macrophages, dendritic cells or remain as monocytes with antigen-presenting abilities. Ly6c^{lo} monocytes on the other hand "patrol" blood vessel walls where they "search" for tissue damage and pathogens and they are directly involved in tissue repair and other endothelial cell-supporting functions.

In terms of monocytes and arbovirus infections, monocytes appear to play different roles during infections depending on the virus. For example, studies with CHIKV have shown that monocytes/macrophages are susceptible to viral infection in infected joints. With the persistence of infected monocytes in the joints, and their continuous release of inflammatory cytokines, chronic inflammation occurs resulting in arthralgia [317]. Similarly, monocytes have been established to be susceptible to infection with DENV and ZIKV [318] [319]. In contrast, monocytes have also been suggested to play a protective role against arboviruses. Utilizing monocyte deficient mice, one study demonstrated higher viral titers of CHIKV and RSS in both the blood and distal organs. In this model, monocytes inhibited infection by triggering a MAVS-dependent upregulation of type I IFN [320]. Similar conflicting roles for monocytes in arbovirus infections have been found in WNV infections where one study observed enhanced WNV infection accompanied by reduction in survival in mice with defective Ly6C^{hi} monocyte recruitment, whilst other studies have shown that blocking monocyte migration to the CNS led to improved survival [321]. These inconsistent findings highlight the need for further research into whether monocytes play an overall protective or harmful role during arbovirus infections.

Macrophages

Macrophages were first described by Elie Metchnikoff in 1884. Macrophages, which originates from the words μακρός (large) and φαγείν (to eat), are large, phagocytic leukocytes residing in almost all bodily tissues. They derive from the embryonic yolk sac and foetal liver during embryonic development and can be continuously replenished by the differentiation of Ly6c^{hi} monocytes [322]. Being one of the most efficient cell types at phagocytosis, macrophages are highly specialized in the removal of cellular debris, apoptotic cells as well as pathogens which are degraded in the phagolysosome.

Macrophage activation occurs following the recognition of PAMPs or DAMPs by the PRRs. Originally, macrophages were believed to differentiate into either classically activated macrophages (M1), alternatively activated macrophages (M2) or regulatory macrophages. However, more recent studies suggest that this division of macrophages is over simplistic as macrophage phenotypes have been found to be mixed and context dependent [323]. Due to the irrelevancy of this conundrum in the context of this thesis, macrophage differentiation shall not be discussed in more detail.

Macrophages can be activated by various stimuli. During infection with a pathogen, macrophages are activated by a combination of signals including IFNγ produced predominantly by natural killer cells (discussed below) and T helper 1 cells (TH1 cells) (discussed in section 1.5.3.1), as well as tumour necrosis factor and IFNβ, both of which are produced by the macrophage via MyD88 or TRIF dependent pathways. Activation of macrophages via these signals will result in the priming of the macrophages in the production of superoxide anions, oxygen and nitrogen radicals to aid in the killing of bacteria, as well as in an increase in the production of specific pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, IL-18, as well as the secretion of inflammatory chemokines such as CXCL10. The production of these specific pro-inflammatory chemokines and cytokines will recruit more monocytes and other immune cells to the infection site. These types of macrophages, also commonly referred to as M1 macrophages, can also produce matrix-metalloproteinases (MMPs) that aid in the process of phagocytosis as they are utilised

for the degradation of extracellular matrix. Finally, these cells can act as a bridge between the innate and adaptive immune response as they will upregulate major histocompatibility class II proteins, enabling antigen presentation on the cell surface and the activation of primed lymphocytes [324].

Alternatively, macrophages can adopt the alternative macrophage phenotype, also commonly referred to as the wound-healing phenotype which occurs via the activation of macrophages by IL-4 and IL-13 secreted by T helper 2 cells (TH2 cells) and polymorphonuclear cells. These wound-healing macrophages secrete minimal amounts of pro-inflammatory cytokines but produce IL-10 and TGFβ as well as extracellular matrix components used for wound healing. Studies have shown that these cell types play a protective role during parasitic infections via the production of chitinase and chitinase-like molecules such as YM1 and YM2 which could potentially degrade the chitin surfaces of some parasites. However evidence supporting this hypothesis remains inconclusive.

Macrophages can also adopt a regulatory function. These regulatory macrophages are activated by a range of factors including prostaglandins, apoptotic cells, dopamine, histamine and IL-10. Once activated they secrete large amounts of the anti-inflammatory cytokine IL-10 and will downregulate the expression of IL-12 [325]. In general, macrophages are known for their high degree of plasticity which allows them to respond to specific environmental ques and quickly adapt their behaviour.

In terms of infection with arboviruses, macrophages have been found to play either protective or pathogenic roles during the course of infection. Skin resident macrophages are ideally situated in close proximity to the initial viral inoculation site where they can quickly detect invading pathogens with their wide range of cytosolic, extracellular and endosomal PRR's and initiate an antiviral response. However, studies have shown that macrophages are one of the key targets for infection by the majority of mosquito borne viruses which could explain the mechanism behind the local replication of arboviruses in the skin [95, 326]. There is also evidence for persistent infection of macrophages in tissues resulting in chronic inflammation. In CHIKV infections, long term infection of joint resident macrophages have been

associated with the pathogenesis of chronic arthralgia and/or arthritis, whilst long term infections of muscle resident macrophages following RRV infection has been linked to chronic pain and muscle damage triggered by uncontrolled cytokine cascades [327].

Dendritic cells

Dendritic cells (DCs), which were originally described by Ralph Steinman in 1973, were named after the branch like structures they develop during specific developmental stages after the Greek word δένδρον meaning tree [328]. Today, DCs are known to be professional antigen presenting cells which means that they are highly skilled at detecting and capturing antigens which will be presented on the cell surface. As DCs are a heterogenous population, they can be sub-divided into groups based on their anatomical location, their origin, and based on their main functions. Dendritic cells derive from either embryonic progenitor cells which will give rise to resident DCs including Langerhan cells (discussed further in section 1.6.1) or from specific myeloid derived hematopoietic stem cells known as the macrophage/DC progenitors which will give rise to conventional DCs as well as plasmocytoid DCs. Ly6Chi monocytes can also differentiate into DCs following tissue migration. These sub-categories of DCs, are commonly referred to as conventional DCs (cDCs) and nonconventional DCs, which are made up of plasmocytoid DCs (pDCs) and monocyte derived DCs. The majority of DCs are derived from the bone marrow[329].

cDCs are highly migratory as they; patrol barrier tissue environments searching for pathogens; migrate from peripheral tissues to lymph nodes to help maintain immune self-tolerance; and separately shuttle between the T-cell and B-cell zones of lymphoid organs. Immature cDCs are competent phagocytic cells, an ability that is significantly reduced following pathogen sampling and the subsequent cell maturation. Mature cDCs present processed antigens derived from pathogens on MHC II, migrate to the T-cell zones of lymphoid organs where they secrete cytokines such as IL-12. Naïve T lymphocytes will then be exposed to the antigen carried by the cDCs as well as DC-derived cytokines resulting in their subsequent activation [330]. This highly migratory nature of cDCs may potentially indirectly aid arbovirus

dissemination, as cDCs, similarly to macrophages, have been found to be primary targets of virus infection. Arboviruses known to infect cDCs include RRV, EEEV, VEEV, CHIKV and DENV [331] [332]. In addition, there is evidence showing that DENV infected DCs are incapable of inducing an antiviral response, as well as unable to prime T-cells. This suggests that some arboviruses have evolved immune supressing mechanisms that inhibit the DC innate immune functions [333] [334].

Plasmocytoid dendritic cells are notably known for their ability to rapidly secrete large quantities of type I interferon in response to the detection of viral RNA through TLR7 signalling. Whilst originally located in the bone marrow, pDCs will migrate to peripheral tissues in response to chemotactic cues that activate CCR2. TLR7 signalling leads the subsequent release of IFNα, IFNβ and IFNλ [335] [336]. pDCs may also act as antigen presenting cells that can recruit leukocytes via the secretion of chemokines. The role of pDCs in arbovirus infections remains unclear, as limited research has been conducted on the topic. However, research on DENV infections have discovered that infections with DENV lead to an enhanced activation of pDCs with higher activation correlating with better clinical outcome in patients suffering from severe dengue fever [333].

Innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently discovered cell type that can be considered the innate counterparts of T cells (discussed in further detail in section 1.5.3.1) which, in contrast to T cells, lack antigen receptors[337] [338]. These ILCs, which are derived from bone marrow lymphoid precursor cells can be split into two separate groups; the cytotoxic ILCs and the non-cytotoxic ILCs. The only known cytotoxic ILC are the Natural Killer cells (NK cells) which were originally identified in 1975 [339]. As suggested by their name, NK cells are "naturally" cytotoxic that do not require previous antigen exposure unlike cytotoxic T cells. Instead, they are filled with cytotoxic granules which are released following their binding to specific target cells. [340]. Similarly to many other innate immune cells that have been discussed previously, ILCs can play either a protective or pathogenic role during arbovirus infection depending on the species and cell tropism of the invading virus. Due to the constraints of this thesis, ILCs will not be discussed in any further detail.

1.5.3 Adaptive immunity

In contrast to the rapid unspecific responses of the innate immune response, the adaptive immune response in naïve animals offers a delayed, but specific, response to invading pathogens that can take several days, following initial encounter with a pathogen, to develop. Importantly adaptive immune responses enable the acquisition of immunological memory to infection. This section will cover the basic mechanisms of the adaptive immune response, the main cell types involved in adaptive immunity as well as the role of the adaptive immune response during arbovirus infections.

1.5.3.1 Cells of the adaptive immune response

Adaptive immune responses can be both humoral and cellular and it is dependent on the clonal selective process of specific B-cells and T-cells. B-cells and T-cell are both lymphocytes that are derived from multipotent hematopoietic stem cells. These cell types will develop into multiple distinct cellular lineages whilst located in separate anatomical compartments within the bone marrow. Developed B-cells and T-cells can then undergo recombination of their antigen receptor genes which will produce new antigen receptors that can bind to essentially any antigen. During this procedure, the T-cells and B-cells that efficiently recognize a foreign antigen, and MHC molecules in the case of T cells, are selected and cloned to enable highly specific responses to pathogens. B-cells and T-cells that have encountered antigen can remain as memory cells in the body, in order to offer a swift and a significantly more efficient response to the same specific pathogen during any subsequent infections. This phenomenon is known as immunological memory which can be viewed as the hallmark of the adaptive immune response.

T-cells

T-cells are bone marrow derived cells which migrate to the thymus where they undergo maturation and selection before they are shuttled to remote tissues. Whilst in the thymus, T-cells with the classical α/β T-cell receptor (TCR) undergo stages of selective pressures. The first selection stage comprises of the positive selection of T-cells with a receptor that can recognize self MHC, where, T-cells with receptors that are unable to do this undergo apoptosis. Approximately 95% of all developing T-

cells will die at this stage. The second selection stage of T-cell maturation involves negative selection during which, T-cells with receptors with high affinity towards self MHC undergo apoptosis. These selective pressures result in antigen specific T-cells that can be divided into two lineages based on their TCR; CD4+ and CD8+ cells where CD4+ T-cells where selected on MHC class II molecules whilst CD8+ cells where selected with MHC class I. Both lineages will migrate into the circulation following maturation as antigen naïve cells .

CD4+ T-cells make up the majority of T cells within the human body. CD4+ T-cells are also commonly called T helper cells due to their main function as producers of specific cytokine signaling targeted to other immune effector cells as well as in order to aid in the production of antibodies of high affinity by B-cells (see section below). In terms of arbovirus infections, research has shown that during CHIKV infection, Th1 T-cells are the main producers IFNγ by producing approximately 50% of all IFNγ detected during acute CHIKV infections [341] [342]. CD8+ T-cells are circulating T-cells who's main function is the killing of infected cells. Due to this, CD8+ T-cells are commonly referred to as cytotoxic T-cells. Cytotoxic T-cells have an antigen specific TCR, with high affinity to MHC class I molecules (primarily derived from cytosolic proteins) which are displayed on the cell surface of infected cells [343]. During arboviral infections, CD8+ T-cells have been shown to play a key role in viral clearance by attacking cells infected with the virus. For example, it has been established that CD8+ T-cells are essential for viral clearance during acute phase CHIKV infection [344]. Additionally, CD8+ T-cells appear to be responsible for viral clearance in the CNS during avirulent SFV infections [80]. In contrast, cytotoxic T-cells can also play a pathogenic role during arboviral infection. Infection with SFV for example, can lead to demyelination in the CNS caused by CD8+ T-cell activity [345].

Finally, there are a small subset of T-cells, most of which do not express neither CD4 nor CD8, known as the γδ T-cells. These T-cells make up less than 5% of lymphocytes in human tissues, although they are more concentrated within the gastrointestinal epithelium. During alphavirus infection with SFV and CHIKV, γδ T-cells contribute to the clearance of infection as research utilising γδ T-cells knock

out mice observed enhanced infection with an increase in inflammation-mediated oxidative damage in the joint [346] [347].

B-cells

B-cells are antigen presenting lymphocytes who's main function is playing a key role in the humoral immunity via the production of antibodies whilst also being able to produce pro-inflammatory cytokines. B-cells, which are bone marrow derived cells, will undergo several developmental stages including pro-B, pre-B and immature B. During this development B-cells will acquire antigen specificity [348]. Mature B-cells express antigen specific B-cell receptors (BCR) which consist of an immunoglobulin as well as CD79a/b adaptor proteins [349]. Similarly to T-cells, B-cells will undergo selective pressures during maturation and the surviving cells will exit the bone marrow and migrate to secondary lymphoid organs such as the spleen, where they will develop into marginal zone B-cells or follicular B-cells [348]. Following B-cells encounter with antigen, in combination with exposure to specific cytokine stimuli, B-cells will become memory B-cells which can be activated in the future, or it can turn in to long lived plasma cells which undergo rapid clonal expansion and which possess the ability to produce large amounts of antibody. Further signalling via B-cell interaction with CD4+ T-cells can aid in the production of antibodies of higher affinity. This interaction allows B-cells to switch their production of antibodies of the immunoglobulin classes IgM and IgD to other isotypes such as IgG, IgA and IgE via a process known as class switching [350]. Class switching is partially regulated by cytokines as expression of IL-4 and IL-13 promote switching to IgE for example [351]. Simultaneously, point mutations occur in the heavy and light chain variable regions of the immunoglobulin via a process known as somatic hypermutation (SHM). SHM is responsible for affinity maturation of antibodies which leads to the production of high affinity antigen binding antibodies [352]. These highly specific antibodies will aid the body's immune response by either direct neutralization of invading pathogen, marking pathogen for phagocytosis by other immune cells or by providing the scaffold required for the activation of the complement system. For example, IgE antibodies are highly associated with hypersensitivity reactions triggered when mast cells coated with IgE-Fc receptor complexes recognise antigen

[353], whilst IgG antibodies, the most common antibody isotype in the body, are known to directly neutralize viruses and toxins [354].

In terms of B-cells role in the immune response towards arbovirus infections, there is evidence that B-cells can either be protective or contribute to pathogenicity. For example, studies have shown that IgM and IgG antibodies activated in response to SFV A7(74) in mice were protective [345]. In contrast, B-cells and B-cell derived antibodies are speculated to be implicated during pathogenic antibody-dependent enhancement of dengue virus infection. During this process, antibodies derived from a previous DENV infection will bind to DENV of another strain during a secondary infection which results in the subsequent infection of monocytes via the antibody directed binding of virus particles to Fc receptors located on monocytes [355].

1.5.4 Cytokines and chemokines of the innate and adaptive immune response

Cytokines and chemokines are small molecular weight proteins which play a fundamental role in the signal communication of both the innate and the adaptive immune response in addition to contributing to the regulation of cell migration and cell positioning during vertebrate development. Whilst cytokines were originally believed to originate exclusively from lymphocytes, it is now known that cytokines and chemokines are secreted by a wide range of cells. Cytokines can act in an autocrine, paracrine or endocrine manner by binding to cell surface receptors, which will in turn initiate a plethora of effects depending on the target cell type. Based on their structure and main function, cytokines can be sub-divided into different groups. This section will briefly cover key cytokines and chemokines implicated in the innate and adaptive immune response with particular focus given to the type I interferon family due to their role in innate immune responses to viruses.

1.5.4.1 IL-1 Family

Interleukin 1 (IL-1), is a family of proinflammatory cytokines that are mainly associated with innate immune responses. The IL-1 family is made up of IL1α, IL1β, IL-1 receptor antagonist (IL-1RA), IL-18 as well as IL-33. During an inflammatory response, IL-1 cytokines are secreted by a wide range of different cell types. IL1α

and IL1β are produced by cell types such as monocytes, neutrophils, macrophages, hepatocytes, as well as tissue resident macrophages located throughout the body [356]. IL1β is one of the members of the IL-1 family that are initially synthesized as a precursor of its active form, commonly referred to as pro-IL1β, which must be subsequently cleaved in order to become activated. Cleavage of the IL1β precursor is carried out by either the IL-1-converting enzyme (ICE), or caspase-1, the activation of which is mediated by a multiprotein cytosolic complex known as the inflammasome. The inflammasome, which was first described in the early 2000s, refers to a group of different cytosolic complexes which are all activated by different PAMPs and DAMPs [357]. However, due to the complexity of the inflammasome and because it does not feature prominently in this thesis, it shall not be discussed in further detail. Secretion of both IL1α and IL1β lead to the activation of neutrophils and macrophages as well as T and B lymphocytes [358]. When bound to IL-1 receptors, these cytokines will also activate the secretion of additional cytokines including IL-6 and TNF, and can help induce Th17 adaptive immune response [359].

1.5.4.2 Common γ Chain Family

Cytokines that bind to cytokine receptors containing the common γ chain are known as the common γ chain family of cytokines. This family consists of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. These cytokines are mainly implicated in the differentiation and activation of lymphocytes whilst also functioning as growth factors for various leukocytes. IL-2, which is mainly secreted by activated T-cells, B-cells, NK-cells and neutrophils, causes cell proliferation whilst also promoting Th1, Th2 and Th9 differentiation. IL-4, which is mainly involved in immune responses against helminths as well as allergy, promotes differentiation of B-cells and class switching to IgE. It also promotes differentiation of Th2 and Th9 as well as triggering proliferation of tissue resident macrophages. IL-7 is a hematopoietic factor responsible for the development and homeostasis of T-cells whilst IL-9 promotes the proliferation of mast cells. Lastly, IL-15 is required for the development and survival of NK cells whilst IL-21 promotes the differentiation of B-cells to plasma cells and influencing immunoglobulin production [360].

1.5.4.3 Common β Chain family

Cytokines that bind to a cytokine receptor that contain the cytokine-specific α chain as well as the common β chain are known as the common β chain family. This family consists of IL-3, IL-5, and GM-CSF. As a family, these cytokines are mainly responsible for the differentiation and regulation of myeloid cells with a particular role in the allergic response. Specifically, IL-3 and GM-CSF are implicated in the development of immune cells such as macrophages, mast cells and polymorphonuclear cells whilst IL-5, which is derived from T-cells, is responsible for promoting the growth and differentiation of B-cells [361].

1.5.4.4 Tumour Necrosis Factor Superfamily

The tumour necrosis factor superfamily (TNFSF) consists of over 20 cytokines and close to 30 cytokine receptors. These receptors and corresponding cytokines make up signalling pathways involved in processes such as immune regulation, cell proliferation and differentiation as well as apoptosis. The best described tumour necrosis factor (TNF), is TNFα. TNFα, which is produced by macrophages and T-cells, can be secreted either as a soluble molecule or in a membrane bound form. It binds to TNF receptor TNFR1 and TNFR2 where TNRF1 is expressed on the cell surface of leukocytes as well as on stromal cells, whilst TNFR2 is expressed on B-cells and T-cells. Binding of TNFα to its receptor triggers the activation of a signalling cascade that leads to NFkB activation or apoptosis initiation [362].

1.5.4.5 Interferons

In 1957, two scientists named Isaacs and Lindenmann discovered a molecule that they observed to be capable of interfering with viral replication. Due to this observation, the molecule in question was named interferon [363]. Today we know that there are different types of interferon (IFN) molecules which can be split into three groups: type I IFN, type II IFN and type III IFN. Interferons can be secreted by the vast majority of cells in the human body in response to an invading pathogen.

In this section, each type of interferon will be discussed with a description of their key roles in the innate and adaptive immune response.

Type I Interferons

Type I IFN family comprises of IFNα and IFNβ, as well as the more poorly described IFNδ (only present in ruminants), IFNε, IFNζ (only present in mice), IFNκ, IFNν (only present in cats), IFNτ (only present in porcine species) and IFNω. Type I IFNs are key players in the mammalian immune response against viruses [364]. Typically activated in response by PRRs in response to specific PAMPs, type I IFNs will bind to the homodimeric interferon α receptor (IFNAR) located on the majority of leukocytes and stromal cells. Binding facilitates signalling of the Janus kinase -signal transducer and activator of transcription (JAK-STAT) pathway which leads to the dimerization, translocation (to the nucleus) and binding of specific molecules to IRF9 which will result in the formation of the interferon stimulated gene factor 3 complex (ISGF3). ISGF3 will bind to interferon stimulated response element (IRSE) inside the nucleus resulting in the subsequent activation of the transcription of numerous interferon stimulated genes (ISGs) (see section below) [365]. IFNα, unlike the other type I IFNs, can be further subdivided into 13 partially homologous subtypes of IFNs (14 distinct subtypes in mice). Despite their highly homologous nature (70-99% homology), each subtype has a different affinity for IFNAR [366].

Despite our limited understanding of the functions of many of these IFNs, it remains clear that type I IFNs are critical in facilitating an effective antiviral response. In terms of arboviral disease, recent studies with SFV have shown that IFNAR knockout mice succumb to infection within 24h post infection in comparison to wild type mice where lethality was delayed for a minimum of another 3 days [286]. In fact, all strains of SFV are lethal in KO mice, including the A7(74) strain which is avirulent in WT mice [93].

Type II Interferons

The type II IFN family consists of only one IFN type; namely IFNγ. IFNγ, which is secreted by NK cells, Th1 cells and CD8+ T-cells and interacts with the interferon gamma receptor (IFNGR) present on the majority of nucleated cells [367]. IFNγ has a range of activities including macrophage maturation and stimulation of macrophage phagocytosis, enhancing antigen presentation molecules of both MHC I and MHC II, induction of apoptosis of infected cells, activation of NK cells, stimulating class switch recombination of B-cell immunoglobulins to IgG and IgE as well as triggering differentiation of CD4+ T-cells into Th1 cells. The versatile activities displayed by IFNγ, demonstrate its importance in facilitating a robust antiviral response whilst also providing a bridge between innate and adaptive responses. Studies utilising IFNγ knockout mouse models observed significant increase in viraemia following WNV infection in comparison to wild type mice, with virus disseminating earlier to the CNS in the knockout mice suggesting that IFNγ plays a protective role during WNV infection [368].

Type III Interferons

Originally discovered in 2003, the type III interferon family consists of IFNλ1, IFNλ2, IFNλ3 and IFNλ4. Whilst functionally similar to type I IFNs, for signalling, it interacts with a different heterodimeric receptor complex which consists of the IFN-λR1 chain and the IL-10R2. Interaction of IFNλ with their receptor results in upregulation of similar antiviral ISGs as Type I IFNs, as well as causing inhibition of epithelial and glioblastoma cell proliferation [369].

1.5.4.6 Interferon Stimulated Genes

Detection of the majority of viral infections via PRR will result in the production of interferons which will subsequently lead to the secretion of specific interferon stimulated genes (ISGs) which can exhibit antiviral effector functions. As mentioned in the section above, Type I and type III interferons play an important role in antiviral immunity, and both will signal via the JAK-STAT pathway in response to

virus infections which will eventually result in the transcriptional regulation of hundreds of ISGs. Whilst the mechanistic activities and anti-viral effector functions of the majority of ISGs remains unclear, it has been established that in general, in response to virus infections ISGs can inhibit infection in a number of ways including; interfering directly with viral replication, arresting protein synthesis machinery, reducing apoptosis threshold of implicated cells, inhibition of cell metabolism, MHC class I upregulation as well as driving cell migration. With each ISG having different effector properties, the profiles of ISG expression in response to virus infections is virus species specific with further variations observed between cell types. The observation of highly specific ISG expression profiles targeted at different viruses was recently described by Schoggins et al, were the expression of 380 different ISGs were monitored in response to a range of different virus infections including HCV, YFV, WNV, CHIKV, VEEV and HIV-1. Via this method, they were able to demonstrate that certain ISGs had a collective antiviral effect, whilst in contrast, the effect of certain ISGs had a pathogenic effect. These contradictive findings, emphasise the complex nature of ISG responses and the necessity of further research investigating ISG responses to viruses. This section will cover key ISGs that have been well described as well as the ISGs investigated for the purpose of this thesis [366].

RSAD2/viperin

Radical SAM domain containing-2 (RSAD2) is a gene of 331 amino acids in length which encodes viperin (virus inhibitory protein, endoplasmic reticulum associated, interferon inducible) which is an interferon inducible protein, 43kDa in size. The expression of viperin is highly conserved with its expression having been detected in a wide variety of mammalian species as well as in certain fish and reptiles [370]. RSAD2 is made up of three domains: a species variable N-terminal domain, a central domain containing a "radical SAM domain" which is highly conserved between species as well as a conserved C-terminal domain. In terms of antiviral activity, studies have observed that the central domain appears to play a key role in viperins' antiviral effector function against HIV and BUNV, although the exact anti-viral mechanisms involved remain unclear [371] [372]. Similarly, the highly conserved C-terminal region of viperin has been demonstrated to play a critical role against

infections with flaviviruses such as HCV and DENV, where DENV budding was inhibited by viperin [373] [374].

Whilst further research is required in order to enable a complete understanding of the antiviral effector functions of viperin, studies suggest that viperin may be responsible for the inhibition of either viral protein function or synthesis as well as inhibiting viral soluble protein transfer from the ER. Recently, it has been demonstrated that viperin can catalyzes the conversion of the previously unheard of molecule, cytidine triphosphate (CTP). In this the catalyzed CTP acts as a chain terminator of RNA-dependent RNA polymerases, thereby inhibiting viral replication of several flaviviruses such as ZIKV [375]. In terms of viperins role in other arbovirus infections, a study looking at transcriptional profiles of PBMCs acquired from CHIKV infected patients, observed an upregulation of viperin in monocytes. In addition, in RSAD2 knock out mice, enhanced viraemia accompanied by exaggerated joint inflammation was observed. In this system, the N-terminal domain of RSAD2 appeared to be the key factor involved in the antiviral response against CHIKV infection [376].

ISG15

ISG15 is widely considered one of the most strongly and rapidly induced ISGs in response to pathogen invasion. ISG15 is a ubiquitin like ISG which can conjugate with other proteins in a formation known as ISGylation. However the function of this conjugation remains elusive. ISG15 can also exist on its own as an unconjugated protein which can function as a cytokine. In terms of antiviral activity during arboviral infections, attenuated ISG15 has been demonstrated to inhibit Sindbis virus replication when given to IFN-αβR knockout mice as well as delay ISG15 knockout mice from succumbing to infection following administration of attenuated ISG15 [377]. In addition, ISG15 has been demonstrated to play an important role during CHIKV infection. A study investigating CHIKV infection in ISG15 deficient mice, observed enhanced lethality in response to infection in the ISG15 knockouts than in wild type mice, with an accompanied by an enhanced induction of

proinflammatory cytokines. This indicates that ISG15 has an anti-inflammatory role during CHIKV infection which inhibits pathogenesis [378].

IFIT

IFIT are a group of ISGs which encode for interferon-induced proteins with tetratricopeptide repeats (IFIT). Whilst humans have 4 separate IFIT genes, mice have only 3, and generally IFIT genes have been found to have major variability between species. IFIT has the ability to bind specific proteins including cellular and viral proteins as well as viral RNA. This ability plays an important role in antiviral immunity as IFIT can bind to proteins essential for translation initiation which ultimately results in the inhibition of viral protein production. Furthermore, IFIT can capture viral RNA by binding to the 5' ends of RNA lacking 2'-O-methylation, a feature which is especially common in RNA viruses; a mechanism that appears to be important in antiviral responses against arbovirus infections [379]. However, recent studies have questioned the occurrence of this in alphaviruses as VEEV and SINV, were demonstrated to have successfully evolved secondary structures in their 5'- UTR region in order to prevent IFIT1 binding [380]. In flavivirus infections, WNV has evolved to incorporate a 2'-O methylated 5' cap in order to successfully avoid IFIT binding [381]. *In vitro* studies utilising A549 cells have also demonstrated that IFIT inhibits translation of ZIKV and DENV [382].

PRRs

Apart from proteins that exhibit antiviral effector functions, multiple pathogen recognition receptors which are stimulated by the interferon response are also incorporated into the ISG classification. Such PRRs include TLR3, TLR7, TLR9, MAVS, and Ddx58 (which encodes RIG-I). These were discussed at greater length previously 1.5.2.2.

1.5.4.7 Chemokines

Chemotactic cytokines (chemokines) are a subgroup of cytokines that implement control over the migration and positioning of immune cells. The immune system is therefore heavily dependent on chemokines to orchestrate the coordination of cell

migration as well as promoting interactions between different immune cells. There are 50 known chemokines in both humans and mice, all of which are 8 to12 kDa in size, with the ability to control cell migration by binding to a group of approximately 20 chemokine G-protein coupled receptors (GPCR's) [383]. These chemokine receptors have varying degrees of specificity and promiscuity with multiple chemokines binding to the same receptors. Chemokines can be further subdivided into four separate groups based on the spacing of their first cysteine residues. These four groups include CC chemokines, CXC chemokines, XC chemokines as well as CX3C chemokines. CC chemokines are therefore distinguishable by their two adjacent cysteine residues whilst CXC chemokines are characterised by the positioning of a random amino acid located in between the first two cysteine residues [384]. As the latter two subgroups will not be part of this thesis they shall not be discussed in further detail.

CC chemokines, which make up the majority of chemokines with their 28 members (CCL1 to CCL28), are mainly responsible for regulating leukocyte migration during the inflammatory response [384]. Out of these 28 chemokines CCL6, CCL9, CCL10 and CCL12 do not exist in humans but can be found in mice, whilst the opposite is true for CCL13 and CCL16[385]. Whilst all CC chemokines have different roles, CCL2 is known to trigger monocyte recruitment to sites of inflammation via its interaction with chemokine receptor CCR2, whilst CCL3 is responsible for polymorphonuclear cell recruitment by interacting with CCR1, CCR4 and/or CCR5 [386]. CCL5, which is secreted by a number of immune cells such as macrophages, plays an important role in the recruitment of a range of leukocytes including anti-viral Th1 T-cells, macrophages, eosinophils and basophils to sites of inflammation. This thesis will mainly look at expressions of CCL2 and CCL5 [387].

The CXC chemokines group contains 17 distinct chemokines (CXCL1 to CXCL17). Similarly to CC chemokines, certain CXC-chemokines exist in humans but not in mice such as in the case of CXCL6 and CXCL8, whilst CXCL15 exists only in mice but not in humans. Certain CXC chemokines play a key role in regulating homeostasis such as in the case of CXCL12 which is ubiquitously expressed in the bone marrow where it is responsible for the retention of hematopoietic progenitor

and stem cells via its interaction with receptor CXCR4. CXC chemokines also play an important role during inflammation as CXCL1 and CXCL2, which are expressed by immune cells such as macrophages, are responsible for neutrophil recruitment which can then go on to produce more CXCL1 and CXCL2 thereby amplifying the immune reaction. In addition, CXCL9 and CXCL10 are both type I and type II IFN inducible in a range of cell types and interact with receptor CXCR3 which is located on a range of anti-viral immune cells such as certain NK cells and Th1 and CD8 T-cells [388]. This thesis mainly studied the expressions of CXCL2 and CXCL10.

In terms of chemokines and arbovirus infections, studies have demonstrated an upregulation of chemokines such as CCL2, CCL5, CCL3, CCL4, CCL7, CCL8, CXCL9 and CXCL10 in response to infections with SFV and WNV. Studies in mice investigating arbovirus induced encephalitis, determined that chemokine receptors CXCR3, CCR2, and CCR5 are key in the triggering of CNS inflammation by coordinating leukocyte migration to the brain during viral infection. In this study, infection with SFV resulted in the upregulation of CC and CXC chemokines which resulted in the directed influx of CD8+ T cells, macrophages and NK cells into the CNS. Contradictory, this influx of immune cells into the CNS, which was originally believed to be essential for viral clearance, has been demonstrated to be contributing to pathogenesis and one of the main causes of lethal encephalitis during SFV infection. The blocking of receptors CCR2 and CXCR3 prevented the influx of immune cells to the CNS thereby preventing encephalitic infection [389]. Furthermore, infections with ZIKV and DENV have shown to upregulate chemokines such as CCL2 and CXCL12 as well as other chemokines which collectively play a role in cell migration of monocytes and T-cells. Specifically, CXCL12 expression was observed to be significantly higher in monocytes during ZIKV infection which could help explain brain inflammation and the associated brain abnormalities observed in infected foetuses as CXCL12 has been associated with the retention of white blood cells in the CNS as well as initiating migration of CD8+ T cells into the brain during WNV infection [319] [390]. Similarly, another study by Pingen et al observed CCL2 production in the skin following exposure to mosquito biting which recruits virus permissive myeloid cells which were observed

to become infected with SFV and BUNV [95]. Mosquito bite dependent enhancement of arboviral infections will be discussed in more detail in section 1.7.

1.5.5 Immune evasion by arboviruses

In order for a virus to maintain a productive infection within a host and potentially develop persistence, it is essential that it avoids detection by the immune system, at least long enough to allow for viral replication to occur. Arboviruses have therefore developed mechanisms specifically evolved for the purpose of suppression and/or evasion of the hosts innate and adaptive immune responses. These mechanisms tend to vary greatly between different virus species. This section will therefore only cover a few key immune evasion mechanisms utilized by arboviruses.

Alphaviruses contain a gene known as nsP2. This gene, in alphaviruses such as SFV and CHIKV, has been discovered to have the ability to inhibit antiviral gene transcription via the degradation of the a subunit of the host cells RNA polymerase II complex known as Rpb1 which is essential for gene transcription. This occurs from 6 hours post infection [391]. Similarly, new world alphaviruses have also evolved to interfere with host cell gene transcription although via a separate mechanism. Studies on viruses such as VEEV and EEEV, have determined that certain new world alphaviruses inhibit gene transcription via the use of an amino-terminal fragment of their capsid protein which interacts with host cell polymerases [392]. Similarly, utilizing a mutant SFV virus where the localization of nsp2 inside the cell nucleus has been inhibited, it was demonstrated that mutated virus resulted in a significantly higher IFN response in comparison to its response to WT virus [393].

Apart from inhibiting gene transcription of host cells arboviruses exhibit a wide range of other mechanisms for immune evasion. For example, some mosquito-borne flaviviruses are believed to evade the immune system by inhibiting the immune responses of specific immune cells. Studies on DENV have demonstrated its ability to supress haematopoiesis in the bone marrow [394] whilst CHIKV and other alphaviruses have been shown to hijack immune mechanisms responsible for the induction of cellular apoptosis as a means of infecting neighbouring cells. This mechanism also enables macrophages to become infected via the process of

phagocytosis of the cellular debris that contains virus particles. In this way, the virus is able to spread without the threat of exposure to immune cells present in the extracellular environment. [395]. In addition, DENV, and other flaviviruses such as WNV, have also been shown to interfere with the phosphorylation of STAT1 in order to prevent the induction of type I IFN which can be induced in a STAT dependent manner [396]. Furthermore, the highly conserved flavivirus protein NS5 which plays an important role in the replication of viral RNA, also produces an N7 and 2′-O methylation viral RNA 5′ cap which is used by flaviviruses as a hiding mechanism as this enables them to resemble mammalian host RNA and thereby go undetected by certain PRRs [397]. Finally, subgenomic RNAs present in flaviviruses have also been demonstrated to inhibit antiviral immune responses by binding to molecules involved in the regulation IFN and ISG response [398].

Numerous more examples could be listed, but from this brief overview of arboviral immune evasion it is possible to conclude that an evolutionary arms race exists between host immune responses and viruses that has enabled the evolution of a range of variable mechanisms that target immune responses.

1.5.6 Antivirals, vaccines and treatments

As of the time of writing this thesis, no specific antivirals targeting arboviral disease have been licensed and few vaccines have been developed. Due to this, the only available treatments for the majority of arboviral infections are analgesics, anti-inflammatory and antipyretic drugs.

There are several obstacles at play that hinder the development of antivirals and vaccines targeting arboviral disease. Such obstacles include the vast number of different arboviruses, with more than 550 described out of which more than a 100 are known to infect humans. As symptoms of disease tend to overlap, accurate diagnosis are problematic. Another major obstacle is the spontaneity and unpredictability of arboviral outbreaks, which complicates development and stockpiling of virus species-specific medications. As was the case for the recent epidemics of CHIKV in the Pacific islands and ZIKV in South America, there is no prior warning for the majority of arboviral epidemics. Spread of disease following

emergence can also occur very rapidly. During the recent Zika epidemic for example, WHO was initially notified of sporadic infections with ZIKV in March 2015. Within 9 months, the virus had already spread widely to an additional 10 countries including El Salvador, Mexico and Paraguay. Due to these factors, the possibility of stockpiling drugs for each arbovirus in case of a potential outbreak is likely to be unfeasible. Furthermore, research and development required for drug discovery, as well as the requirement for extensive clinical trials, makes drug development a time consuming laborious process, estimated to take approximately 10 years, which makes the emergence of sporadic new outbreaks difficult to deal with. In addition, the entire process of developing a new drug is very expensive with an estimated total cost of $2-3 billion USD per drug [399]. As, the majority of incidences affect people in less affluent countries, there has been a historical lack of incentive for pharmaceutical companies to develop vaccines and treatments.

The accumulation of all these obstacles have hindered the development of effective vaccines and treatments and so novel ideas are required for treatment discovery. Currently, much hope is given to broad-spectrum antivirals. Such putative compounds would have the ability to inhibit a broad range of distinct virus infections by targeting different aspects of the virus life cycle such as inhibition of viral cell entry/exit, disruption of viral replication or even by directly lysing the virus particle. One such example for arboviruses is compound 3′-fluoro-3′-deoxyadenosine which can supress infection of several viruses including SFV and VEEV, whilst 2,6-diaminopurine derivatives have been found to prevent infections with ZIKV and DENV *in vitro* [400] [401] [402]. One of the major benefits of broad-spectrum antivirals would be eliminating the need for accurate diagnosis, reduce the number of drugs required for stockpiling as well as the potentiality that already developed drugs could be efficacious against newly emerged diseases as well.

In terms of vaccine development, the only licensed vaccine currently available against mosquito borne viruses is the JEV, DENV and Yellow fever vaccine, known as the 17D vaccine [403]. Despite the vaccines efficacy however, the cost and time associated with its development in combination with lack of education of the public regarding its existence, current vaccine cover falls far below the estimated 80% coverage recommended by the WHO [404]. However, vaccine development

targeting other mosquito borne viruses has so far been largely unsuccessful. Attempts made for the development of an effective DENV vaccine have been complicated by the existence of 4 separate viral serotypes. As immunity to one serotype does not automatically confer immunity to the remaining 3 serotypes, and as immunity to one serotype is known to cause enhanced infection via antibody dependent enhancement if ever infected with another serotype, a DENV vaccine must confer immunity to all 4 serotypes simultaneously without instigating antibody dependent enhancement. A recently developed DENV vaccine known as Dengvaxia, which was approved in 2015 following a phase 3 clinical trial, was recently re-evaluated as it appears as if the vaccine is only effective in individuals who have already been infected with DENV previously, whilst it simultaneously aggravates DENV infection in DENV naïve individuals. Therefore, all individuals vaccinated with Dengvaxia have to be tested for seroprevalence [405].

Lastly, following the ZIKV epidemic, attempts have been made for the development of a Zika vaccine. However, due to the reduction of ZIKV cases world-wide many vaccine development projects have been cancelled for economic reasons [406]. Also, instigating a phase III clinical trial is problematic without an active outbreak. On the positive side, the efficiency with which alternative vaccine targets were developed is promising for future vaccine developments during future outbreaks.

1.6 Skin Biology

Arboviruses are initially deposited into the skin of the host by a blood feeding vector. Host inflammatory responses to the bite, and virus inoculum in the skin, appear to be an important factor in facilitating virus spread and infection (discussed in more detail in section 1.7). Therefore it is important to understand the fundamental aspects of cutaneous biology, which will be the focus of this section, which will cover skin structure, skin vasculature and basic skin immune responses as well as the current understanding of immunological reactions against mosquito bites.

1.6.1 Skin structure

As the bodies largest organ, the skin is a dynamic system comprised of an extensive ligament, nerve, blood vessel and lymphatics network that effectively protects the body from the external environment. But it does not simply act as a static barrier to the outside world; there is continuous communication between epithelial, stromal and immune cells which help regulate homeostasis and inflammatory responses towards pathogens [407]. The skin is comprised of three main layers; the epidermis, the dermis, and the hypodermis; with a layer of subcutaneous fat underneath (see figure 1.11) [408].

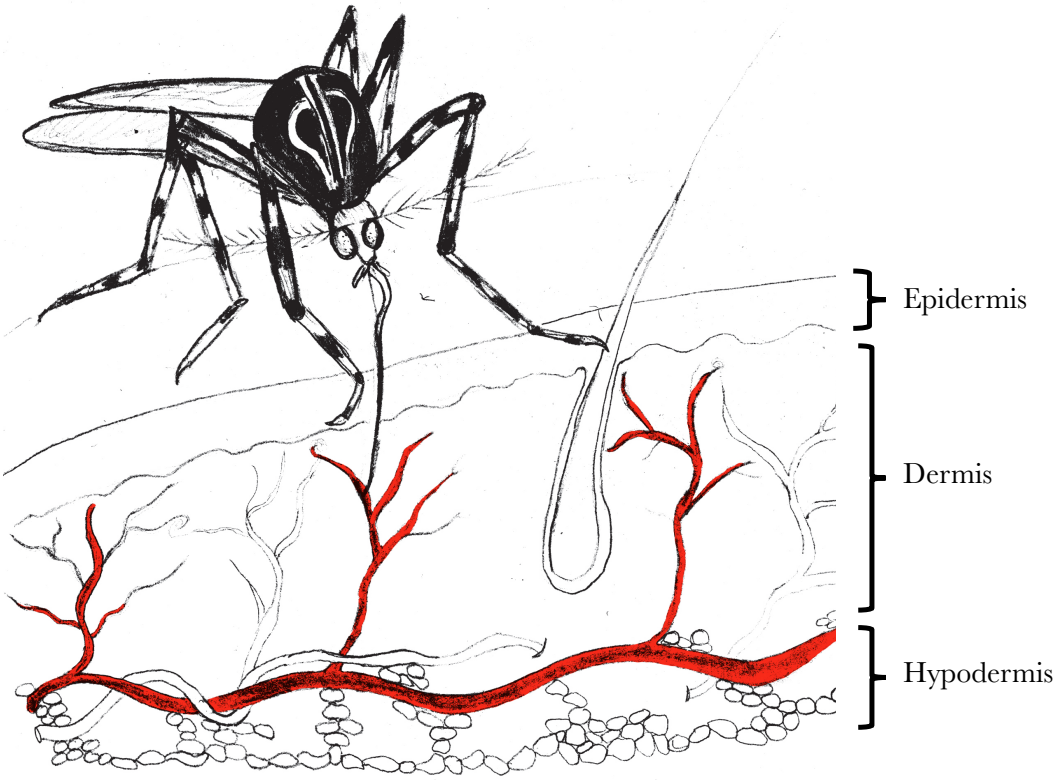


Figure 1.11. Mosquito biting.

Diagram illustrating how mosquitoes will probe through the epidermis and deposit saliva and virus into the dermis [408].

The epidermis, which refers to the uppermost layer of the skin, is 75-150μm thick in humans and can be further subdivided into the basal cell layer, the spinous cell layer, the granular cell layer, and the stratum corneum. It is mainly made up of keratinocytes which make up approximately 95% of all cells and are continuously undergoing self-renewal [409]. Professional antigen presenting Langerhans cells are also situated throughout the epidermis [410]. The dermis, which is the skin layer that

provides mechanical strength and elasticity, can be further subdivided into two distinct layers; the superficial papillary dermis and the deeper reticular dermis. These are made up mainly of dermal fibroblasts as well as adipocytes which are mainly distributed throughout the superficial papillary as well as surrounding blood vessels, hair follicles and the nerve endings which are also located in this area. Lastly, the hypodermis is mainly made up of connective tissue and adipose tissue with the majority of cells being fibroblasts and adipocytes [409].

1.6.1.1 Cells of the Epidermis

As mentioned above, the most numerous cell type of the epidermis is the keratinocyte. These cells are required for structural support and will differentiate as they migrate from the basal layer to the stratum corneum where they stop proliferating, increase in size and die in order to form the water impermeable dead skin layer [411]. They also play a role in innate immune recognition mainly via TLR1-6 and TLR9 whilst also playing a role in anti-bacterial and anti-viral immunity via the secretion of β-defensins and type I IFN [412]. Due to their numbers, keratinocytes have historically been regarded as one of the key cell susceptible to arbovirus infection as they have been shown to susceptive to infection with a range of different viruses including WNV, SFV and DENV [413]. However, during probing, mosquitoes will probe right through the epidermis and deposit arbovirus within the dermis which lacks keratinocytes [291]. Therefore it remains unclear if keratinocyte susceptibility to arbovirus infection is of any relevance. Another prevalent cell type located in the epidermis are the Langherans cells which possess a range of PRRs including TLR1-3, TLR5-6 and TLR10, as well as foetal derived γδ T-cells [305] [412] [414]. As in the case of keratinocytes, as virus inoculum is deposited into the dermis, it remains controversial whether the cells of the epidermis play a role in the immune response during arboviral infection.

1.6.1.2 Cells of the Dermis

Anatomically, the dermis consists of an extracellular matrix which is has collagen and elastic fibres filling any extracellular spaces, functioning as a scaffold for the migration of immune cells. The main mesenchymal cell type of the dermis are the

fibroblasts which are responsible for the production of matrix proteins such as elastin and collagen, as well as responding to inflammatory signals. Dermal fibroblasts have been shown to be susceptible to arboviral infections of viruses such as CHIKV and DENV, where fibroblasts will induce an IFNβ response [106] [332] [415]. In addition, the dermis possess at least 5 distinct populations of dendritic cells; the most common being CD11b + DC which make up approximately 50% of the dermal DC population. Dermal cDCs are implicated in phagocytosis and MHC class II presentation of antigen. DCs will then migrate to the lymph nodes where they will activate T cells resulting in the subsequent production of pro-inflammatory cytokines. Furthermore, populations of dermal macrophages are also located within the dermis which play an important role in pathogen recognition via PRR, triggering the release of proinflammatory cytokines and chemokines, responsible for neutrophil recruitment, upon activation [416]. The role of dermal DCs and macrophages in arbovirus infections remain largely unclear. Studies have however shown that CD1+ DCs are responsible for the initiation of a type I IFN response during DENV infection whilst macrophages were observed to have a pathological effect [417]. Similarly, ZIKV can successfully infect immature dendritic cells generated from human derived peripheral blood mononuclear cells, suggesting an important role for dermal DCs in sustaining infection in the skin [418].

Populations of mast cells can also be found within the dermis (discussed in section 1.5.2.3) which play an important role in the immune and allergic response by releasing histamines and proinflammatory cytokines (discussed more in section 1.5.4). Finally, the dermis also contains a γδ T-cell population which are capable of producing IL-17 and IL-22 [419].

1.6.2 Skin blood vasculature and oedema

Throughout the extracellular matrix of the dermis, there is a network of blood and lymph vasculature which play an important role in inflammatory responses as the recruitment of immune cells to sites of inflammation occurs via the vascular systems. Blood vessels in the dermis can be split into four separate groups; arteries, capillaries, postcapillary venules and collecting venules. The majority of blood endothelial cells lining the blood vessels have tight and adherence junctions between them which

restrict the crossing of any plasma proteins exceeding 70kDa. However, during inflammation, endothelial cells lining the postcapillary venules become separated as their tight junctions and adherence junctions diminish, allowing for the crossing of fluid, along with albumin and immunoglobulins, to accumulate in the tissue at the site of inflammation which inevitably leads to tissue swelling and the formation of oedema. As this only occurs in the postcapillary venules, they are considered the most important dermal blood vessels in terms of humoral immunity (see figure 1.12).

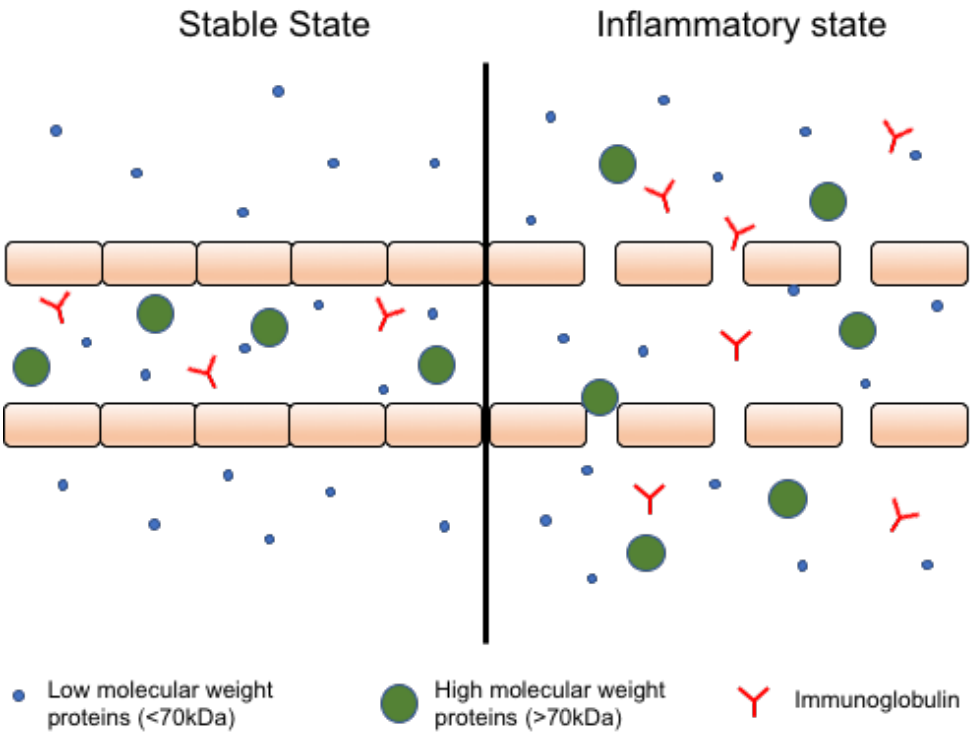


Figure 1.12 Endothelial cell permeability.

Endothelial cells lining the blood vessels have tight adherence junctions between them restricting the crossing of any plasma proteins exceeding 70kDa. During an inflammatory state, endothelial cells become separated as their tight junctions and adherence junctions diminish, allowing for the crossing of higher molecular weight proteins (green) and immunoglobulins (red), to accumulate in the tissue at the site of inflammation which leads to tissue swelling and oedema formation.

Surrounding the endothelial cells of the postcapillary venules are pericytes and the basement membrane, with macrophages and mast cells located in close proximity. These components function as the perivascular extravasation unit. Recent studies have shown that perivascular macrophages play a vital role in neutrophil recruitment as they will secrete large quantities of CXCL1 and CXCL2 which are neutrophil

chemoattractant [420]. T-cell recruitment and activation is also dependant on postcapillary venules. Following their recruitment in response to inflammation in the skin, T-cells will form clusters with DCs and perivascular macrophages, known as iSALT. iSALT is essential for initiation of the adaptive immune response as it functions mainly as an antigen presentation site [421]. The formation of oedema during the acute inflammatory response can be split into two sections; immediate reaction and late-phase reaction.

1.6.2.1 Immediate reaction

The immediate reaction, which occurs within seconds following antigen exposure, is instigated by the activation of mast cells, usually triggered by IgE recognising its cognate allergen in antigen-experienced individuals. In the absence of IgE (i.e. as occurs with non-allergic oedema), mast cell activation can occur in response to polycationic compounds including 48/80, substance P, bradykinin, mastoparan, polyethyleneimine as well as in response to pathogens. Activation of mast cells leads to degranulation which results in the release of large quantities of bioactive amines, proteoglycans, proteases and histamine as well as the synthesis of leukotriene C4, prostaglandin D2 and platelet-activating factor in addition to specific cytokines and chemokines [422] [423]. Prostaglandins, which are a group of active lipid compounds known as eicosanoids, are located in the majority of tissues where they function as locally acting vasodilators [424]. Leukotrines, which are also part of the eicosanoid family, are responsible for cell signalling regulating the inflammatory response [425].

Histamine, which is arguably the most important oedema-activating amine, is a known mediator of the allergic response and the key instigator of the early phase reaction. Following its synthesis in the golgi apparatus, it is transported for storage inside granules. Mast cells, which are the only histamine carrying cell type (with the exception of basophils), contain approximately 2-5 pg of histamine per cell. In the skin, released histamine will act on the H1 receptors of local blood vessels which triggers the immediate increase in vascular permeability and fluid extravasation. Histamine will also act on the H1 receptors of local nerve endings which leads to vasodilation of dermal blood vessels which has of consequence the characteristic

reddening of the skin and the potential formation of skin lesions known as the wheal and flare reaction [426]. Apart from its effects on dermal blood vessels and nerves, studies have shown that histamine can also bind to cell receptors which can in turn activate and attract neutrophils and eosinophils. Histamine also induces the expression of CXCL8 [427]. The combined actions of histamines, prostaglandins, leukotrienes cause a rapid increase in extravasation allowing for the movement and activation of leukocytes, including neutrophils and monocytes, to cross from the blood vessels into the infected tissue guided by chemokines mainly expressed by macrophages. This contributes to the development of the late-phase reaction.

1.6.2.2 Late-phase reaction

The late-phase reaction will develop sometime between 2-6h following initial allergen exposure and will peak sometime between 6-9 hours following exposure depending on allergen dose. In contrast to the early phase reaction, which is characterised by the formation of a wheal and flare lesion, the cutaneous late phase reaction is characterised by the oedematous, firmer lesion which usually resolves within 24 hours (occasionally swelling can be observed up to 72h post allergen exposure) [428]. It is triggered by the continuous synthesis of mediators of the inflammatory response. These mediators, which are mainly produced by mast cells, include the calcitonin gene related peptide (CGRP) and the vascular endothelial growth factor (VEGF) which cause vascular permeability and the formation of oedema. CGRP, which is part of the calcitonin family of peptides, is a highly efficient vasodilator inducing endothelial permeability indirectly via the aid of histamine [429]. VEGF, the expression of which is partially regulated by CGRP, mainly acts upon endothelial cells by promoting cell proliferation, migration and maintenance. VEGF is also known to be chemotactic for monocytes [430]. Recent studies have demonstrated that CGRP and VEGF can be secreted by immune cells infiltrating the skin during the inflammatory response. CGRP is expressed by neutrophils in response to inflammatory cytokines such as IL-1 and TNF-α, and CD3+ T cells, whilst VEGF is expressed by eosinophils, T-cells, and macrophages [431]. In the human skin, immune cells commonly recruited to the site during late-phase reaction include leukocytes such as Th2 and Th1 cells, granulocytes such as eosinophils, neutrophils and basophils, as well as monocytes [432].

1.6.2.3 Chronic allergic inflammation

Continuous or repeated exposure to allergens can lead to chronic or persistent inflammation. Persistent inflammation is often accompanied by phenotypic and functional alterations in structural cells and in the extracellular matrix in the affected area which can have implications on organ functions. Due to study limitations, it remains unclear how repeated or prolonged allergen exposure shifts the inflammatory response from early and late phase reactions towards a chronic allergic reaction. However, it has been established that whilst the main features of the acute inflammation continue to occur during chronic inflammation, such as vasodilation, extravasation and neutrophil migration, during chronic infection, neutrophils tend to become replaced by macrophages and lymphocytes. These immune cells will secrete large quantities of pro-inflammatory cytokines and growth factors that will eventually result in tissue damage and the subsequent formation of granuloma and fibrosis by secondary tissue repair [433] [434]. Examples of chronic allergic pathologies include atopic dermatitis, allergic rhinitis and asthma; all of which are characterised by the remodelling of affected tissues. For example, in atopic dermatitis, the skin barrier becomes weakened, which subsequently results in an increased risk of skin infections [435].

1.6.3 Skin immune reaction to mosquito bites

The immune responses to arbovirus infection by mosquito differs to the immune responses elicited by non-vector borne viruses, as arboviruses are always accompanied by a bite from a vector. This means that the bite itself will also elicit an immune response on its own. In the case of mosquito-transmitted viruses, during mosquito feeding, mosquitoes will probe the skin whilst simultaneously injecting saliva in order to acquire a blood meal. This will eventually trigger an immune response targeting the tissue trauma from the probing of the skin, as well as, against the saliva injected by the mosquito, which contains a range of proteins and potentially pathogens (discussed further in section 1.7) [95, 436, 437]. Depending on the mosquito species, a mosquito bite will typically trigger a skin reaction that can range from small papule formations, to larger pruritic lesions. Whilst the specific immune responses elicited against mosquito bites, and the pharmacological and

potentially immunomodulatory effects of mosquito saliva are poorly understood, there are some studies investigating these responses and how they modulate the course of arbovirus infection. Therefore, as it is important to understand the effects the bite has on the host immune response, this section will focus on the current understanding of the immune responses triggered in response to mosquito bites.

1.6.3.1 Innate immune responses to mosquito bites

The mosquito bite quickly triggers immune responses in the skin including the expression of cytokines, degranulation of mast cells as well as the expression of neutrophil attracting chemokines [95, 438]. Reactions to mosquito bites are highly dependent on mast cell activation and degranulation and the accompanied vasodilation, extravasation and the recruitment of polymorphonuclear cells. In the absence of mast cells, mice no longer exhibit upregulation of the neutrophil chemoattractant CXCL2 in response to mosquito bites. Neutrophils attracted to the site of inflammation secrete high levels of IL1β, and studies utilising neutrophil deficient mice observed reduced expression of pro-inflammatory cytokine and chemokines chemotactic for CCR2 expressing myeloid cells [95].

Interestingly, mast cell activation in response to mosquito bites does not seem to rely exclusively on IgE mediated activation, as studies have demonstrated mast cell degranulation occurrence in naïve mice in response to *Anopheles* mosquito bites. In this study, *An. stephensi* saliva directly induced mast cell degranulation in connective tissues in the absence of IgE [315]. TNFα and MIP-2 were also significantly upregulated in this system as they were secreted by mast cells [439]. A similar study investigating cytokine and chemokine responses against *Anopheles* bites in naïve mice observed upregulation of MIP-2 in the skin as well as upregulation of IL-10 expression in the draining LN [440].

A recent study investigating the effect of *Ae.aegypti* mosquito bites on host immune responses observed an increase in NK cells, and myeloid cells such as DCs, monocytes, macrophages and neutrophils as well as CD8 + T-cells 6 hours post bite in the serum [441]. In terms of cytokines, an upregulation of pro-inflammatory IL-1α was observed in the serum, accompanied by the downregulation of other pro-inflammatory cytokines such as pro-inflammatory cytokines IL-12p40 and TNFα. In

addition, enhanced quantities of VEGF was observed [441]. 24h post bite, Tregs were observed to have migrated from the spleen to the serum. The authors suggest that this could result in an enhanced secretion of anti-inflammatory IL-10. Interestingly, *in vitro* studies have shown that mosquito saliva appears to inhibit cytokine production such as CCL5 in human PBMCs.

In terms of oedema, mosquito salivary gland extract induces endothelial permeability of the skin when injected into mouse ears, as well as disrupting human endothelial cell junctions *in vitro* when given at high doses. This, in combination with the observed degranulation of mast cells discussed earlier, suggests that mosquito bite induced oedema formation occurs from the direct action of saliva on endothelial cells as well as via its instigation by mast cells [310]. The relevance that each has for modulating host susceptibility to arbovirus infection is not known.

1.6.3.2 Adaptive immune responses to mosquito bites

It has been observed that humans bitten by mosquitoes for the first time do not always display an obvious reaction. With exposure to repeated biting however, an allergic reaction with an accompanied itch can develop. In mice, studies have shown that repeated injections of recombinant mosquito salivary allergen caused an increase in detectable IgE and IgG in serum and caused the induction of an immediate skin reaction [442]. Similarly, other studies in mice have observed enhanced extravasation following repeated exposure in comparison to naïve mice [443].

Whilst mosquito saliva will be discussed in greater detail in the following section, it should be noted that mosquito saliva is very complex as it contains hundreds of molecules with varying, and often unknown, functions. There is a lot of controversy regarding whether mosquito bites elicit a pro-inflammatory or putative anti-inflammatory response in the host, with certain compounds exhibiting either pro-inflammatory or anti-inflammatory responses. In summary, exposure to mosquito bites induce mast cell degranulation and neutrophil recruitment with a subsequent influx of monocytes. More studies are necessary in order to gain a better understanding of the immune response to mosquito bites and in order to determine how these responses vary between mosquito species and individuals.

1.7 Vector saliva and infection

Bloodsucking arthropods have evolved to overcome specific host physiological responses in order to better acquire a successful blood meal. These include haemostasis, inflammation and immunity to saliva. To aid them, biting vectors will inject a cocktail of molecules present in their saliva during feeding.

In terms of arboviral infection, it has been established that arboviruses inoculated via a mosquito bite, or administered with mosquito saliva cause a more severe infection compared to virus inoculated in the absence of a mosquito bite (or mosquito saliva) [95, 444, 445]. This is also true for other vector borne diseases transmitted by different vector species as the saliva of hematophagous arthropods contains a wide range of compounds, the majority of which are used to facilitate blood feeding. For example, mosquito saliva contains a mixture of anti-haemostatic, anti-coagulant, vasodilatory, as well as compounds that can elicit or inhibit host immune responses. Whilst transcriptome analysis have been conducted, the function of the vast majority of these compounds remains unknown.

Originally it was hypothesized that the enhancement of arbovirus infection by mosquito bites is caused by a similar mechanism observed in tick-bites. Biting ticks have been found to immunosuppress its host at the bite site by suppressing mammalian chemokine function [446]. It has been postulated that if this was also the case for mosquito-borne virus infection, this would then explain why mosquito bites are able to enhance viral infections in the host. However, in comparison to ticks that have evolved a range of immunosuppressive factors (such as Evasins which bind to chemokines), these factors appear to be absent in mosquitoes. This difference is perhaps not surprising as ticks must remain attached to a mammal's skin for days, while mosquitoes feed for only a brief few minutes. Therefore, host immune responses triggered in response to mosquito bites are unlikely to have a major effect on the efficiency of mosquito-feeding to push for the evolution of mosquito-derived immunomodulatory factors. In fact, for mosquitoes, the majority of evidence for immunosuppression by mosquito saliva has been found *in vitro* [445, 447]. In the previous section, focus was given on the current understanding of immune responses elicited towards mosquito bites and mosquito saliva. This section will focus on the effect of vector bites and saliva on arboviral infection.

1.7.1 Mosquito saliva

The co-injection of virus alongside a mosquito bite, in comparison to the inoculation of virus (in an exclusively experimental setting) in the absence of a bite, results in an enhanced and more rapid dissemination of virus to remote tissues. This has been demonstrated for a range of arboviruses including DENV, WNV, SFV and BUNV [447] [95]. More specifically, the co-administration of mosquito saliva alongside virus is sufficient to enhance infection, suggesting that saliva is responsible for arboviral enhancement. Although mosquito saliva dependent viral enhancement has been established, the mechanisms involved facilitating the enhancement remain unknown. This section will therefore focus on our current knowledge to date of the mechanisms believed to be involved.

1.7.1.1 Inflammatory responses elicited against mosquito bites enhance arbovirus infection

Mosquito bites and mosquito saliva trigger an inflammatory response that triggers an influx of immune cells to the site of inoculation. However, as has been described previously, many of these immune cells are susceptible to arboviral infection. Recent studies in mice have demonstrated that mosquito bites upregulate the expression of neutrophil chemoattractant chemokine CXCL2 as well as IL1β which result in an enhanced influx of neutrophils, and subsequently macrophages, to the site of infection [95]. In addition, neutrophil depletion and the inhibition of IL1β expression has been observed to inhibit viral enhancement by bites. Similarly, another study demonstrated that SGE (salivary gland extract) triggers the recruitment of inflammatory neutrophils and monocytes which may cause enhanced infection with DENV, as some recruited immune cells become infected [310].

1.7.1.2 Vascular response against mosquito saliva may also enhance viral infection and dissemination

As discussed in section 1.6, mosquito bites will trigger early and late phase cutaneous inflammation with the associated formation of oedema and pruritic lesion that can persist for up to 72h. Studies have suggested that the formation of oedema in

response to mosquito bites also occur at the same time as arbovirus enhancement. For example, a study investigating SFV infection in an *in vivo* mouse model observed delayed viral dissemination to draining lymph nodes when virus inoculation was accompanied by a mosquito bite and the formation of oedema. This suggests that oedema may retain SFV in the skin for prolonged period of time, thereby facilitating the infection of dermal cells [95]. Importantly however, the role, and relative importance, that bite/saliva-induced oedema has in enhancing SFV infection is not clear.

In addition, it has been hypothesised that the disruption of endothelial barriers by mosquito saliva may facilitate virus dissemination. As has already been mentioned, mosquito bites and saliva induce vascular leakage, potentially via the use of serine proteases which can break down the extracellular matrix of fibroblasts which increases the permeability of endothelial cells [310] [444]. Studies have suggested that the enhanced permeability of endothelial barriers may aid virus dissemination; in terms of DENV, enhanced endothelial permeability may increase the association of virus particles to potential circulating antibodies, which can facilitate DENV antibody dependent enhancement. In addition, enhanced permeability of blood vessels allows for an increase in the extravasation of monocytes and neutrophils to the site of infection which can indirectly aid the virus by providing new cells that are susceptible to infection by virus [310].

1.7.1.3 Inhibition of innate immune responses by mosquito saliva

Another hypothesis involving mosquito saliva dependent enhancement, suggests that mosquito saliva may aid virus infection by inhibiting host immune responses at the site of infection. Specifically, this has been suggested to occur via the inhibition of the expression of type I IFN by mosquito saliva. One study, investigating the effect of salivary gland extract (SGE) on DENV infection of human keratinocytes observed inhibition of type I IFN and IFNγ by SGE [448]. Similarly, a 34-kDa protein, found to be ubiquitous in *Ae.aegypti* mosquito saliva, also inhibited the expression of type I IFN and IFNγ in human keratinocytes via the suppression of IRF-3 and IRF-7[449]. However this mechanism is unlikely to be responsible for DENV enhancement by

mosquito saliva, as studies have demonstrated that mosquito saliva enhances DENV infection in the absence of type I IFN [310] [450].

In addition, the suppression of T-cell responses by mosquito saliva has also been hypothesised to be responsible for arbovirus enhancement. As mentioned in section 1.6, mosquito saliva has been found to enhance Th2 related cytokines as well as IL-10 [440] [451] [436]. In addition, the addition of recombinant IL-4 has been found to enhance DENV infection *in vitro* [417]. However, as mosquito bite dependent enhancement occurs very quickly in mosquito bite-naïve mice, it is unlikely that the adaptive immune responses would play a significant role in arboviral enhancement in these models. This, in addition to the fact that mosquito bite driven enhancement of arboviruses has been observed in mice that lack adaptive immune responses, suggests that alternative mechanisms must be responsible for mosquito bite dependent arboviral enhancement [95].

1.7.1.4 Alternative mechanism suggestions to arboviral enhancement

Apart from the hypothesis mentioned above, there are a few other speculations on the mechanisms by which mosquito saliva enhances arbovirus infection. It has been speculated that the microbiota present in mosquito saliva may elicit the inflammatory response required for arboviral enhancement. This idea was strengthened by the discovery that the salivary glands of *Anopheles culicifacies* mosquitoes harbour a larger diversity of microbiota than the gut [452]. Whilst this is an interesting theory, it has yet to be investigated. Another study suggested that serine proteases present in mosquito saliva may alter virus infectivity by proteolyzing extracellular matrix proteins that aid infection of cells by increasing cell attachment and cell entry [450].

1.7.1.5 Pre-existing immunity to vector saliva

In terms of the adaptive immune response, it remains unclear whether it plays a role in mosquito bite dependent arboviral enhancement. Whilst a couple of studies have demonstrated that mosquito bites trigger a Th2 immune response which enhances arboviral infection [440] [451] [436], the majority of research implicated suggests

that bite mediated enhancement occurs too early for the adaptive immune response to play an important role in naïve individuals. Furthermore, a study investigating the pre-sensitization of mice to *Culex tarsalis* mosquito saliva and its subsequent effect on WNV infection by mosquito saliva, observed no significant difference between naïve and pre-sensitised mice [447].

1.7.1.6 Mosquito saliva composition

Mosquito saliva is a complex cocktail of molecules. Transcriptome analysis of female *Ae.aegypti* mosquito salivary gland transcripts have identified hundreds of individual genes; with the function of many remaining unknown [453]. As a general rule, all hematophagous arthropods contain at least one compound each with anticlotting, antiplatelet, and vasodilatory properties; with the majority containing multiple molecules with similar properties which can vary greatly between species. For example, *Aedes* mosquito saliva has been found to contain a vasodilatory tachykinin decapeptide named sialokinin [454], whilst instead, *Anopheles* mosquito saliva has been found to contain a ~65 kDa vasodilatory peroxidase, which is known to disrupt norepinephrine and serotonin which can be skin vasoconstricting agents [455]. In terms of anticlotting molecules, the majority of the *Aedes* genus utilises an inhibitor of factor Xa which is a member of the serpin family [456], whilst *Anopheles* mosquitoes utilise a different smaller anticoagulant which is unrelated to other known peptides [455]. There are numerous differences between similarly acting compounds found in different hematophagous vector species; differences that can probably be attributed to early lineage diversions. For example, as was discussed in section 1.4.1.4, *Aedes* and *Anopheles* lineages diverged approximately 150mya which is approximately 100 million years prior to the expansion of mammals. This is of significance as it has been suggested that any hematophagous insects that diverged prior to the radiation of mammals have a considerably higher variation in their sialome than species that diverged afterwards [457].

Interestingly, certain transcripts appear to exhibit differential expression between male and female mosquitoes. In the case of *Ae.aegypti* mosquitoes, 207 transcripts upregulated in the salivary gland were discovered to be female specific. Several of these transcripts have an undetermined function but can be presumed to be

implicated in the act of blood feeding and the alteration of host physiology [458]. Complicating things further is the observation that infection of mosquito salivary glands, as well as the act of blood feeding, appears to alter the levels of transcription of certain transcription factors [459] [460] [461] [462].

Following the discovery that mosquito saliva enhances arboviral infection several studies have attempted to specify which compound of mosquito saliva is responsible for this phenomenon. Therefore, I will briefly mention a few examples of individual proteins that have been suggested so far, as well as discuss their effects on the host host response.

LTRIN

One factor that has been suggested to play a role in facilitating arboviral enhancement is a factor the authors named LTRIN due to its ability to interfere with lymphotoxin-β receptor (LTβR) signalling. LTRIN was discovered to be upregulated in the salivary glands of mosquitoes following a blood meal. LTβR is involved in the homeostatic maintenance of lymphatic and lymph node structures and has previously been found to be implicated in chronic inflammatory disease. However, due to the early time point of arboviral enhancement, it remains unclear if LTRIN can be playing a key role as the study in question only investigated viral enhancement at day 7 following co-injection of Zika with LTRIN [463].

NeSt1

Utilizing a yeast-display library accompanied by an antigenic salivary gland screen, a recent study discovered a previously undescribed SG protein; NeSt1 (neutrophil stimulating factor 1). NeSt1 was found to upregulate IL1β, CXCL2 and CCL2 *in vivo*. Interestingly, passive immunization of mice with NeSt1 antiserum resulted in a reduction of the expression of IL1β, CXCL2 and CCL2 whilst simultaneously preventing the infiltration of immune cells such as neutrophils to the site of inflammation. Pre-immunization to NeSt1 also significantly reduced the number of mice succumbing to ZIKV infection [464]. This study further supports the theory put forward in the Pingen et al paper [95], that host responses to bites/saliva are important in the enhancement of arbovirus infection.

AgBR1

Similarly to the discovery of NeSt1, AgBR1 was also discovered using a yeast surface display screen. AgBR1, or *Ae.aegypti* bacteria responsive protein 1, which is homologous to *An.gambiae* bacteria responsive protein 1 (also called AgBR1) was found to upregulate IL-6. Injections with AgBR1 antisera also appeared to confer some protection against ZIKV infection. However, AgBR1 appeared to have minimal effect on ZIKV pathogenesis when co-injected alongside the virus [465].

D7

Another molecule that has been suggested to be implicated in viral enhancement is D7. Unlike the previous examples D7 has not been suggested to be implicated in host responses to saliva. Instead the 45-kDa sialylated saliva glycoprotein, belonging to the D7 protein family, was discovered to form complexes with DENV *in vitro* thereby enhancing its internalization into host cells [466]. However, a contradicting study has demonstrated that D7 inhibits DENV infection *in vitro* and *in vivo* by direct neutralization of virus particles via binding, or, by inhibiting immune cell infiltration to the site of inflammation [467].

CLIPA3

CLIPA3 is a serine protease which has been demonstrated to enhance the dissemination of DENV. Serine proteases are capable of proteolyzing the extracellular matrix proteins of cells, and thereby enabling viral attachment. Authors also demonstrated that knocking down CLIPA3 in mosquitoes utilizing siRNAs resulted in a reduction of SGE dependent DENV enhancement *in vitro* [450].

As mosquito salivary components are numerous and highly diverse, some proteins that appear to be implicated in mosquito saliva dependent arboviral enhancement, when tested in isolation, appear to counter act each other; with some factors enhancing or inhibiting immune responses. Therefore, this suggests that it is unlikely that one single salivary factor is responsible for viral enhancement, but instead the collective whole of factors and the subsequent total effect they have on the host immune response.

1.7.2 Tick saliva

Similarly to mosquitoes, ticks will inject saliva from their salivary glands during feeding, and like mosquito saliva, tick saliva also aids the transmission of arboviruses; a phenomenon commonly referred to as saliva assisted transmission (SAT)[468]. Tick saliva has been found to consist of a mixture of water, ions, non-peptidic molecules, a range of tick peptides and tick proteins, several host proteins as well as exosomes. This cocktail of molecules has been attributed to aid in tick water balance, ensuring maintained attachment and minimal leakage, modulation of host responses, mate guarding and SAT. In order to circumvent host responses, ticks, like mosquitoes, also inject a mixture of antihemostatic, vasoconstriction modulators and anticoagulants [469].

Initially, the ability of tick saliva to assist viral transmission, was first discovered in the transmission of an influenza like virus known as Thogoto, in guinea pigs. Unlike mosquito borne viruses, tick borne virus transmission relies on the co-feeding of uninfected and infected ticks on non-viremic hosts. This non-viremic/co-feeding transmission mechanism relies on tick saliva for successful transmission between ticks [470]. In terms of immune responses triggered in response to tick bites, tick attachment results in the activation and degranulation of mast cells. In order to circumvent this response, ticks utilise compounds that will remove released histamine and serotonin. Tick saliva also directly inhibits the expression of pro-inflammatory cytokines via the use of evasins [471]. In addition, tick saliva has been found to interfere with DC differentiation, maturation, function and migration [471] [472] [473]. Importantly, tick saliva also inhibits the expression of IFNs which indirectly aids arboviral infection [474] [475]. Overall, tick saliva appears to predominantly supress the immune responses of its host, most likely in order to avoid detection and to facilitate blood feeding. This immunosuppression appears to indirectly aid the transmission of tick borne disease [476].

1.7.3 Sandfly saliva

Sandfly saliva, specifically that of *Lutzomyia longipalis*, was the first type of hematophagous saliva that was demonstrated to possess the ability to enhance

arthropod borne disease infection; specifically that of *Leishmania major* [477]. The composition of sandfly saliva differs between species, but differences can also be detected between distinct populations. Similarly to the saliva of other hematophagous insects, sand fly saliva contains molecules that will aid in its acquisition of a successful blood meal including anticoagulant, vasodilatory and immunomodulatory compounds [478]. In terms of immunomodulatory effects, saliva of several sandfly species, have been observed to enhance influx of macrophages to site of infection which can aid transmission of Leishmania, as this parasite preferentially infects and grows within macrophages [479] [480]. Sand fly saliva has also been found to enhance the production of anti-inflammatory cytokines whilst the secretion of pro-inflammatory cytokines is inhibited [481].

Generally, it appears as if arthropod borne pathogen enhancement by the accompanied saliva during their transmission, is a phenomenon that applies to the majority of hematophagous insects. However, evidence shows that saliva composition varies greatly between species, and even between different genus, populations and between the sexes. This is reflected by the observation that different hematophagous insects facilitate pathogen enhancement via different mechanisms. Further research is required to improve our understanding of arboviral enhancement.

1.8 Thesis aims

Mosquito borne viruses make up a significant medical and economic burden on human society. Despite this, very few effective vaccines and treatments are available for the majority of arbovirus associated diseases. The unpredictable nature of these types of viruses (discussed in section 1.5.6) which makes future outbreaks problematic to foresee, in addition to the difficulty in the accurate diagnosis of specific disease due to the similarities in clinical manifestations, highlights the need for the development of novel treatments targeting arbovirus infections.

Previous studies, as has been discussed in detail in section 1.7, have shown that arbovirus infection of mammals is enhanced by the presence of a mosquito bite at the inoculation site, in comparison to the experimental administration of virus via needle inoculation in the absence of a mosquito bite; with inflammatory responses

elicited against the bite appearing to play a key role in this enhancement effect. Furthermore, the experimental inoculation, via a needle, of mosquito saliva alongside virus inoculum, in the absence of the trauma caused by a mosquito bite, also has the ability to enhance viral infections. Considering that mosquito bites/saliva modulate the infection of many distinct viruses, investigating and subsequently targeting these common aspects of mosquito borne virus infections, could provide a strategy that prevents the occurrence of serious disease for multiple genetically distinct arboviruses. Therefore in this thesis we have studied the mechanistic basis of these observations.

Whilst it has been demonstrated that mosquito bites/ saliva enhance virus infection, it remains unclear which specific components of the saliva that are responsible for viral enhancement. In addition, whilst previous studies have indicated that host responses elicited against mosquito bites/ saliva are important for virus enhancement to occur, it remains unclear which specific host responses are required for viral enhancement. Determining the factor within saliva that instigates virus enhancement, as well as the specific host responses elicited against saliva that will trigger virus enhancement, may provide us with potential treatment targets preventing serious disease development.

Furthermore, whilst viral enhancement of several mosquito borne viruses has been demonstrated to occur by the bites/saliva of their corresponding mosquito vector species, to date, no one has investigated whether mosquito bites/ saliva from mosquitoes enhance virus infection of viruses that they do not transmit. Investigating this would help us understand whether all mosquito species are capable of enhancing virus infection, and whether the host mechanisms and salivary components implicated are the same for all mosquito species. It is important to understand whether this is the case if we are to successfully develop a treatment targeting multiple arbovirus infections. It is possible that the enhancement of virus infections are highly species/virus specific. This factor could then contribute to the determination of mosquito vector competence.

In this thesis we hypothesized that extracted mosquito saliva enhances mosquito borne virus infection of mammals in the absence of a bite.

Therefore the aims of this thesis are:

1. **To investigate which components of the mosquito saliva are important for facilitating saliva dependent virus enhancement.**

2. **To investigate which aspects of the host immune response against mosquito saliva are important in facilitating arbovirus enhancement.**

3. **To investigate whether the ability of mosquito saliva to modulate infection has an impact on mosquito vector competence**.

With mosquito bites and the accompanied injection of mosquito saliva alongside viral inoculation in the skin being the common aspect shared by the transmission of all mosquito borne viruses, a better understanding of the mechanisms responsible for arboviral enhancement could lead to the development of a novel treatment that blocks the enhancing effect from occurring in the first place; thereby reducing the risk of more serious illness commonly associated with mosquito-borne virus infection.

CHAPTER 2: Methods

Male Aedes Aegypti

2.1 General reagents

Plastics: All tissue culture plastics used for experiments were purchased from WVR (WVR, USA) or Corning (Corning, Loughborough, UK) unless indicated otherwise. 1.5 ml microcentrifuge tubes were purchased from Eppendorf (Stevenage, UK). All tips were filter tips and purchased from starlabs (UK). QPCR plates were purchased from Sigma Aldrich (USA).

PBS: Dulbecco's Phosphate-Buffered Saline (DPBS) without magnesium was purchased from Gibco (ThermoFisher Scientific, UK).

PBSA: 0.75% bovine serum albumin (BSA) (Invitrogen) with PBS (Gibco, UK).

PFA: 10% PFA (paraformaldehyde) was purchased from ThermoFisher Scientific (ThermoFisher, USA).

10x TAE Buffer: 10x TAE buffer (Tris base, acitic acid and EDTA) was purchased from ThermoFisher Scientific (ThermoFisher, USA).

Toluidine Blue: Crystalized toluidine blue was purchased from Merk (Sigma-Aldrich, USA). Toluidine blue was used as a 0.1% solution by mixing 0.1g of toluidine blue in 100ml of deionized water.

Tissue culture media:

DMEM – Dulbecco's Modified Eagle Medium (DMEM) (Gibco, life technologies, UK) was supplemented with 10% FCS (Gibco, life technologies, UK), 10% tryptose phosphate broth (TPB) (Sigma Aldrich, USA), 5 ml Pen/Strep (Penicillin/streptomycin) (Gibco, life technologies, UK) and 5ml Glutamine broth (Sigma Aldrich, USA).

GMEM – Glasgows Minimum Essential Medium (GMEM) (Gibco, life technologies, UK) was supplemented with 5% fetal bovine serum (Thermo Fisher, USA), 10% TPB (Thermo Fisher, USA) and 2% Pen/Strep (Thermo Fisher, USA).

RPMI – Roswell Park Memorial Institute (RPMI) media was supplemented with 5ml Glutamax (ThermoFisher, USA), 10% FCS (Gibco, Life Technologies, UK) 1.4 ml 1M HEPES, 550µl 10-5M β-mercaptoethanol (Sigma Aldrich, USA), 5ml Penicillin/streptomycin (Thermo Fisher, USA), 500µl gentamycin (Thermo Fisher, USA).

L-15 – L-15 medium was supplemented with 10% FBS (Thermo Fisher, USA), 10% TPB (Thermo Fisher, USA) and 2% Pen/Strep (Thermo Fisher, USA).

DMEM/F12 – DMEM (Gibco, life technologies, UK) and Ham's F12 nutrient mixture (Gibco, life technologies, UK) were mixed 1:1 and supplemented with 10% FCS, 1% Pen/Strep, 0.1% gentamycin (Thermo Fisher, USA) and 1% Glutamine broth (Sigma Aldrich, USA).

Tissue digestion buffer: Tissue digestion media for flow cytometry was made up from 0.9ml of Hanks balanced saline solution (HBSS) (Sigma Aldrich, USA), 1mg/ml of Collagenase D (Roche/Sigma Aldrich, USA) (skin and spleen only), 0.5mg/ml of Dispase II (Roche/Sigma Aldrich, USA) (skin and spleen only), 0.1mg/ml DNase I (Roche/Sigma Aldrich, USA) and 0.1mg/ml liberase (Roche/Sigma Aldrich, USA) (lymph node only).

FACS buffer: 500 ml PBS without Ca2+ and Mg2+ was mixed with 0.5% FCS and 2mM EDTA (Invitrogen).

RNase free water: RNase free water was purchased from Invitrogen.

2.2 Mice

Wild type C57BL/6j mice bred in-house at the SBS at the University of Leeds were used in all *in vivo* experiments unless stated otherwise. Mice were maintained at SBS under specific pathogen free conditions and used between 4 and 12 weeks of age unless stated otherwise. BALB/C mice were purchased from Charles River Laboratories. Mice were age and sex matched in all *in vivo* experiments. All

procedures were carried out in accordance with the United Kingdom Home Office regulations under the authority of the appropriate project and personal license.

2.3 Cell Culture

BHK-21 cells were used to grow-up virus stock and determining viral titres via plaque assays. Cells were kept at -195°C for long term storage. For de-thawing, 37°C water bath was used following immediate transfer to a T75 flask containing media. BHK-21 cells were cultured at 37°C with 5% CO2 in GMEM media supplemented with 10% TPB, 5% FCS (Gibco Life technologies) and 1% Pen/strep.

Aedes albopictus mosquito derived C6/36 cells were used for growing virus stocks. Cells were kept at -195°C for long term storage. For de-thawing, 37°C water bath was used following immediate transfer to a T75 flask containing media. C6/36 cells were cultured at 28°C at 0% CO2 in L-15 media (Gibco Life technologies) supplemented with 10% TPB, 10% FCS and 1% Pen/strep.

Mouse Embryonic Fibroblasts from C57BL/6 mice (cell biologics, USA) were kept at -195°C for long term storage. For de-thawing, 37°C water bath was used following immediate transfer to a T25 flask pre-coated with 0.2% gelatine (cell biologics, USA) and containing media. MEF cells were cultured at 37°C at 5% CO2 in DMEM media supplemented with 10% FCS, 1% Pen/Strep and 1% Glutamax (Gibco Life technologies).

2.3.1 M-CSF Macrophages

Macrophages were extracted from C57BL/6 mouse bone marrow by flushing cells from the femur using a 26-gauge needle with cold PBS. Cells were then passed through a 40µm cell strainer in order to remove any potential debris. Cells were cultured at 37°C at 5% CO2 in DMEM/F12 media (Gibco Life technologies) supplemented with 10ng/ml M-CSF (Peprotech) to ensure monocytes differentiate into macrophages, 10% FCS, 1% Pen/Strep, 1% Glutamax and 0.1% Gentamycin. $4x10^5$ cells were seeded in 10ml media per sterile plastic petri dish used. New media was added to the cells after 3 days. 7 days after extraction cells were pooled by washing with PBS (Gibco Life technologies) and adding 3ml Cellstripper (non-

enzymatic cell dissociation solution) to each dish. Plates were then incubated in the fridge for 5min to make cells constrict. Cells were then gently scraped of the plastic whenever necessary. All cells were then pooled, counted and centrifuged at 300g for 5 minutes before being seeded at a concentration of $2x10^5$ per well in a 24-well plate or at $5x10^4$ per well in a 96-well plate using complete DMEM/F12 media.

2.4 Viruses

Semliki Forest virus 4 (SFV4) and Semliki Forest virus 6 (SFV6) stocks were generated from plasmids containing the genomic sequence provided by Andres Merits (University of Tartu). ONNV-2SG-ZsGreen with ZsGreen cloned between native and duplicated SG promoters of ONNV icDNA using AvrII (5', ligated to Nhe I) and Eco RI (3') restriction sites and pCMV-SFV6-2SG-GLuc (Gaussia luciferase) plasmid were kindly provided by Margus Varjak (CVR, Glasgow). SFV6 Gluc has a Gluc marker inserted under a duplicated subgenomic promoter positioned at 3' direction of structural reading frame. Plasmids containing the genomic sequence of all viruses were electroporated into BHK cells to generate infectious virus with 2 pulses at 250V for 0.8S. Infectious virus of SFV6 Gluc was aliquoted with cellular debris to allow for improved virus uptake by macrophages *in vitro*. Wild type Zika virus from Recife, Brazil was kindly provided by Prof. Alain Kohl at the university of Glasgow. Virus was grown in Vero cells and BHK-21 cells, supernatant was collected then centrifuged to remove cell debris and virus titers were determined by plaque assays on BHK-21 cells. All viruses used *in vivo* were passaged once through C6/36 cells. Supernatant from C6/36 cells was collected and infectious virus present in the supernatant was titrated via plaque assay in BHK-21 cells.

2.5 Plaque Assays

For titrating virus stocks and for the quantification of viraemia plaque assays were performed. For the plaque assays BHKs were used at an 80% confluency in a 12-well plate with virus serial dilutions made with 0.75% PBSA (PBS with 0.75% bovine serum albumin). 200µl virus was added to each well and left for an hour whilst rocking occasionally. 2 ml 2 x MEM medium (Gibco Life technologies) with 4% FCS (Gibco Life technologies), 200 units/ml penicillin and 0.2 mg/ml streptomycin

mixed with 1.2% Avicel (FMC Biopolymer, UK), which is a low-viscosity overlay medium used in viral plaque assays, was then added to the cells. Cells were incubated for 2 days at 37°C with 5% CO_2. After 2 days cells were fixed in PFA for an hour and stained with 0.1% Toluidine Blue (Sigma Aldrich) for at least 30min. PFU was calculated per ml using the following equation:

$$\text{PFU/ml} = \frac{\text{average number of plaques (in duplicate)}}{\text{amount of inoculum x dilution factor}}$$

2.6 Mosquito rearing

Aedes aegypti (Liverpool strain) mosquitoes were kept in incubator with 80% humidity at 28°C with a 12-hour light/dark cycle. Eggs on filter paper, kindly provided by Dr. Emilie Pondeville at the University of Glasgow, were placed in trays (Dutscher Scientific) containing approximately 1.5 cm of water to hatch overnight. Larvae were then fed with Go-cat cat food until pupation. *Ae.albopictus* (La Providence strain) and *Cx.pipiens* (slab strain) were reared under the same conditions as *Ae.aegypti*.

Anopheles gambiae (Kisumu strain) mosquito eggs were kindly provided by Dr. Francesco Baldini at the University of Glasgow. The eggs were hatched the same day of arrival by placing in water. Ground Tetramin fish flakes were fed to the larvae the first days following which the larvae were fed with tetramin pellets until pupation. When pupae formed these were picked and placed in small water filled containers and left to emerge into BugDorm mosquito cages (Watkins and Doncaster). All adult mosquitoes were fed a 10% sucrose solution. Mosquitoes were ready for salivations and biting experiments 21 days post hatching. *An.stephensi* (SDA-500 strain) were reared under the same conditions as *An.gambiae*.

2.6.1 Mosquito Antibiotic Treatment and validation

An antibiotic treatment was developed and validated. Mosquitoes were given a 10% sugar solution containing Penicillin/streptomycin (Gibco) at 200 U per ml, Gentamycin at 200 µg/ml Gentamycin (Sigma), and Tetracycline at 100 µg/mL for 1 week. The treatments efficiency was then validated by dipping treated and untreated control mosquitoes in 70% ethanol, then placing them in 400µl PBS. They

were then shaken with one 7mm stainless steel bead on the Tissue Lyser for 10min at 50Hz. The samples were then plated on agar plates and left overnight in an incubator at 28°C to allow for bacteria to grow before counting. [482]

2.7 *In vivo* model

Mice were anesthetized with 0.1ml/10g of Sedator/Ketavet via intraperitoneal (I.P.) injection and placed on foil on top of the mosquito cages with the dorsal side of one or both hind feet exposed to allow no more than 5 mosquitoes to feed. Great care was taken to cover the toes with tape to prevent mosquitoes biting. Mosquitoes were left to feed until fully engorged. Virus injections of either C6/36 derived SFV6 (250 PFU) or SFV4 (10000 PFU in 1µl) were then made accordingly directly at the bite site with a 5µl 75N syringe, 26ga (Hamilton) using small RN ga33/25mm needles (Hamilton). The viruses were diluted in PBSA to $1x10^7$ PFU/ml. Saliva injections were made at a concentration of 5 mosquitoes-worth of saliva per injection.

2.7.1 Survival and mice monitoring

Mice subjected to neurotropic virus infections were monitored 4 times daily and weighed every morning for the entire duration of the experiment. Mice demonstrating 2 or more of the symptoms listed in table 2.1 were immediately culled. Surviving mice were culled at day 15 post infection via schedule 1.

Moderate	Severe
Loss of body weight of up to 20%	Loss of body weight greater than 25%
Reduction in food and water consumption of up to 40% less than normal for 72 hours	Reduction in food and water consumption of up to 40% less than normal for 7 days or anorexia (complete inappetence for 72 hours)
Staring coat-marked piloerection	Marked piloerection accompanied with other signs of dehydration including skin tenting
Subdued even when provoked. Limited peer interaction	Unresponsive to activity and provocation
Hunched intermittently	Hunched persistently

Vocalisation if provoked	Distressed – persistent vocalisation
Persistent oculo-nasal discharge	Persistent and abundant oculo-nasal discharge
Intermittent abnormal breathing	Laboured respiration
Intermittent tremors	Persistent tremors
Intermittent convulsions	Persistent convulsions
No self-mutilation	Self-mutilation
Intermittent prostration (< 1 hour)	Prolonged prostration (> 1 hour)

Table 2.1. Common symptoms of mice infected with SFV and the associated severity category.

2.7.2 Dissections

Mice were culled via a schedule 1 method. Tissues dissected depended on the experiment but most commonly included, skin from foot, popliteal lymph node and spleen. Blood samples were also collected from the ventricles. Tissue samples collected were stored in 0.5ml RNAlater (Sigma Aldrich, USA) in 1.5ml tubes, with the exception of spleen and brain samples that were cut in half and stored in 1ml of RNAlater to enable complete permeabilization of the RNAlater in to the tissue. All samples were left in RNAlater for a minimum of 16 hours to prevent RNA degradation. Samples were then stored at 4^{o}C short term storage or at -80^{o}C for long term storage. Blood samples were centrifuged and serum was collected and stored at -80^{o}C until use.

2.7.3 Oedema

Vascular permeability, vascular leakage and the formation of oedema can be successfully measured by the use of Evans Blue (EB) dye. EB is a dye with a molecular weight of 961Da which binds strongly to serum albumin when injected into a mouse [483, 484]. The EB that's bound to albumin will then remain stable in the blood and can then spread throughout the entire body of the mouse often causing it to turn blue. Selectively permeable barriers, such as the endothelial cell barrier of the blood vessels, normally only allow specific micromolecules to pass and not macromolecules such as albumin. Therefore, when this physiological function is disrupted it can lead

to an increase in the permeability of the barrier allowing the EB-bound albumin to cross causing a blue-stained oedema to form [485]. In order to determine the amount of fluid accumulation and vascular leakage in the skin, mice were injected subcutaneously with 200µl of 1% Evans Blue.

Skin samples were acquired 30min, 3 hours or 6 hours post challenge and placed in 250µl of formamide and left to soak overnight at 4^{o}C. Skin samples were then removed from the solution and the dye-stained formamide solution was taken and a 10-fold serial dilution was created by mixing the samples with water. Levels of fluid accumulation was then determined using colorimetric measurement of dye concentration at 620nm using the Multiskan Ex (Thermo scientific). Blood samples were acquired and centrifuged. Amount of dye present in the serum was used as a control for amount of dye present in each mouse.

In order to ensure the complete removal of any residual dye from the blood in the skin tissue perfusions were carried out immediately after acquiring blood samples. During this process, using a 50ml syringe of PBS with a 26-gauge needle which was inserted into the ventricles and the PBS pumped in to ensure the flush out of blood from the entire circulation.

2.7.3.1 Inhibition and induction of oedema

Oedema was induced in the absence of any mosquito factors via the use of Histamine dihydrochloride. For this purpose Soluprick (ALK, UK) was injected as a 1µl sub-cut injection at a concentration of 10mg/ml using a 5µl 75N syringe, 26ga (Hamilton) with a small RN ga33/25mm needles (Hamilton). Oedema was inhibited using Adrenaline (Epinephrine) Xylocaine 1% containing lidocaine and adrenaline 1:200,000 (Aspen, Ireland). 4µl of the substance was given as a local subcutaneous injection prior to exposure to potential oedema inducing agents such as mosquito bites, saliva or histamine. An additional 4µl of adrenaline were administered 3h post exposure. Antihistamines were administered as an IP injection 1 hour prior to challenging mice with mosquito saliva or histamine. Antihistamines were administered as a mixture of 0.5mg/kg Cetrizine hydrochloride (sigma), 5mg/kg Loratadine (Alfa aesar) and 10mg/kg Fexofenadine hydrochloride (Alfa aesar).

2.7.4 Sensitization of mice to mosquito saliva

For sensitization experiments BALB/c mice were utilised. Sensitized mice were subjected to 5 mosquitoes worth of saliva injections in 1 µl of PBSA weekly for 4 consecutive weeks. Injections were made on dorsal side of left hind foot. A group of mice were exposed exclusively to PBSA injections weekly to rule out sensitization to BSA.

2.8 Saliva extraction

Mosquito saliva was acquired via forced salivation of *Aedes aegypti* and *An.gambiae* mosquitoes which had been starved for one day prior to salivation. Salivation was then done by aspiring mosquitoes with a custom made hand-held hoover and sedating them on ice. Females and males were separated based on certain characteristics including fluffier antennae of males, larger torso of females (see figure 2.1).

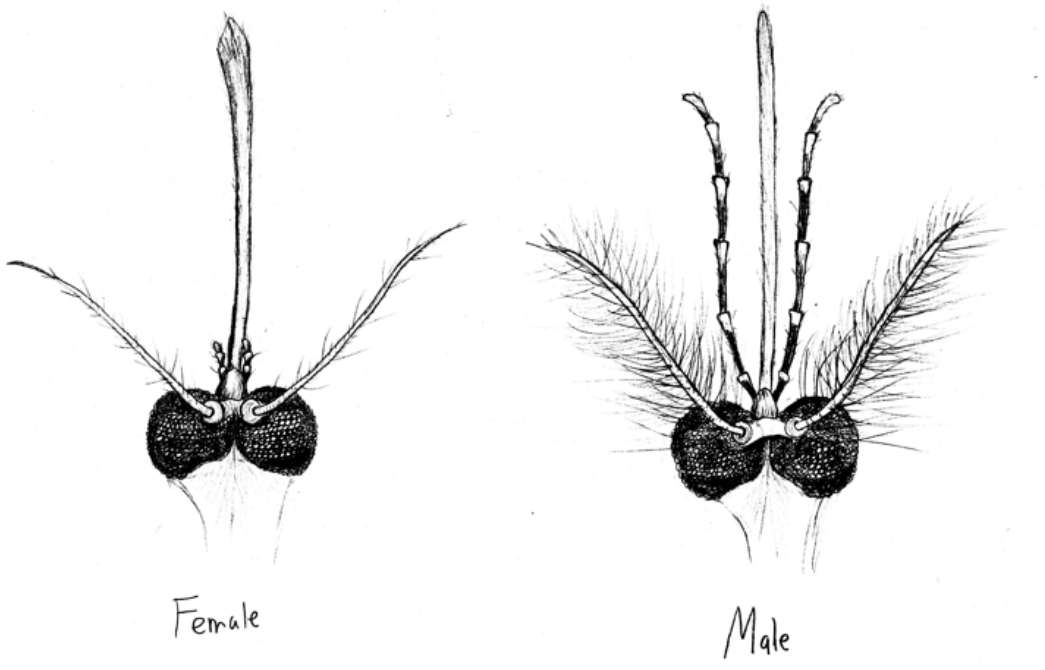


Figure 2.1. Physical differences between male and female *Ae.aegypti* mosquitoes.

Illustration highlighting characteristic differences between male and female *Ae.aegypti* mosquitoes. Note the fluffier antennae of the males and the considerably longer palps. These physical differences allow for easy sexing of mosquito species.

Wings and legs were then removed under a dissection microscope and their proboscis was placed in a p10 tip containing 0.5µl immersion oil (Cargille Laboratories, USA). Mosquitoes were then left to salivate for up to an hour before tips were placed in an Eppendorf tube and centrifuged (see figure 2.2). Saliva droplets were then pooled and stored at -80°C. Before use, droplets of saliva were carefully pipetted out of the oil under microscope and diluted in PBSA. 5 mosquitoes worth of saliva was utilised per injection unless stated otherwise. Majority of saliva samples were kindly provided by Dr. Emilie Pondeville at the University of Glasgow whilst the remainder were extracted at the University of Leeds.

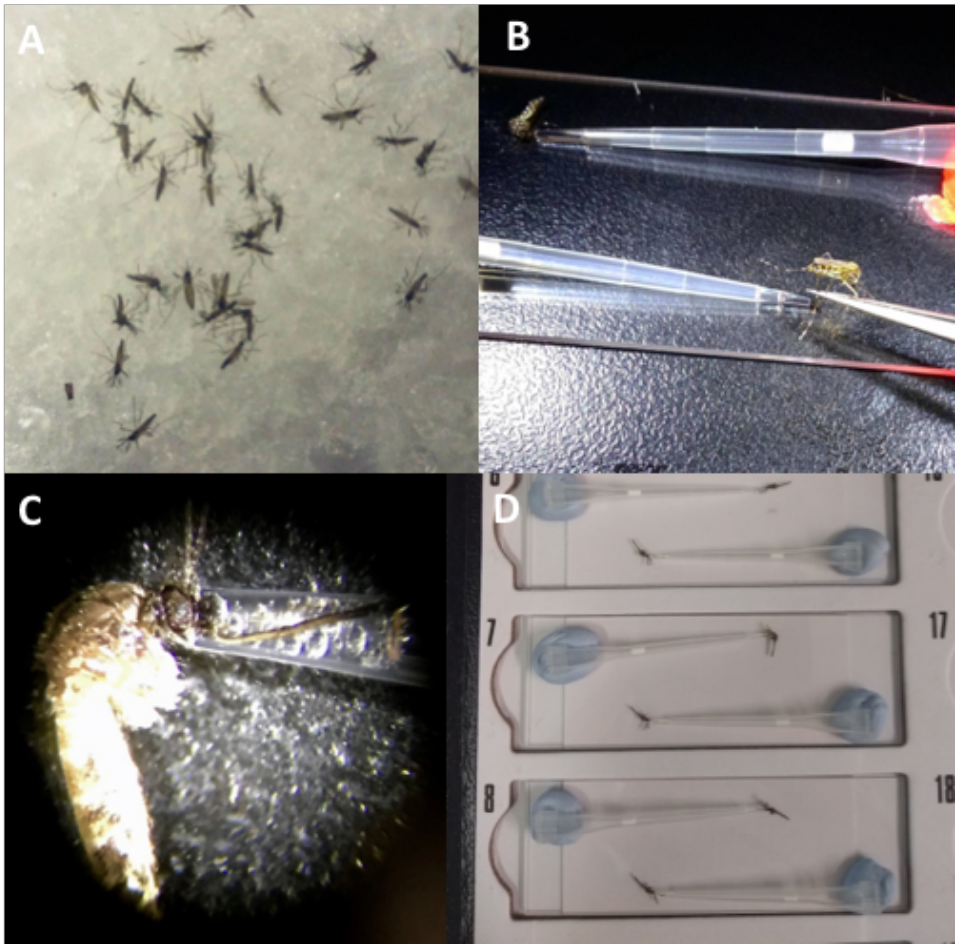


Figure 2.2. Images illustrating the mosquito salivation process.

2.9 Infection of mouse skin explants

Mice were culled via a schedule 1. Skin was then dissected from the hind feet and transferred into a 24 well tissue culture plate containing complete DMEM (Gibco, life technologies, UK) supplemented with 10% FCS (Gibco, life technologies, UK), 10% tryptose phosphate broth (TPB) (Sigma Aldrich, USA), 5 ml Pen/Strep

(Penicillin/streptomycin) (Gibco, life technologies, UK) and 5ml Glutamine broth (Sigma Aldrich, USA). Explants were kept at 37°C with 5% CO2.

2.10 Infection and saliva treatments of cells in cell culture

Extracted and differentiated macrophages and MEF cells were seeded in 24 well or 48 well plates. Cells were either pre-treated with saliva and then infected, or saliva was pre-mixed with the virus for 20min prior to its addition to the cells. 1.8 mosquitoes worth of saliva was used per well. Cells were infected with SFV6 Gluc virus at an MOI (multiplicity of infection) of 0.01, 0.1, 1 or 5. Supernatant was then acquired at 6, 24 and 48 hpi in order to monitor the progression of infection.

2.11 RNA purification

2.11.1 Tissue samples

RNA extractions were performed using the RNA mini purification kit by life technologies by following the protocol provided with the kit. All tissue samples were lysed in 1ml Trizol reagent (QIAGEN, UK) and shaken with 7mm stainless steel beads (QIAGEN, UK) on a Tissue Lyser (QIAGEN, UK) at 50Hz for 10 minutes to ensure complete lysis of all tissues. 0.2ml chloroform was then added to all samples which were then inverted 15 times to allow for gentle mixing of the solutions. Afterwards, samples were centrifuged at 12,000g for 15 minutes at 4°C in order to separate the mixture into a lower red phenol-chloroform phase and a colourless upper aqueous phase. The upper aqueous phase aqueous phase, containing the RNA, was transferred to a new tube containing an equal amount of 70% ethanol (Sigma Aldrich, USA). The samples were vortexed briefly to ensure no precipitate was formed prior to transferring of the sample to the RNA column. 2 wash steps each of wash buffer I and wash buffer II were then conducted, were 350µl or 500µl respectively was added to the column which was then centrifuged for 15 seconds at 12,000g and the flow through discarded. An on column DNAse step was included in between the first and second wash with wash buffer I to ensure all genomic DNA contamination was degraded to prevent SYBR green from binding to it. During this step 80µl of DNase mixture (10µl DNase, 70µl reaction buffer) was added to each spin column before incubating for 15 minutes at room temperature. After the washes

the column was centrifuged for a minute to ensure the column was dry before eluting the RNA in RNAse-free water by the addition of 30µl of RNase free water for lymph node samples, 60µl for skin samples and 100µl for brain and spleen samples and left to incubate at room temperature for 1 minute prior to centrifugation at 12,000g for 1 minute. Purified RNA was then stored at -80^{o}C.

2.11.2 Cells

All cell samples were lysed using the lysis buffer contained in the RNA micro kit (Life Technologies) with 1% β-mercaptoethanol (Sigma Aldrich, USA). Samples were then centrifuged using QIAshredders (QIAGEN) to ensure complete lysis of cells. RNA extractions were then made through on column purifications in the same way as tissue samples, but using the Purelink RNA micro kit.

2.11.3 Measurement of RNA quality and degradation

RNA samples were routinely analyzed via nanodrop (Spectrophotometer, ND-1000) blanked with 1µl. Purified RNA was stored at -80°C.

2.12 cDNA synthesis

Approximately 1µg of RNA in a volume of 9µl of RNAse free water was moved to a 96 well plate (StarLab, UK) and kept on ice. Using the "Applied Biosystems High Capacity RNA to cDNA" kit (AppliedBiosystems, ThermoFisher, USA), an enzyme-buffer mix was made consisting of 1µl of 20X RT Enzyme Mix which was mixed with 10µl of 2X RT Buffer Mix per reaction in a 2ml microcentrifuge tube. 11µl of the enzyme-buffer mix was then added to each well containing RNA resulting in a final reaction volume of 20µl. After a brief centrifugation of the plate in order to ensure the removal of any bubbles, the plate was sealed with a StarSeal® aluminum foil cover (StarLab, UK). The plate was then transferred to the GeneAmp® PCRsystem2700 (AppliedBiosystems, ThermoFisher, USA) PCR machine where it was incubated at 37°C for 60 minutes then stopped by heating to 95°C for 5 minutes. The final cDNA was then stored at 4°C for short term use and -20°C for long time storage.

2.13 RT-qPCR

The method of quantitative polymerase chain reaction was utilized in order to measure the relative expression of a range of transcripts during this thesis. A list of all primers targeting these transcripts can be found in table 2.2. RT-qPCR combines the polymerase chain reaction (PCR) technique with detectable fluorescent molecules such as SYBR green (used in this thesis) which binds to double stranded DNA. Real time detection of fluorescence allows us to correlate the concentration of DNA with the florescence intensity. This correlation is measured via the cycle threshold (Ct) which indicates the number of PCR cycles required in order for the fluorescence to exceed background values. Generally this means that the higher DNA concentration, the less PCR cycles are required before the reaction reaches the Ct cycle threshold.

2.13.1 Primer design

All primers used in this thesis are listed in table 2.2. Primers were designed using Primer3 software, version 4.0 (bioinfo.ut.ee); all of which were designed to meet the following criteria:

1. 18 and 23 base pairs (bp) in length
2. 40% and 65% guanine (G) and cytosine (C) content
3. Melting temperatures (Tm) of primers between 59.5^{o}C and 61^{o}C
4. maximum self-complementarity of 2
5. maximum 3' prime end self-complementarity of 1
6. amplicon size of less than 150 base pairs
7. no GC clamp at the 3' end

Exceptions to these rules were made if no primers were suggested by the software. For example the minimum Tm was altered to 59°C or the maximum self-complementarity was altered to 3 if no primer was found. 2 sets of primers were designed for each gene; the first was used for quantitative PCR and the second was used to generate PCR products to be used for the making of standard templates. The standards' primers were designed to amplify a section where the qPCR primers could bind. Primers were purchased from Integrated DNA Technologies (IDT, USA) and

Sigma Aldrich (USA) and reconstituted upon arrival to a final concentration of 0.1nm/µl.

2.13.2 Generation of DNA standards for absolute quantitative QPCR

In this project relative quantities of the specific genes of interest were used as absolute quantities were not necessary due to the fact that all experiments were specifically designed to be comparable to internal controls via a fold change analysis. Relative quantification was calculated via the use of a standard curve which functions as a calibrator where the values estimated for each sample are divided by the arbitrary value that has been assigned to the standard. The standards used were generated via a PCR reaction of a random sample known to contain the gene of interest in combination with specific primers specifically designed for the gene of interest. 4µl of the sample were mixed with 0.5µl each of the forward and the reverse primers as well as 45µl RED PCR master mix Rovalab, VH-Bio, UK) containing MgCl2, 1mM dNTPs and Taq DNA polymerase. A PCR reaction was then undertaken using a GeneAmp 9700 (Applied Biosystems, ThermoFisher, USA) or Venti 96 (applied Biosystems, ThermoFisher, USA) using the following programme:

1. 3 minutes at 94°C
2. 15s at 94°C for denaturing of DNA
3. 10s at 60°C for annealing of primers
4. 50s at 72°C for extension
5. 7 minutes at 72°C
6. Held at 4°C

×40 cycles

The PCR product was run on a 2% agarose gel with ethidium bromide at 80 -100V for 1 hour. A DNA ladder was used to determine the molecular weight of the product and the bands were visualized using the ChemiDoc XRS+ gel imager (BioRad, USA). Primers were only purified if clear single bands were detected at the correct molecular weight. In the cases were no or multiple bands were observed new primers were designed and the old ones were discarded.

QIAQuick PCR product purification kit (Qiagen, Germany) was used for the purification of the PCR products. During this procedure the product was mixed with 5 x volume of buffer PB. The mixture was then added to the spin column before being centrifuged. 750µl buffer PE was then added before a second centrifugation. After discarding the flowthrough the membrane was dried by the spinning of the column for 1 minute at 13,000g. The product was finally eluted in 50µl of elution buffer. The purified standards were diluted 100-fold and stored in a -20^{o}C freezer.

Gene Name	Orientation	Sequence	Product size (bp)	NCBI Reference
18S	Forward	gactcaacacgggaaacctc	124	NR_003278.1
	Reverse	taaccagacaaatcgctccac		
18S Standard	Forward	cgtagttccgaccataaacga	443	NR_003278.1
	Reverse	acatctaagggcatcacagacc		
CCL5	Forward	ctgctgctttgcctacctct	124	NM_013653
	Reverse	acacacttggcggttcctt		
CCL5 Standard	Forward	ccctcaccatcatcctcact	280	NM_013653
	Reverse	tcagaatcaagaaaccctctatcc		
CXCL10	Forward	tgccacgatgaaaaagaatg	182	NM_021274
	Reverse	aggggagtgatggagagagg		
CXCL10 Standard	Forward	atccctgcgagcctatcc	524	NM_021274
	Reverse	aaacttagaactgacgagcctga		
IFN-α	Forward	aggacaggaaggattttgga	186	NM_010504
	Reverse	gctgctgatggaggtcatt		
IFN-α Standard	Forward	tggctaggctctgtgctttc	385	NM_010504
	Reverse	ggaggttcctgcatcacac		
IFN-γ	Forward	agcaaggcgaaaaaggatg	66	NM_008337
	Reverse	ctggacctgtgggttgttg		
IFN-γ Standard	Forward	atctggaggaactggcaaaa	597	NM_008337
	Reverse	agatacaaccccgcaatcac		
IFN-β	Forward	cacagccctctccatcaact	152	NM_010510
	Reverse	gcatcttctccgtcatctcc		
IFN-β Standard	Forward	ggcttccatcatgaacaaca	399	NM_010510
	Reverse	tcccacgtcaatctttcctc		
Rsad2	Forward	tgaagcgtggcggaaagtat	73	NM_021384.4
	Reverse	tccttcccatctcagcctca		
Rsad2 Standard	Forward	ctgtgcgctggaaggttttc	583	NM_021384.4
	Reverse	cactggaccttgctcctctg		
IFIT2	Forward	tgcaccacactagcttgca	96	NM_008331.3
	Reverse	gggatggaagcactcacagt		
IFIT2 Standard	Forward	gcacctctatgtttgagcagtt	290	NM_008331.3
	Reverse	gcagaaaagtcaaggcaggaa		
ISG15	Forward	cgcagactgtagacacgctta	80	NM_015783.3
	Reverse	ctcgaagctcagcagaact		

ISG15 Standard	Forward	gtccgtgactaactccatgac	504	NM_015783.3
	Reverse	tcccaaaagtcctccatacc		
SFV E1	Forward	cgcatcaccttcttttgtg	173	DQ_189086
	Reverse	ccagaccacccgagatttt		
SFVE1 Standard	Forward	aagtgaagacagcaggtaaggtg	446	DQ_189086
	Reverse	tatgagttgccccgagtttc		
Zika ENV	Forward	ggaggctgagatggatggt	148	KX_197192.1
	Reverse	cagtgtttcagccgggatct		
Zika ENV Standard	Forward	aggcaaactgtcgtggttct	679	KX_197192.1
	Reverse	tcagacccaaccacatcagc		
CXCL2	Forward	aagtttgccttgaccctgaa	129	NM_009140
	Reverse	tctctttggttcttccgttg		
CXCL2 Standard	Forward	cgcccagacagaagtcatag	484	NM_009140
	Reverse	actcaccctctccccagaaa		
IL-1β	Forward	cgctcagggtcacaagaaac	67	NM_008361.3
	Reverse	gaggcaaggaggaaaacaca		
IL-1β standard	Forward	aaagtatgggctggactgtttc	410	NM_008361.3
	Reverse	atgtgctggtgcttcattca		
16s[452]	Forward	aagtttgccttgaccctgaa	129	NM_009140
	Reverse	tctctttggttcttccgttg		
16S standard	Forward	cgcccagacagaagtcatag	484	NM_009140
	Reverse	actcaccctctccccagaaa		
IL-5	Forward	tcctgcctcctcttcctgaa	147	NR_003278.1
	Reverse	accctgatgcaacgaagagg		
IL-5 standard	Forward	acagagtgggcaatggaagg	422	NM_010558.1
	Reverse	gggtatgtgatcctcctgcg		
IL-13	Forward	tgccatctacaggacccaga	146	NM_008355.3
	Reverse	cgtggcgaaacagttgcttt		
IL-13 standard	Forward	gtgtctctccctctgaccct	358	NM_008355.3
	Reverse	tgagtccacagctgagatgc		
CCL2	Forward	ctcacctgctgctactcattca	153	NM_011333.3
	Reverse	ccattccttcttggggtca		
CCL2 standard	Forward	caccagcaccagccaact	519	NM_011333.3
	Reverse	gcatcacagtccgagtcaca		
ONNV E1	Forward	acgctccttccatcacagac	72	AF192890.1
	Reverse	cggcacctccaaaatcag		
ONNV E1 standard	Forward	gcagtgggcaacataccag	544	AF192890.1
	Reverse	cggatagtgaccgcatttgt		

Table 2.2. Primer list. List of primers, primer orientation, sequences, product size and NCBI reference for all qPCR primers used in this thesis.

2.13.3 qPCR using SYBR green

All RT-qPCR reactions in this project, were done using SYBR® green I (Quanta, ThermoFisher, USA) for the fluorescent labelling of DNA. SYBR® green is a fluorescent dye which binds nonspecifically to double stranded DNA whilst unbound SYBR® does not fluoresce. Unbound DNA can fluoresce when in a complex with double stranded DNA – with an excitation (λ_{max}) of 497nm and an emission (λ_{max}) of 520nm. cDNA was diluted 1 in 5 in RNAse free water. This was done in order to prevent the interference of the RT buffer with the PCR reaction. Following this, a master mix was created made up of cDNA, primers, water and SYBR® green mix. For each individual sample, 1µl of cDNA was mixed with 4µl of RNAse free water, 5µl SYBR® green I and 0.15µl of primer mixture containing both the forward and reverse primers; making a final volume of 10.15µl. 9µl of this mixture was then transferred to a 384 well plate (StarLab, UK). A triplicate or a quadruplicate technical replicate was made for each biological replicate. The generation of a standard curve was accomplished by the dilution of the 10^{-2} PCR standard in a10-fold serial dilution. A non-template control (NTC) consisting of RNAse free water and the master mix was also included. After the loading of all the samples into the 384 well plate the plate was briefly centrifuged at 200G for 15-30 seconds in order to ensure the removal of any potential air bubbles. Following centrifugation the plate was sealed with StarSeal Advance Polyolefin seals (Starlab, UK). The plate was kept at 4°C in the dark until the reaction.

The PCR plates were finally run on an Applied Biosystems 7900HT or Applied Biosystems quantstudio 7 flex machine (AppliedBiosytems, USA). The PCR protocol consisted of the following steps:

1. 94°C for 10 minutes
2. 94°C for 3 seconds
3. 60°C for 30 seconds

×25-40 cycles

4. Disassociation and melt curve (records fluorescence between the final temperature increase of 60°C to 94°C)

Ct value was calculated automatically by the quantstudio software which detects the logarithmic phase of the PCR reaction. The threshold calculated was equal to the cycle where fluorescence exceeded background levels. Each samples relative quantity was calculated based on their position on the standard curve. The standard curve had to have an efficiency close to 100%, which was indicated by the coefficient $R^2 \geq 0.998$ and a slope of 3.3. Melt curves were conducted in order to investigate the specificity of each primer as a single peak on a melt curve indicates that the primers are specific.

2.13.4 Normalization of qPCR data

In order to control for any potential differences in absolute quantities of nucleic acid between samples, the normalization of samples to a housekeeping gene was conducted. Samples can exhibit variations in nucleic acid quantities due to a range of different factors including differences in tissue size, partial degradation of sample (RNA or cDNA) during freeze thaw cycle. The housekeeping gene used on all occasions, unless stated otherwise, was the 18S gene which consists of ribosomal rRNA from the 18S ribosomal subunit. Using 18S as a housekeeping gene has the advantage that the gene remains stable and ubiquitously expressed in all tissues during arbovirus infection. The relative expression of the gene of interest was then normalized to that of the housekeeping gene by the division of the quantity of the gene of interest with the quantity of the 18S of each sample. The quotient is then multiplied by the arbitrary value of $1x10^7$ in order to scale up the values.

2.13.5 Analysis

Analysis of qPCR data was done with Microsoft Excel by the use of the median of the technical replicates and normalizing them to the median of the technical replicates of the housekeeping genes.

2.14 Luciferase assay

There are a range of different luciferase types used in research. In this project Gaussia luciferase (Gluc) was utilised at all times. Gluc is a protein naturally expressed by *Gaussia princeps*. The production of light from the use of luciferase is

based on the oxidative decarboxylation of coelenterazine by Gluc which leads to the production of coelenteramide, carbon dioxide, and light. As Gluc is secreted into the culture medium by infected cells quantification of Gluc was made in the supernatant of infected cell culture. The enzymatic activity detected in the supernatant correlates with the amount of infectious particles that are released from the virus infected cells.

2.14.1 Infection of cells *in vitro* with SFV6 expressing Gaussia luciferase

Luciferase assays were performed with SFV6 virus modified to express Gaussia luciferase. Bone marrow derived M-CSF macrophages or MEF cells were seeded at a known concentration in 24 or 96 well plates and infected with a known amount of SFV6. Cells were either pre-treated with mosquito saliva, or saliva was added pre-mixed with the virus.

2.14.2 Detection of luciferase

For detection of luciferase in macrophages and fibroblasts infected *in vitro* Renilla Luciferase Assay System (Promega) kit was used and samples were run on Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Germany). 20µl of cell supernatant was diluted with an equal amount of 1:5 lysis buffer supplied with the kit. Luciferase substrate was mixed with the Luciferase buffer at 1:100. Samples were loaded onto white bottomed 96 well plates (Starlab, UK).

2.15 ELISA

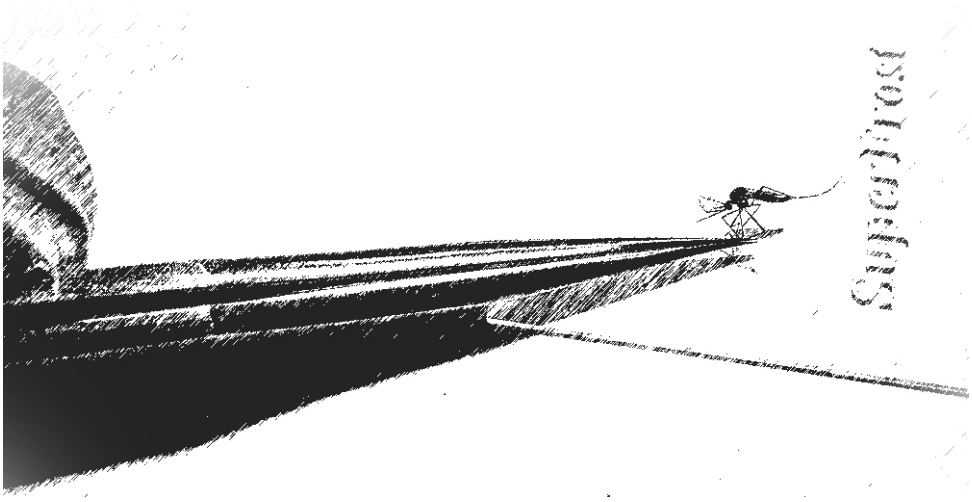
ELISA (Enzyme-linked immunosorbent assay) was conducted using Mabtechs ELISA development kit. High protein binding ELISA plates were coated with 2µg/ml of capture antibody diluted in PBS and incubated overnight at 4°C. Plates were washed twice with PBS the following day and plates were then blocked with reagent diluent containing PBS with 0.05% Tween 20 and 0.1% BSA for 1 hour at room temperature to prevent non-specific binding. Plates were then washed 5 times with wash buffer containing PBS with 0.05% Tween 20. Standard were reconstituted to a concentration of 0.5µg/ml. Serial dilutions were made 1:2 ranging from the highest concentration to 10pg/ml. 100µl of sample or standards diluted in incubation buffer was then added to the wells in duplicates for 2 hours. Plats were then washed

5 times as previously and then incubated with 100 µl of detection antibody at 200 ng/ml for 1 hour at room temperature. Following another washing step the plates were incubated with 100 µl of streptavidin-HRP (1:200 diluted) for 20 minutes in the dark. The plates were then washed again 5 times and 100µl substrate solution was added. The reaction was then stopped utilising Stop Solution (Invitrogen). The plate was read on the Multiskan EX microplate reader (Thermo scientific) set to 450 nm to measure optical density (OD). Measurement was also taken at 540 nm and values were subtracted from 450nm measurements in order to correct for possible optical imperfections in the plate.

2.16 Statistical analysis

All data was analysed with GraphPad Prism software (San Diego, CA, USA). Non-parametric Kruskal-Wallis test was used for comparisons between more than two groups whilst non-parametric Mann-Whitney was used for comparisons between two groups. Ordinary-ANOVA was performed for comparisons between more than two groups of normally distributed data. All differences were considered significant at $P < 0.05$. All plots have statistical significance indicated as follows: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns=not significant. Analysis of survival curves was conducted using the logrank (Mantel Cox) test.

CHAPTER 3: Extracted mosquito saliva enhances virus infection *in vivo* in the absence of a mosquito bite



3.1 Introduction

Emerging and re-emerging infectious arthropod borne viruses have a tremendous impact on global health. As was discussed in chapter 1, arthropod borne viruses constitute an important threat due to continuous emergence and re-emergence events as well as due to the limited availability in effective treatments and vaccines. Development of novel treatments are required imminently. The discovery of viral enhancement by mosquito bites allows for the development of potential treatments that specifically target the key aspect of mosquito bites, thereby reducing the likelihood of more serious disease. Research investigating the mechanisms responsible for viral enhancement triggered by mosquito bites is therefore required to pinpoint potential therapeutic targets.

Investigating how mosquito bites enhance arbovirus infection could allow for the development of a treatment that blocks this enhancing effect thereby reducing the risk of more serious illness associated with mosquito-borne virus infection. Previous work, as was discussed in detail in section 1.7 have shown that mosquito bites enhance arbovirus infection of mammals with studies suggesting that the injection of mosquito saliva specifically into the skin is the key factor responsible for viral enhancement. Therefore, in this section **we hypothesized that extracted mosquito saliva retained the ability to enhance arbovirus infection in the absence of a bite with the same efficacy.** Here, we established a model system that enabled the investigation of the effect of mosquito saliva in an *in vivo* setting.

In order to study this hypothesis, the neurotropic alphavirus Semliki forest virus (SFV) was used. SFV, which is a BSL2 human pathogen, is a well-established *in vitro* and *in vivo* alphavirus infection model. Previous work of the lab have established an *in vivo* model utilising the immunocompetent C57BL/6 mice and the avirulent strain SFV4. This work determined the *in vivo* kinetics of SFV4 replication and dissemination following inoculation in the skin in the absence and presence of a mosquito bite. Under these circumstances, following subcutaneous inoculation of SFV in the absence of a bite, SFV4 replicates rapidly within the skin with dissemination of virus to the nearest lymph node occurring 6-24h post infection and

to remote tissues including non-draining lymph nodes occurring 48 hours and at 96 hours post infection. Occasionally detection of viral RNA can occur in the brain from 48 hours onwards.

Therefore, this *in vivo* model more closely mimics natural infection by incorporating a vital aspect of mosquito borne virus transmission, exposure to *Ae.aegypti* mosquito bites. In particular, a significant increase in viral RNA was detected at 24h post infection if inoculated at a mosquito bite compared to resting skin. Furthermore, SFV4 disseminated more readily to the brain resulting in enhanced virulence and reduced mice survival. However, it remains unclear how the injection of mosquito saliva alongside virus, in the absence of a mosquito bite, affects the severity of SFV4 infection. In the studies in this thesis, infected mosquitoes were not used as infected mosquitoes can inoculate a wide range of viral doses whilst probing [486]. In order to control for quantity of viral dose as an experimental parameter, virus was administered via needle inoculation.

Therefore, in this initial chapter the aims and objectives are:

1. **To establish a mouse model that includes injection of mosquito saliva with virus. By determining the efficacy of mosquito saliva at enhancing arbovirus infection, this work aims to establish quantities of saliva required, method of salivary extraction and relevant time point post infection for assessing viral titres.**

2. **Utilising the model system developed, determine the effect of mosquito saliva on viral dissemination and animal survival in comparison to mice inoculated with virus at mosquito bites.**

3.2 Establishing method of mosquito saliva delivery

It has been established that mosquito bites enhance arbovirus infection of mammals in comparison to inoculation of virus in the absence of a mosquito bite and it has been suggested that the key factor facilitating this enhancement is mosquito saliva. Previous work investigating the effect of mosquito saliva on mosquito borne

arbovirus infection have utilised a couple of different approaches in terms of saliva administration; dissection and homogenization of whole salivary glands to generate 'salivary gland extract' (SGE), or forced mosquito salivation of non-dissected mosquitoes. Whilst both methods have their pros and cons, the use of SGE has been criticized for the inclusion of proteins and molecules that are never injected by the mosquito, as the entire tissue is homogenized. On the other hand, the use of mosquito saliva acquired via forced salivation has been criticized for potentially not containing the same contents as the saliva injected by the mosquito during a blood feed. Here, both methods were initially tested and their effect on SFV4 infection compared.

To investigate this, SGE was acquired via the removal of the mosquito salivary gland which was then processed according to the method utilised by Conway et al 2014 [450]. This involved freeze thawing the salivary glands three times on dry ice and the subsequent removal of insoluble debris via centrifugation at 5000g for 10min. Mosquito saliva was acquired via forced salivation of mosquitoes by placing the mosquito proboscis in a tip containing a drop of immersion oil. All saliva samples were then pooled and spun down. Saliva droplet was pipetted out of oil under a dissection microscope. Aliquots of extracted saliva were initially quantified by measuring protein concentration via a nanodrop which showed that on average, 1 female *Ae.aegypti* mosquitoes worth of saliva contains 0.3714µg/ml of protein (see appendix 1).

To investigate whether extracted saliva and salivary gland extract could successfully enhance arbovirus infection, C57BL/6 mice were infected with 10,000 plaque forming units (PFU) of SFV4 administered subcutaneously in the left foot in 1µl of PBSA only, or in combination with either 5 mosquitoes worth of saliva, or SGE from 5 mosquitoes. In addition, 5 homogenized mosquito ovaries used as a negative control of a random non-salivary associated organ. As a positive control, virus was inoculated into skin bitten by 5 mosquito bites. 24 hours post infection, mice were sacrificed and dissected for tissues of interest as well as the collection of serum. Tissue samples were then analysed via RT-qPCR for gene expression analysis of the SFV viral glycoprotein E1, which is a gene encoded by SFV4 that we have previously

established as a good indicator of viral RNA levels (see figure 3.1 A). Similarly, serum was analysed for viral titres via plaque assays (figure 3.1 B).

At 24h post infection significant increase in viral RNA could be detected at the inoculation site in mice exposed to mosquito bites immediately prior to infection or mosquito saliva co-injected alongside virus as well as significant increase in viral titres detected in the serum in comparison to mice with virus injected on its own in the absence of a mosquito bite or mosquito saliva. In comparison, injected salivary gland extract did not significantly increase virus RNA levels at the inoculation site, although significant enhancement was observed in the serum. As injected SGE was not as potent at enhancing SFV4 infection in these experiments as extracted mosquito saliva, and because it is hard to dissect out the role for injected unrelated proteins, saliva derived from forced salivation into oil was used for all future experiments.

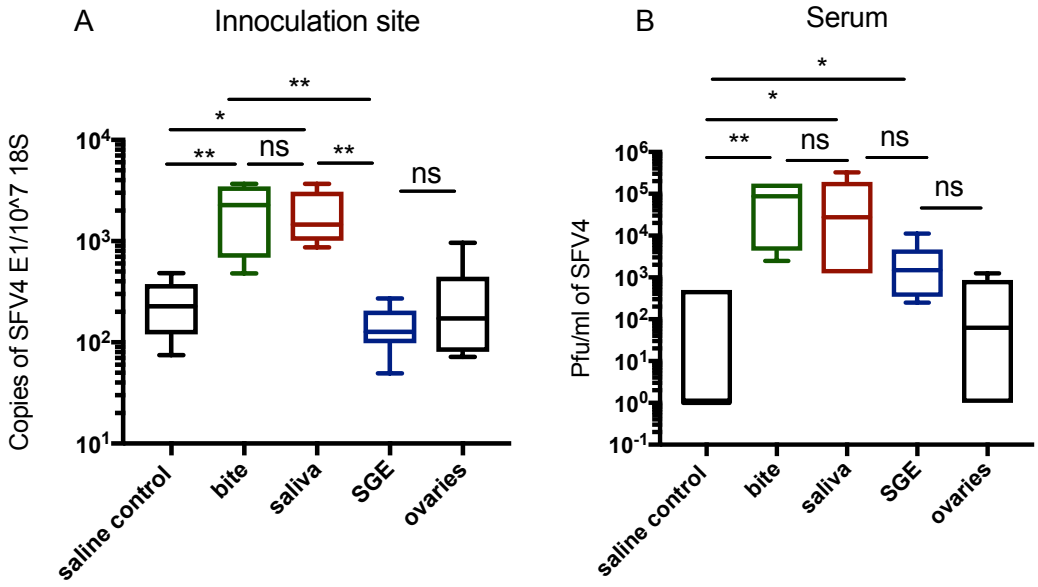


Figure 3.1. Extracted saliva and SGE effect on SFV4 infection *in vivo*.

In order to compare the viral enhancing effect of mosquito bites to extracted mosquito saliva and SGE, mice were infected with 10,000 PFU SFV4 subcutaneously on its own, following exposure to up to 5 *Ae.aegypti bites* (green), alongside 5 mosquitoes worth of *Ae.aegypti* saliva (red), alongside 5 SGE (blue) or alongside 5 ovaries. Expression of viral gene SFV E1 was measured using RT-qPCR in the skin (A) at 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

To ensure that the trauma caused by the needle stick during injection did not affect viral enhancement by causing an inflammatory response to needle trauma, an experiment was conducted comparing viral titres in mice subjected to inoculation in the skin with or without exposure to 10 additional needle sticks. At 24h post infection viral RNA was quantified at the inoculation site and virus titrated from the serum. No significant difference in viral RNA or viral titres was detected between mice subjected to 10 needle jabs at the site of inoculation prior to virus injection in comparison to mice injected with virus only (see figure 3.2), meaning that tissue trauma caused by the needle injection does not impact arbovirus infection. Note that injection of skin with needle was undertaken while mice were anesthetised.

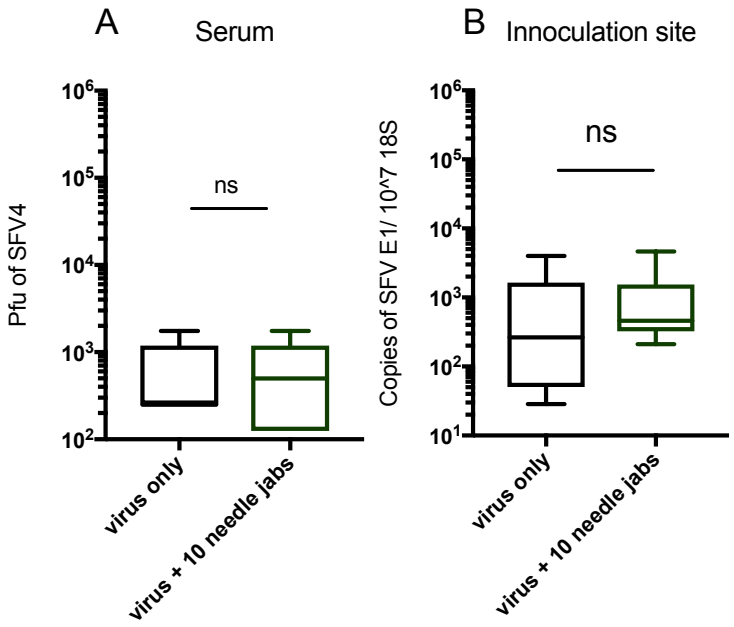


Figure 3.2. Impact of needle jabs on SFV4 infection

To investigate whether tissue trauma from needle injection has an impact on SFV4 infection mice were infected with 10,000 PFU SFV4 subcutaneously as a single injection (black) or following exposure to 10 needle jabs (green). Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral gene SFV E1 was measured using RT-qPCR in the skin (B) at 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Mann Whitney test).

3.3 Establishing the efficacy of mosquito saliva in enhancing SFV *in vivo*, by investigating the timeline of virus dissemination and salivary dosage response required

In the previous section saliva extracted via forced salivation of mosquitoes was observed to significantly enhance SFV4 by 24h post infection. Following this, we needed to assess the quantity of saliva required to reach max viral enhancement. To investigate this, a dosage curve experiment was conducted where C57BL/6 mice were subjected to inoculations of 10,000 PFU of SFV4 in saline or pre-mixed with 1, 5 or 25 mosquitoes worth of extracted mosquito saliva. Mice were sacrificed 24h post infection and viral RNA quantified at the inoculation site by qPCR whilst serum was utilised to determine viral titres. Whilst the co-injection of saliva alongside virus always enhanced infection regardless of quantity, larger amounts of saliva correlated with a larger increase of viral enhancement, with virus titres reaching a plateau at approximately 5 mosquitoes worth of saliva (see figure 3.3). Therefore, 5 mosquitoes worth of saliva was utilised in all experiments going forward unless stated otherwise.

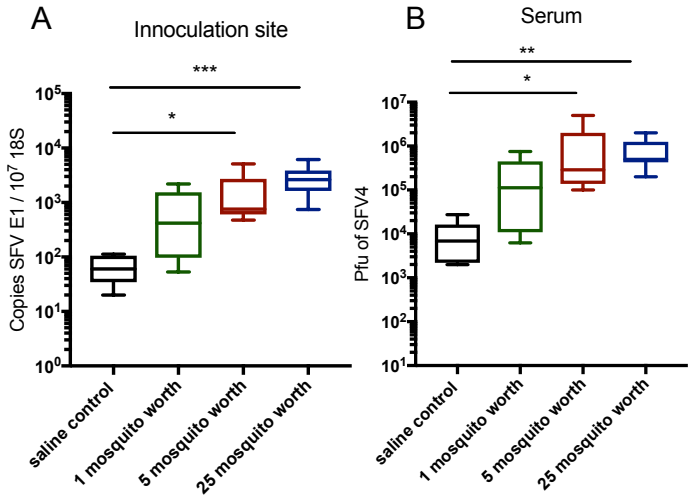


Figure 3.3. Quantity of extracted mosquito saliva required for peak viraemia

Mice were infected with 10,000 PFU SFV4 subcutaneously on its own (black) or alongside 1 mosquitoes worth of *Ae.aegypti* saliva (green), 5 mosquitoes worth of *Ae.aegypti* saliva (red) or 25 mosquitoes worth of *Ae.aegypti* saliva (blue). Expression of viral gene SFV E1 was measured using RT-qPCR in the skin (A) at 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

After determining the quantity of saliva required to reach maximum viral enhancement, the timepoint of peak viraemia and the timepoint of viral dissemination to draining lymph node was investigated. As Pingen et al 2016 [95] determined that peak viraemia in mice, injected with SFV4 following a mosquito bite occurs 24h post infection, we needed to determine whether this also applies for virus co-injected with extracted mosquito saliva. Therefore, mice were subjected to injections of 10,000 PFU of SFV4 into either resting skin, skin pre-exposed to mosquito bite or virus co-injected with 5 mosquitoes worth of saliva. Mice were sacrificed at 5h, 10h and 24h post infection. The impact of mosquito bite/ saliva on SFV4 infection was assessed by RT-qPCR for the viral gene E1 in tissues such as draining lymph node and skin at the inoculation site.

Viral RNA at the inoculation site revealed that viral titres were highest at 24h post infection for both mosquito bitten mice and in mice injected with saliva. Both mosquito bites and mosquito saliva enhanced virus infection to a similar extent at 24h (see figure 3.4). Interestingly, virus disseminated much more rapidly to the draining lymph node in mice were virus was co-injected with mosquito saliva in comparison to resting/mosquito bitten mice, with viral RNA detected in the draining lymph node at 5h post infection. Virus disseminated to the draining lymph node was slowest in mosquito bitten mice suggesting that mosquito bites retain virus in the skin as was also observed by Pingen et al 2016 [95].

Regardless of the rapid dissemination of virus co-injected with mosquito saliva to the draining lymph node, higher quantities of viral RNA was still observed at the inoculation site compared to virus injected alone, suggesting that saliva facilitates enhanced viral replication at the inoculation site whilst simultaneously increasing viral dissemination. This suggests that increased virus at inoculation site is not just due to retained virus, but due to enhanced viral replication. Differences in quantity of viral RNA are therefore most likely due to differences in replication levels.

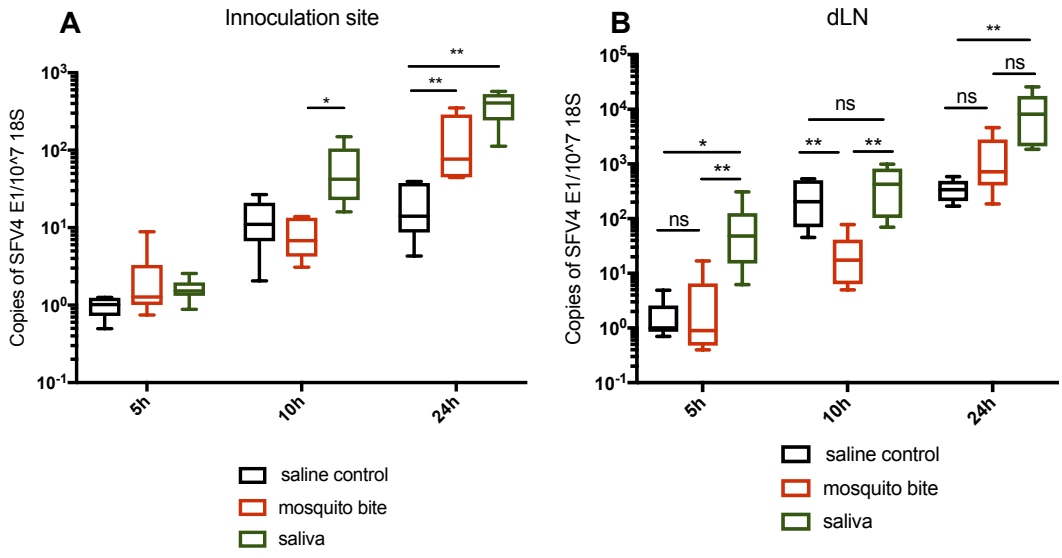


Figure 3.4. Kinetics of SFV4 infection *in vivo*

In order to compare the time required for SFV4 to disseminate to draining lymph nodes following infection in the absence or presence of either mosquito bite exposure or saliva injection, mice were infected with 10,000 PFU SFV4 subcutaneously on its own (black), following exposure to up to 5 mosquito bites (red) or alongside 5 mosquitoes worth of *Ae.aegypti* saliva (green). Expression of viral gene SFV E1 was measured using RT-qPCR in the skin (A) and in the dLN (B) at 5, 10 and 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

3.4 Mosquito saliva causes upregulation of key inflammatory genes in the skin

All previously published studies have assessed host immune response to either SGE or mosquito bites. Importantly, the effect on host responses of extracted mosquito saliva, in the absence of a mosquito bite, are largely unknown. Therefore, we wanted to investigate whether extracted mosquito saliva modulates expression of inflammatory genes in the skin of the inoculation site. Mice were injected with 1 mosquitoes worth of saliva in the dorsal side of their left foot and the expression of inflammatory genes compared to mice injected with equal amounts of PBSA. Mice were culled and samples acquired 6 hours post inoculation. This time point was selected as it has previously been demonstrated that expression of many pro-inflammatory gene transcripts, in response to *Ae.aegypti* bites, peak at 6 hours post exposure [95]. QPCR was conducted on the skin samples utilising primers targeting CXCL2, IL1β, CCL2, CCL5 and ISG15. These genes were chosen to represent genes previously identified to be upregulated by *Aedes* mosquito bites (with the

exception of CCL5 and ISG15, which are instead prototypic ISGs activated by virus sensing). As can be observed in figure 3.5, CXCL2, IL1β and CCL2 were significantly upregulated by *Ae.aegypti* saliva whilst CCL5 and ISG15 were not. This is somewhat similar to the findings of Pingen et al, that observed a similar pattern of inflammatory gene modulation by mosquito bites, suggesting that mosquito saliva is responsible for activating expression of these cytokines following bites.

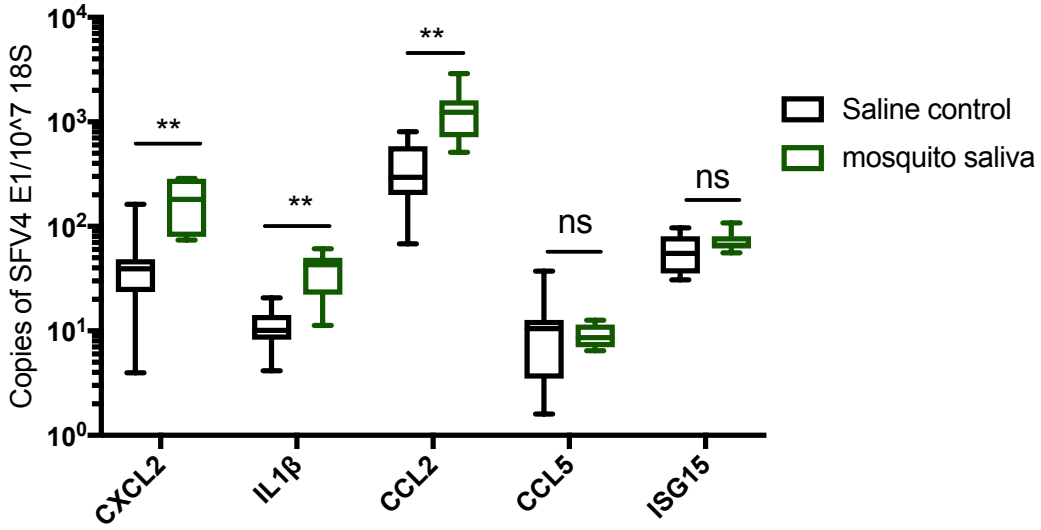


Figure 3.5. Upregulation of key inflammatory genes in the skin in response to *Ae.aegypti* saliva

In order to investigate whether *Ae.aegypti* upregulates key inflammatory genes in the skin, mice were inoculated subcutaneously in the the dorsal side of their left foot with 1µl PBSA to control for upregulation of genes in response to the trauma of inoculation (black), or 5 mosquitoes worth of *Ae.aegypti* saliva in 1µl (green). Expression of CXCL2, IL1β, CCL2, CCL5 and ISG15 were measured using RT-qPCR in the skin 6 hours post inoculation. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$.)

3.5 Extracted mosquito saliva, like mosquito bites, reduce mouse survival following SFV4 infection

Following the confirmation that mosquito saliva enhances arbovirus infection in the absence of a mosquito bite at an early time point, we wanted to determine if mosquito saliva in the absence of a bite, affected mouse survival with SFV the same as mosquito bites. For this purpose, mice were injected with 40.000 PFU of SFV4 with or without 5 mosquitoes worth of saliva, or pre-exposed to up to 5 mosquito bites. Mice where then left for up to 14 days and monitored for signs of defined

clinical signs. When mice reached a clinically defined and established endpoint (see chapter 2) they were euthanized in accordance with home office regulations. In this experiment, mice subjected to mosquito bites, or injected with mosquito saliva exhibited a significantly higher mortality rate than mice that were infected with SFV4 on its own in the absence of any salivary factors (see figure 3.6). Also, there was no significant difference in the mortality rates between mice subjected to mosquito bites or mosquito saliva. This supports the hypothesis that mosquito saliva alone is the factor responsible for the mechanism of arbovirus enhancement caused by mosquito bites. Mice were also weighed each morning in order to monitor changes in weight during the progression of the experiment (see figure 3.6). Monitoring of mice weights demonstrated that whilst the majority mice inoculated with SFV4 alongside *Ae.aegypti* saliva or bite exhibited a significant loss in weight following infection (the majority sometime between day 2 and 5 post infection), the majority of mice infected with SFV4 on its own retained a stable, or an increase in weight.

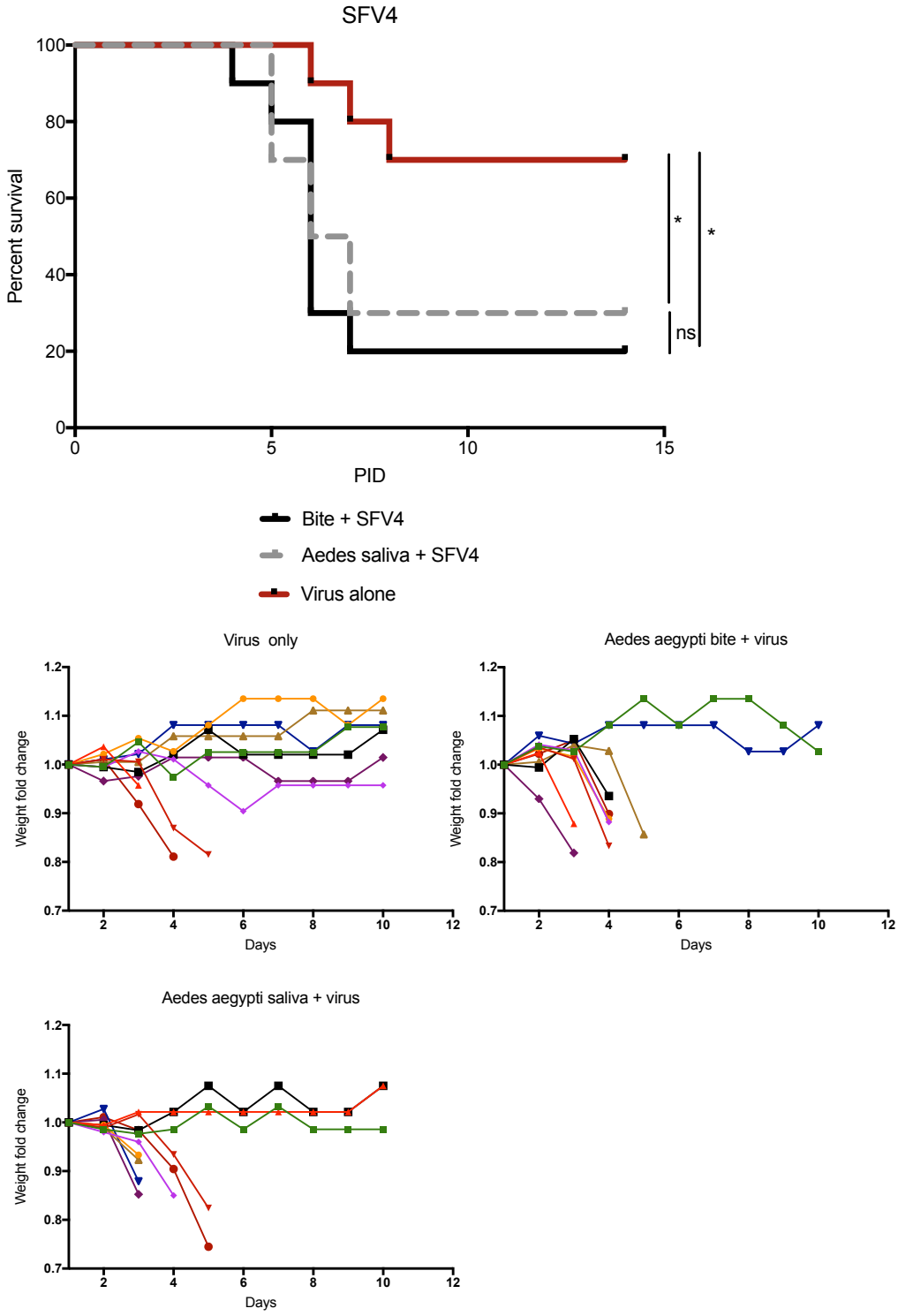


Figure 3.6. Survival to SFV challenge following mosquito bite/saliva exposure

To compare impact on mouse survival following exposure to mosquito bite or saliva mice were infected with 40,000 PFU SFV4 subcutaneously on its own (red), following up to 5 mosquito bites (black) or alongside 5 mosquitoes worth of *Ae.aegypti* saliva (grey). Mice were then monitored for signs of a clinically defined endpoint. Mice were left for a maximum of 14 days post infection. Graph (A) demonstrates the survival percentages whilst (B) illustrated the weight curves of acquired from daily weighing of each individual mouse. (n=10) (Mantel Cox test * $p<0.05$).

3.6 Mosquito saliva enhances infection of the flavivirus Zika virus independently of the type I IFN response

Whilst the previous sections determined that mosquito saliva has the ability to enhance infection of the alphavirus SFV, we wanted to investigate whether mosquito saliva can enhance infection of an arbovirus of a different family. For this purpose, we utilised the flavivirus ZIKV. A ZIKV mouse model was established by Marieke Pingen and Clive McKimmie which encompasses the use of C57BL/6 mice injected with 1.5mg (per mouse) InVivoMAb anti-mouse IFNAR-1, a day prior to virus inoculation. This antibody is well known as an effective inhibitor Type I IFN receptor signalling *in vitro* and *in vivo* [152].

As the ZIKV model relies on the blocking of the type I IFN response, we wanted to ensure that *Ae.aegypti* mosquito bites were still effective at enhancing SFV infection independently of an effective type I IFN response. Therefore, mimicking the developed ZIKV mouse model, mice were injected with 1.5 mg anti-mouse IFNAR-1 1 day prior to infection. The following day, mice were exposed to up to 5 mosquito bites with 10,000 PFU of SFV4 injected immediately following biting. Quantity of virus was compared between mice subjected to virus injection in the presence and absence of mosquito bites 24 hours post infection. Titration of virus in the serum as well as quantification of viral RNA at the site of inoculation demonstrated that there was a significant enhancement of virus quantity when virus was injected alongside mosquito bites in comparison to when virus was injected on its own in the absence of a bite (see figure 3.7). This further suggests that the mechanism behind mosquito bite viral enhancement occurs independently of host type I IFN response.

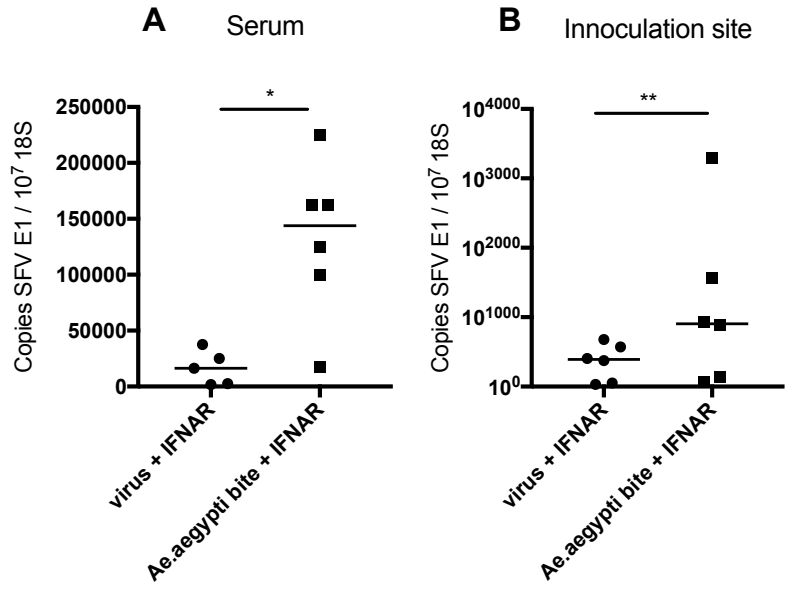


Figure 3.7. Bite mediated viral enhancement independent of type I IFN response

To investigate whether mosquito bites retain their ability to enhance SFV4 infection devoid of a functional type I IFN response, mice treated with IFNAR-1 a day prior to infection were infected with 10,000 PFU SFV4 subcutaneously on its own or following exposure to up to 5 mosquito bites. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral gene SFV E1 was measured using RT-qPCR in the skin (B) at 24 hours post infection. Data are presented as dot plots where each dot represents an individual mouse, with a line at the population median; ($n \leq 6$) (Mann Whitney test * $p<0.05$, ** $p<0.01$)

As mosquito bites enhanced SFV infection in the absence of the type I IFN response, next, using the same mouse model, we wanted to investigate whether *Ae.aegypti* saliva alone, in the absence of mosquito bites, could also successfully enhance ZIKV infection. In this experiment, the day following injection with anti-mouse IFNAR-1, mice were inoculated with 1000 PFU of ZIKV on its own, or pre-mixed with 5 mosquitoes worth of saliva. Mice were sacrificed 24 hours later. qPCR data from skin from inoculation site, and plaque assays of viral titres in serum demonstrated that extracted *Ae.aegypti* saliva enhances ZIKV infection in this model, as significantly higher quantities of viral RNA was detected in the skin of mice which were injected with virus pre-mixed with saliva, in comparison to virus inoculated on its own. Similarly, higher titres of ZIKV were detected in mice which had been inoculated with mosquito saliva (see figure 3.8).

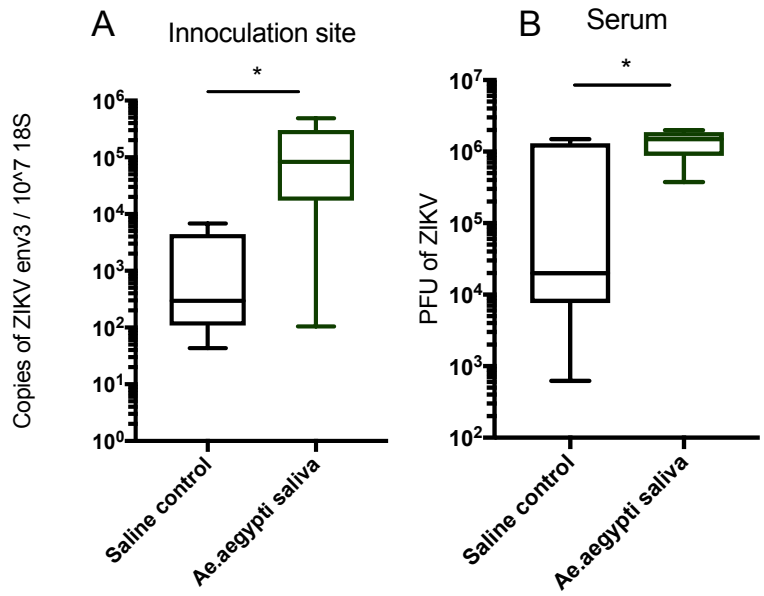


Figure 3.8. Extracted *Ae.aegypti* saliva enhances ZIKV infection *in vivo*

To investigate whether extracted mosquito saliva enhances ZIKV infection, mice were infected with 1000 PFU ZIKV subcutaneously on its own or alongside 5 mosquitoes worth of saliva. Expression of viral gene ZIKV Env3 was measured using RT-qPCR in the skin (A) at 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (B). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Mann Whitney test * $p<0.05$.)

3.7 Summary and Conclusions

In this initial chapter we hypothesized that extracted mosquito saliva retained the ability to enhance arbovirus infection in the absence of a bite with the same efficacy. Mosquito probing of skin causes trauma in addition to the deposition of saliva, and it was not clear what role each had in modulating host susceptibility to virus infection. In order to investigate this, we set out to establish a mouse model utilising mosquito saliva which would then be utilised to determine the effect of mosquito saliva on viral dissemination and animal survival in comparison to that of mosquito bites. Whilst previous studies have investigated the effect of mosquito bites or extracted mosquito saliva or SGE on arboviral infection, to date no one has directly compared the efficacy of mosquito saliva in modulating arbovirus infection, to that of the mosquito bite.

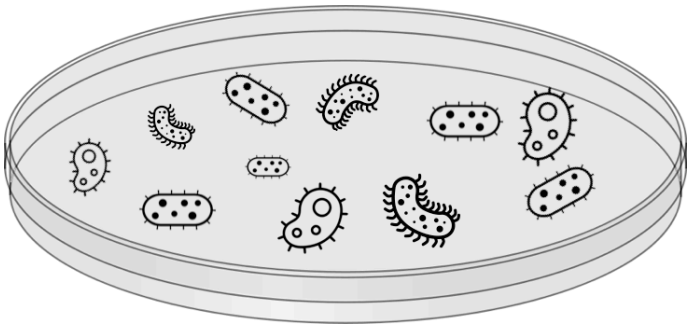
Comparing the ability of SGE, mosquito saliva derived via forced salivation and mosquito bites, we demonstrated that extracted mosquito saliva enhances SFV

infection to a similar extent as mosquito bites. Therefore, even though the exact composition of the extracted mosquito saliva may differ slightly from that of the saliva injected by mosquitoes during probing, the factors responsible for viral enhancement are still present in the extracted saliva, thereby allowing us to use this in order to study mosquitoes saliva's effect on arbovirus infection.

Following this, a range of quantities of saliva were tested, as well as different time points in order to determine quantity and time required to reach peak viraemia of SFV4 in this model system. 5 mosquitoes worth of saliva resulted in high-titre viraemia with SFV4 by 24hpi. In concurrence with previously published work from the lab [95], SFV4 co-injected with mosquito saliva resulted in enhanced replication of virus in the skin whilst also resulting in enhanced dissemination to the draining lymph node. In addition, *Ae.aegypti* mosquito saliva injected in the absence of a mosquito bite, causes upregulation of key inflammatory genes previously determined to be upregulated by *Ae.aegypti* mosquito bites including CXCL2, CCL2 and IL1β. Additionally, a survival experiment concluded that mosquito saliva reduced mouse survival of SFV4 infection to a similar extent as mosquito bites. Also, mosquito saliva was found to significantly enhance infection of mice with ZIKV. Finally, by investigating the effect of needle trauma on virus infection we concluded that physical trauma did not affect virus infection. This, in combination with the saliva enhancing experiments, suggest that factors present in saliva are necessary and sufficient to explain enhancement of infection by bites, and that the skin trauma from the bite is not implicated in enhancing virus infection.

In conclusion, we have established an *in vivo* mouse model utilising extracted mosquito saliva in order to study the mechanism of modulation of arbovirus infection by mosquito saliva. With this model system we have determined that *Ae.aegypti* mosquito saliva is responsible for the arbovirus infection enhancing mechanism displayed by *Ae.aegypti* mosquito bites. This model allows for a more controlled setting for the studying of arbovirus enhancement as quantity of mosquito saliva and virus inoculated are controlled for.

CHAPTER 4: Mosquito saliva inhibits virus infection *in vitro* in a microbiota dependent manner, although microbiota has no effect *in vivo*.



4.1 Introduction

It has been established that mosquito saliva enhances arbovirus infection. However, the mechanisms implicated in this remain unknown. In order to study the mechanisms involved further we wanted to establish an *in vitro* model system. *In vitro* models are high tractable and require the use of less animals. Here, we utilised cell types known to be key targets for alphavirus infection in the skin; macrophages and dermal fibroblasts [437]. It has been suggested that mosquito saliva may enhance virus infection of cells by either; directly aiding virus attachment to susceptible cells; or potentially by making cells more susceptible to infection [450]. Therefore, we decided to investigate whether mosquito saliva enhances arbovirus infection *in vitro* in cells as well as in explants of whole skin *ex vivo*.

In addition, host inflammatory responses to mosquito bites have also been suggested to be necessary for enhancement of arbovirus infection *in vivo*. Accordingly, it has been hypothesized that pro-inflammatory microbiota present in mosquito saliva may contribute to the immune responses elicited against saliva. Thus, immune responses to microbiota in saliva could also indirectly aid virus infection. This hypothesis has been strengthened by the discovery that the salivary glands of *Anopheles culicifacies* mosquitoes harbour a larger diversity of microbiota than the gut which demonstrates the quantity and variety of microbes that are injected in to the skin during mosquito probing [452]. Therefore, bacteria injected into the skin alongside the mosquito saliva could be responsible for initiating the immune response that is responsible for enhancing virus infection. Therefore, we wanted to test this hypothesis using an *in vitro* model.

Whilst the previous chapter determined that extracted mosquito saliva enhances arbovirus infection *in vivo*, this chapter asks the question whether mosquito saliva also enhances infection in isolated cell cultures, or in explanted skin as well as whether microbiota present in mosquito saliva modulates arbovirus infection.

Therefore, the aims of this chapter was:

1. **To determine whether mosquito saliva enhances SFV infection *in vitro*.**

2. **To determine if extracted mosquito saliva enhances SFV infection in skin explants.**
3. **Determine whether microbiota plays a role in modulating arbovirus infection *in vitro* and *in vivo*.**

4.2 Mosquito saliva inhibits virus infection in macrophages and fibroblasts

As it has been suggested that mosquito saliva can enhance viral infection by either directly aiding virus attachment to susceptible cells or potentially by making cells more susceptible to infection, we decided to investigate whether mosquito saliva enhances SFV infection *in vitro* utilising cell types that are known as key targets for SFV infection; macrophages and dermal fibroblasts.

For this purpose, a Gaussia Luciferase expressing SFV6 strain was used in order to more efficiently measure changes in virus quantities. As Gaussia luciferase is secreted by infected cells into the supernatant, it allows for an efficient method for tracking progression of viral infection over time. Also, this is an exceptionally sensitive system, with a high dynamic range, allowing detection of very low and high levels of virus replication. Initially MEF (Mouse Embryonic Fibroblasts) cells from C57BL/6 mice were treated with approximately 1.8 mosquitoes worth of saliva for 1 hour prior to infection with SFV6 with an MOI of either 1 or 5. Cells were left for 6 hours before supernatant was acquired and a luciferase assay conducted. Interestingly, the luciferase assay demonstrated that cells that had been pre-treated with mosquito saliva, had a significantly lower expression of luciferase than cells that had not received a pre-treatment with saliva, which is the opposite phenotype of what was expected. No difference between the two groups was observed in the cells infected with an MOI of 5, probably due to the large quantity of virus added (see figure 4.1).

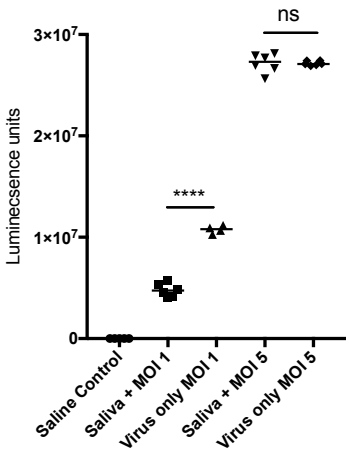


Figure 4.1. Effect of mosquito saliva on SFV infection of MEF cells

In an attempt to develop an *in vitro* model to study saliva mediated viral enhancement in MEF cells, MEF cells were infected with an MOI of 1 or 5 of SFV6 on its own or following pre-treatment with 1.8 mosquitoes worth of saliva per well for 1 hour prior to infection. Supernatant was collected 6 hours post infection and luminescence was measured via luciferase assay. Data is presented as dot plots with each dot representing a separate biological sample with a line at the population median. (n=6) (One-Way ANOVA test **** $p<0.0001$).

Therefore, to determine the effects of saliva on virus infection at lower MOI, we then repeated with an MOI of 0.01, 0.1 and 1. In addition, to help elucidate how the mechanisms by which saliva modulates infection *in vitro*, cells were either pre-treated with saliva and then infected (as above, called "saliva to cells"), or saliva was pre-mixed with the virus for 20min prior to its addition to the cells (referred to as "saliva to virus"). Supernatant was then acquired at 6, 24 and 48 hpi in order to monitor the progression of infection. Interestingly, in most cases mosquito saliva had an inhibitory effect on the early stages of SFV6 infection (see figure 4.2). At 48 hours post infection, all groups had reached the maximum detectable level of luciferase activity. This was surprising as a number of previous studies have demonstrated an enhancing effect of mosquito saliva *in vitro*. However, several of these studies utilised SGE rather than extracted mosquito saliva, which could potentially result in a different outcome [450].

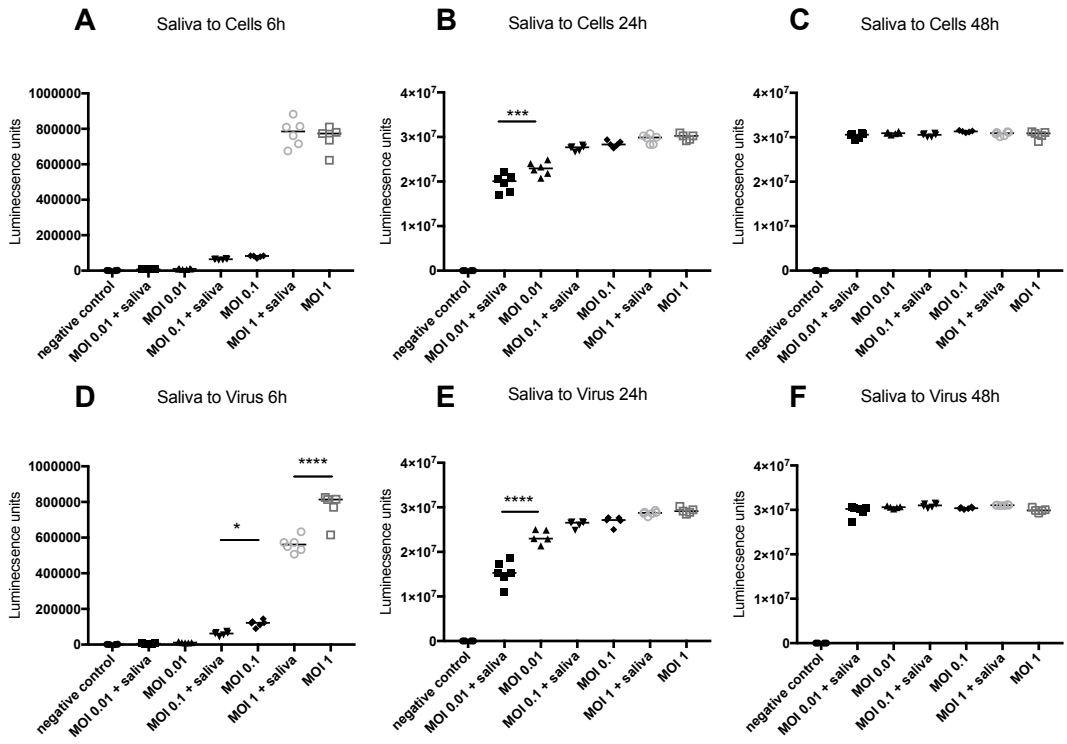


Figure 4.2. Mosquito saliva inhibits SFV infection of MEF cells at lower MOI

In order to investigate the effect of extracted mosquito saliva on the infection of MEF cells, MEF cells were infected with an MOI of 0.01, 0.1 or 1 of SFV6. In "Saliva to cells" groups, cells were treated with 1.8 mosquitoes worth of saliva 1 hour prior to infection (A, B and C) or, in "Saliva to virus" groups saliva was incubated with the virus 20 minutes before infection (D, E and F). Supernatant was collected 6, 24 and 48 hours post infection and luminescence was measured via luciferase assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (One-Way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

Wanting to compare these findings to another cell type, the experiment was repeated utilising macrophages derived from mouse bone marrow precursors (differentiated with M-CSF). In this experiment, SGE was also used as an additional group in order to determine whether saliva acquired via forced salivation and SGE had different effects on virus infection. Mosquito ovaries were included to represent a random mosquito organ as a control for SGE in order to help control for any modulations in response to unrelated non-salivary proteins. Macrophages were either; 1.) pre-treated with mosquito saliva, SGE or ovaries extract for 1 hour prior to infection with an MOI of 0.1; or 2.) infected with SFV pre-mixed with extracted mosquito saliva, SGE or ovaries extract. In concurrence with the data acquired from the MEF studies, extracted mosquito saliva inhibited SFV6 infection of macrophages (see figure 4.3). Interestingly, so did SGE, but only when macrophages were pre-treated with SGE prior to the addition of virus. Interestingly, the effect of saliva was more

pronounced when given to macrophages prior to infection. This suggests that a factor in saliva was modulating cell susceptibility to infection, rather than having a direct effect on virus.

These data are in direct contrast with the findings of a study that demonstrated that SGE enhanced infection with DENV in macrophages and fibroblasts [450], as well as in keratinocytes [487]. We deliberately chose not to study keratinocytes, as their relevance in arbovirus infection is disputable as mosquitoes probe right through the epidermis and deposit arbovirus within the dermis which lacks keratinocytes.

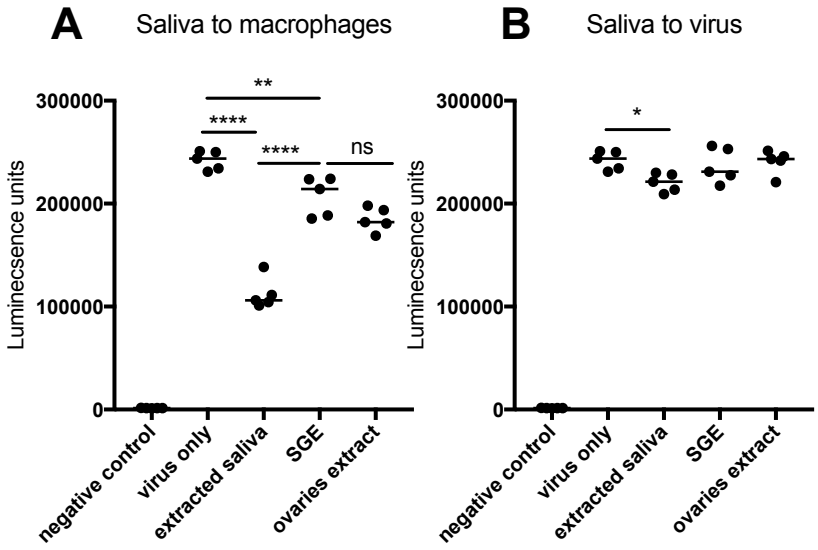


Figure 4.3. Impact of extracted saliva and SGE on SFV infection of macrophages

To investigate the effect of extracted mosquito saliva and SGE on the infection of macrophages, macrophages were infected with an MOI of 0.1 of SFV6. Cells were treated with 1.8 mosquitoes worth of saliva, 2 SGE or 2 ovaries extracts 1 hour prior to infection (A) or saliva/SGE/ovaries extract were pre-incubated with the virus for 20 minutes prior to infection (B). Supernatant was collected 6 hours post infection and luminescence was measured via luciferase assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=5) (One-Way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

4.3 Mosquito saliva does not enhance SFV infection in skin explants

As cell culture models failed to mirror the phenotype observed *in vivo*, where mosquito saliva enhances infection, we developed an *ex vivo* model involving explanted mouse skin. In this model, biopsies of sanitised mouse skin are taken and

placed in tissue culture media for *ex vivo* culture. Explanted mouse skin contains a mixed cell population of all relevant cell types that are present at the inoculation site, but lacks cell types that are recruited following infection e.g. neutrophils. Therefore, by investigating whether extracted mosquito saliva enhances infection in explanted skin, we would answer the question of whether mosquito saliva dependent viral enhancement can occur in isolated skin, in the absence of functional circulation, and recruited immune cells.

For this purpose, mice were injected with either 10,000 or 100,000 PFU of SFV4 on its own, or virus mixed with 5 mosquitoes worth of saliva. Injection was done into the dorsal side of their hind feet immediately post cull in order to ensure cessation of circulation. Skin samples were then acquired 15min later and placed in complete DMEM media for 24h. qPCR from the explanted skin samples demonstrated that there was no difference in quantity of viral RNA between the 2 samples (see figure 4.4) which means that mosquito saliva does not enhance SFV infection when skin is isolated from the circulation system. This suggests that mosquito saliva dependent arbovirus enhancement is dependent on factors and/or processes that only occur *in vivo* and can therefore not be observed in cultured skin explants.

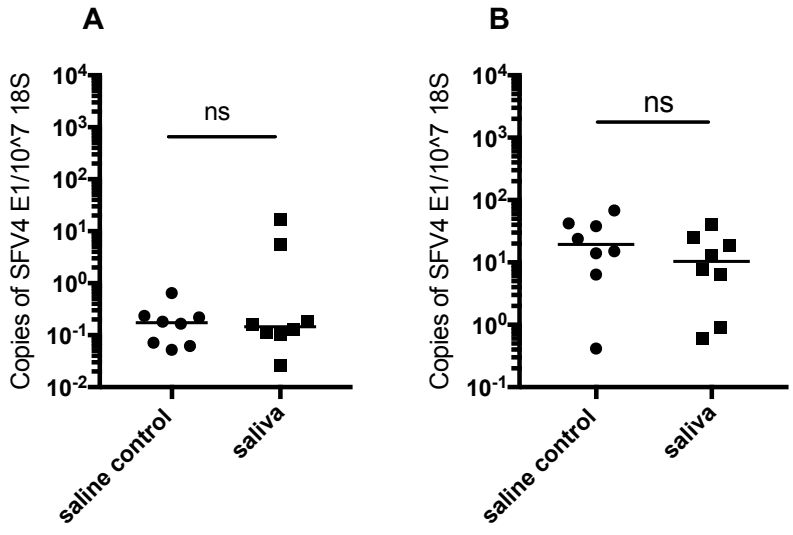
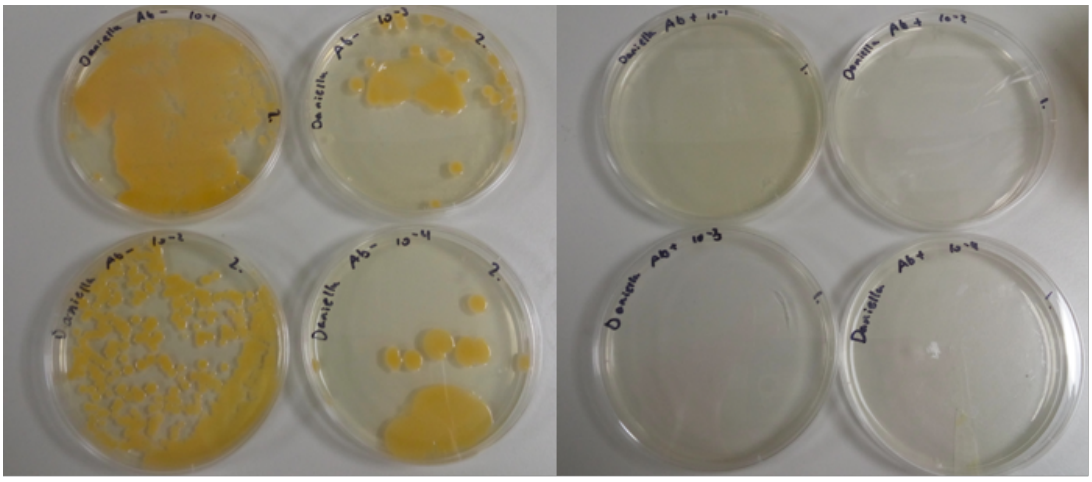


Figure 4.4. Saliva does not enhance SFV infection of skin explants

To investigate whether extracted mosquito saliva enhances infection of skin explants, recently culled mice were infected with wither 10,000 PFU SFV4 (A) or 100,000 PFU SFV4 (B) on its own or alongside 5 mosquitoes worth of saliva. Skin was explanted 15 minutes following infection. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=8) (Mann Whitney test).

4.4 Validation of antibiotic treatment

It is not clear which factors present in saliva are responsible for modulating level of virus infection, either *in vitro* or *in vivo*. The presence of microbiota in mosquito saliva may influence severity of virus infection. In order to test whether microbiota present in mosquito saliva modulates viral infection *in vitro* or *in vivo* we had to firstly establish a treatment that effectively removed microbiota from adult *Ae.aegypti* mosquitoes. This treatment consisted of a mixture of penicillin/streptomycin at 200 U per ml, gentamycin at 200 µg/ml, and tetracycline at 100 µg/mL. Treatment was placed in water of pupae and added to sugar from which adult mosquitoes fed on for 1 week prior to salivation/use (larval stage mosquitoes could not be treated with antibiotics as we found this to be lethal). Treatment was validated via the collection of several randomly selected female mosquitoes which were then dipped in ethanol to remove external microbiota, dried and placed in PBS. After shaking with metal beads, samples were plated on agar plates in serial 10-fold dilutions. An example of this can be seen in figure 4.5 where plates of untreated mosquitoes exhibited large quantities of 10^3 CFU/ml of bacteria (average of 28,000 CFU counted per mosquito), whilst no bacteria grew on plates of antibiotic treated mosquitoes. Previous studies using similar antibiotic treatments have counted up to 40,000 CFU per mosquito [488] whilst others have counted similar CFUs as observed here (ie. 24000 CFU) [213]. Mosquitoes were also collected for qPCR analysis investigating quantities of 16S RNA conducted which also demonstrated a significant decrease of 16S RNA in antibiotic treated mosquitoes (see appendix 2).



Untreated Treated

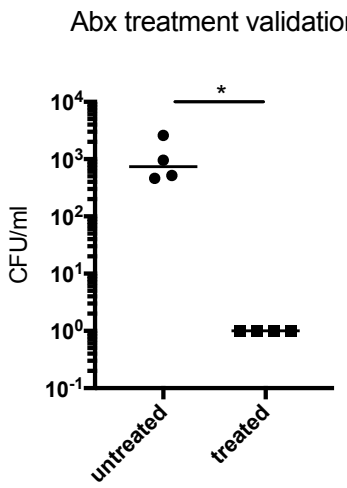


Figure 4.5. Validation of mosquito antibiotic treatment

To validate the efficacy of the developed antibiotic treatment which comprised of penicillin/streptomycin at 200 U per ml, gentamycin at 200 µg/ml, and tetracycline at 100 µg/mL, mosquitoes were taken from the untreated control group (A) and the Abx treated group (B) and dipped in ethanol, dried and placed in PBS. Mosquito extract was plated on agar plates in 10-fold dilutions. CFU/ml was calculated 24 hours later. Data is presented as selected representative pictures (A and B) and dot plots (C) with each dot representing a separate biological sample, with a line at the population median. (n=4) (Mann Whitney test).

4.5 Microbiota present in mosquito saliva is responsible for inhibition of virus infection *in vitro*

Following the validation of the efficiency of the removal of microbiota by the developed antibiotic treatment of the *Ae.aegypti* mosquitoes, we wanted to investigate whether microbiota present in mosquito saliva modulates arbovirus infection. Initially this was investigated *in vitro* with macrophages and MEF cells. As previously, virus was pre-mixed with saliva acquired from untreated or antibiotic treated mosquitoes before infecting the cells with an MOI of 0.1 of SFV6 Gluc virus. Cells were left for 6 hours before tissue culture supernatant was acquired. Surprisingly, luciferase assays demonstrated that whilst saliva acquired from untreated microbiota-sufficient mosquitoes inhibited infection of both macrophages and fibroblasts, saliva from antibiotic treated microbiota depleted mosquitoes did not inhibit infection of fibroblasts, and in macrophages it inhibited infection significantly less than untreated saliva (see figure 4.6). This suggests that microbiota present in mosquito saliva is responsible for inhibition of virus infection of macrophages and fibroblasts *in vitro*.

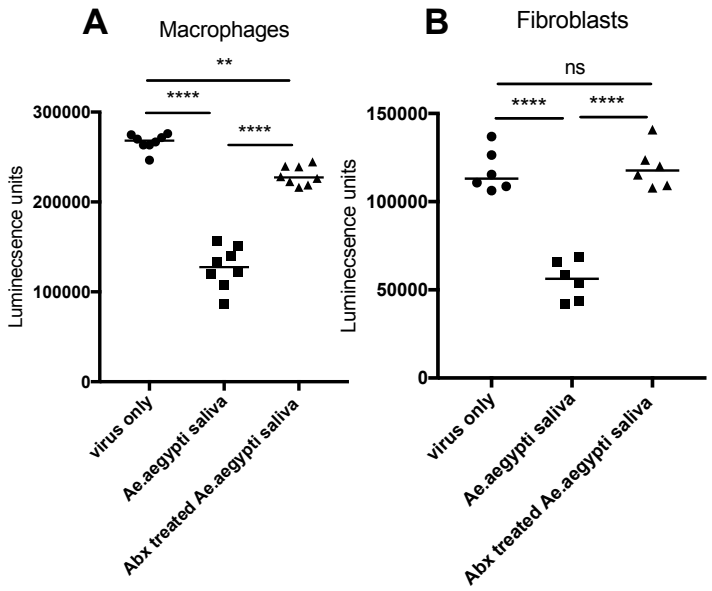


Figure 4.6. Microbiota depleted saliva does not inhibit virus infection of macrophages and MEF cells

To investigate whether salivary microbiota modulates virus infection *in vitro*, macrophages (A) and MEF cells (B) were infected with an MOI of 0.1 of SFV6 on its own or alongside 1.8 mosquitoes worth of saliva from untreated or Abx treated *Ae.aegypti* mosquitoes. Supernatant was collected 6 hours post infection and luminescence was measured via luciferase assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=8 macrophages) (n=6 MEF cells) (One-way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

4.6 Inhibition of virus infection *in vitro* by microbiota is reversed by inhibiting type I IFN signalling

As microbiota present in mosquito saliva was discovered to inhibit arbovirus infection of key cell types, we wanted to further investigate the mechanisms behind this. We hypothesized that microbiota in saliva helps trigger an immune response which could also inhibit the infection of the virus. To test this, macrophages were treated with either 0.1 MOI of SFV6 Gluc, saliva of untreated mosquitoes or saliva from antibiotic treated mosquitoes in the absence of any virus. Cells were lysed 6 hours post treatment. QPCR analysis of the lysed cells demonstrated that CXCL2 was significantly upregulated by saliva acquired from untreated mosquitoes but not from antibiotic treated mosquitoes. CXCL10, CCL5, IL1β and ISG15 were all upregulated to a similar extent by both untreated and treated mosquitoes saliva whilst no IFNβ could be detected, although this is perhaps not unusual as this gene is expressed at low levels in the skin and is often hard to detect (see figure 4.7) [489].

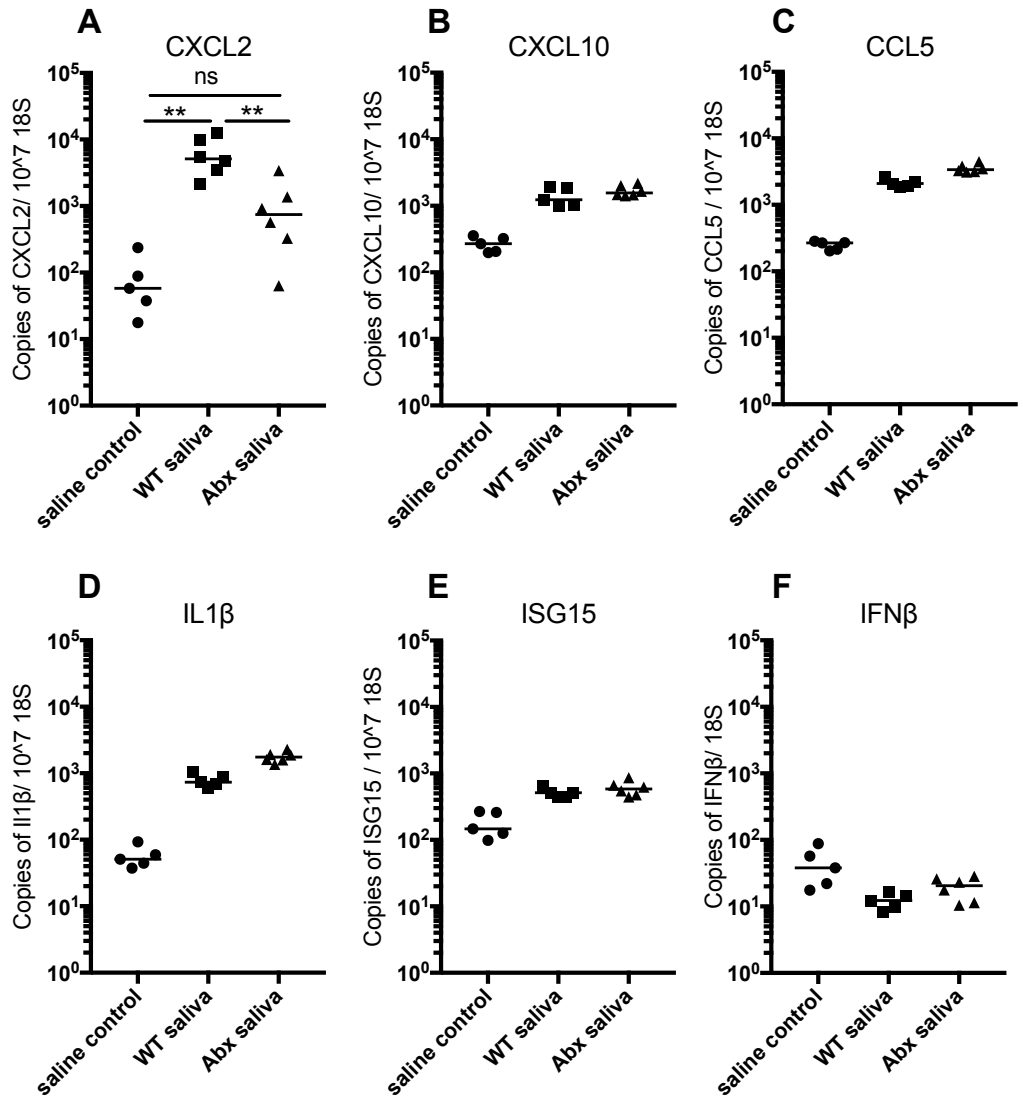


Figure 4.7. Microbiota in extracted saliva causes upregulation of CXCL2 in macrophages

To investigate whether salivary microbiota causes upregulation of key inflammatory genes in macrophages, macrophages were treated with saliva from untreated (WT saliva) or antibiotic treated (Abx saliva) mosquitoes. Expression of CXCL2, CXCL10, CCL5, IL1β, ISG15 and IFNβ were measured using RT-qPCR 6 hours post treatment. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

We hypothesised that despite the inability to detect obvious IFN-β expression, microbiota may be triggering innate immune IFN responses that make them more refractory to infection with virus. Therefore, as a second experiment, IFNAR-1 antibody was used to block expression of type I IFN signalling in macrophages. Macrophages were seeded at 20,000 cells per well in a 96 well plate and infected

with an MOI of 1 of SFV6 Gluc with 1.5 mosquitoes worth of saliva added to each well. Anti-mouse IFNAR-1 was added at a concentration of 1µg/ml for 30min prior to infection. Cells were either infected with; SFV6 on its own; SFV6 infection with mosquito saliva (plus/minus Abx); all with or without IFNAR pre-treatment of cells. Cells were left for 6 hours following infection. As previously, a luciferase assay on media supernatant collected 6 hours post infection demonstrated that mosquito saliva significantly inhibited SFV6 infection, whilst saliva acquired from antibiotic treated mosquitoes did not. However, by blocking the expression of type I IFN in macrophages prior to the addition of extracted mosquito saliva, inhibition of virus infection by mosquito saliva was prevented (see figure 4.8). In fact, there was no significant difference in luminescence between the virus alone group and the SFV6 + extracted mosquito saliva following IFNAR treatment. This suggests that microbiota present in mosquito saliva triggers a type I IFN response which results in inhibition of virus replication *in vitro*.

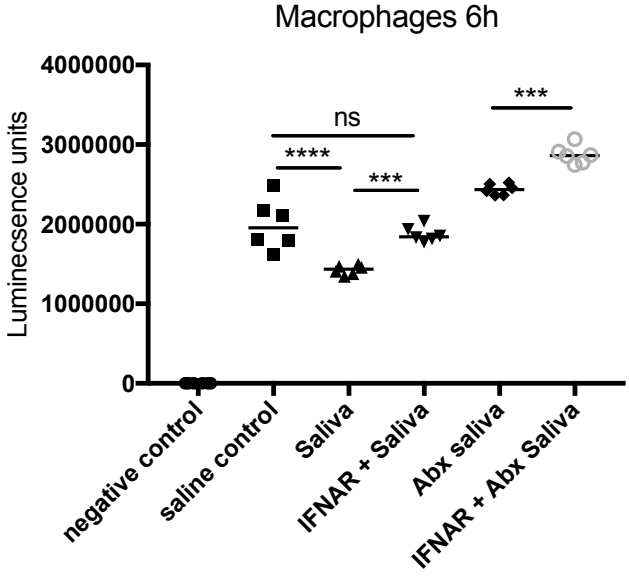


Figure 4.8. Salivary microbiota upregulates type I IFN responses resulting in inhibition of virus infection *in vitro*

To investigate whether salivary microbiota inhibits virus infection via a type I IFN response, macrophages were pre-treated IFNAR-1 for 1 hour prior to infection. Cells were then infected with an MOI of 0.1 of SFV6 on its own or alongside saliva from untreated or Abx treated mosquitoes, with or without pre-treatment with IFNAR-1. Supernatant was acquired 6 hours post infection and luminescence measured via plaque assays. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (One-Way ANOVA test * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

4.7 Microbiota depleted saliva is less inflammatory than untreated saliva *in vivo*

Following the discovery that salivary microbiota causes inhibition of virus infection *in vitro* in macrophages and fibroblasts, we wanted to investigate whether salivary microbiota has an impact on the modulation of arbovirus infection *in vivo*. Initially, in order to investigate whether depletion of salivary microbiota alters the upregulation of inflammatory genes in the skin, mice were exposed to up to 5 mosquito bites of antibiotic treated or untreated mosquitoes on a small section on the dorsal side of their foot and left for 5 hours. Gene expression of a selection of genes previously identified to be upregulated by mosquito saliva (section 3.4) was compared to that of unbitten resting skin via qPCR. Data showed that whilst bites from untreated mosquitoes resulted in a significant upregulation of CXCL2, IL1β and CCL2, mosquito bites from antibiotic treated mosquitoes only significantly upregulated CCL2; the expression of which was still lower than that of untreated mosquito bites (see figure 4.9). This suggests that at least some of the upregulation of inflammatory genes in response to mosquito saliva, is due to the microbiota present in the saliva.

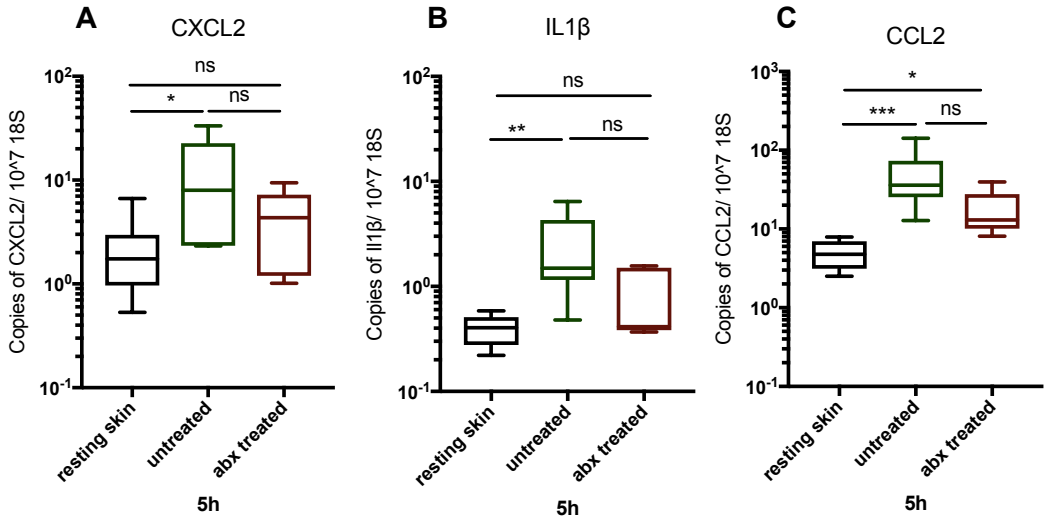


Figure 4.9. Upregulation of key inflammatory genes in the skin in response to salivary microbiota

In order to investigate whether salivary microbiota upregulates key inflammatory genes in the skin, mice were inoculated subcutaneously in the dorsal side of their left foot with 1µl PBSA to control for upregulation of genes in response to the trauma of inoculation (black), or 5 mosquitoes worth of saliva from untreated (green) or Abx treated (red) *Ae.aegypti* in 1µl. Expression of CXCL2, IL1β and CCL2 were measured using RT-qPCR in the skin 5 hours post inoculation. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$.)

4.8 Microbiota does not play a role in facilitating saliva-induced arbovirus enhancement

Following the discovery that microbiota depleted saliva is less inflammatory than untreated microbiota-sufficient saliva, we wanted to investigate whether salivary microbiota modulates levels of SFV infection *in vivo*. For this purpose, mice were anesthetised and exposed to 3 to 5 mosquito bites of either untreated or antibiotic treated *Ae.aegypti* mosquitoes in the dorsal side of their left foot, before being injected with 250 PFU of SFV6. SFV6 was used for data to be comparable to *in vitro* data where SFV6 had been used. 24 hours post infection mice were culled and skin, serum and spleen were collected. Quantification of viral RNA at the inoculation site and spleen demonstrated no significant difference in amount of viral RNA between mice exposed to antibiotic treated or untreated mosquito bites (see figure 4.10). Similarly, no difference was observed in viral titres detected in the serum as both antibiotic treated and untreated mosquito bites significantly enhanced viral infection. This suggests that salivary microbiota does not play a significant role in modulating viral infection *in vivo*.

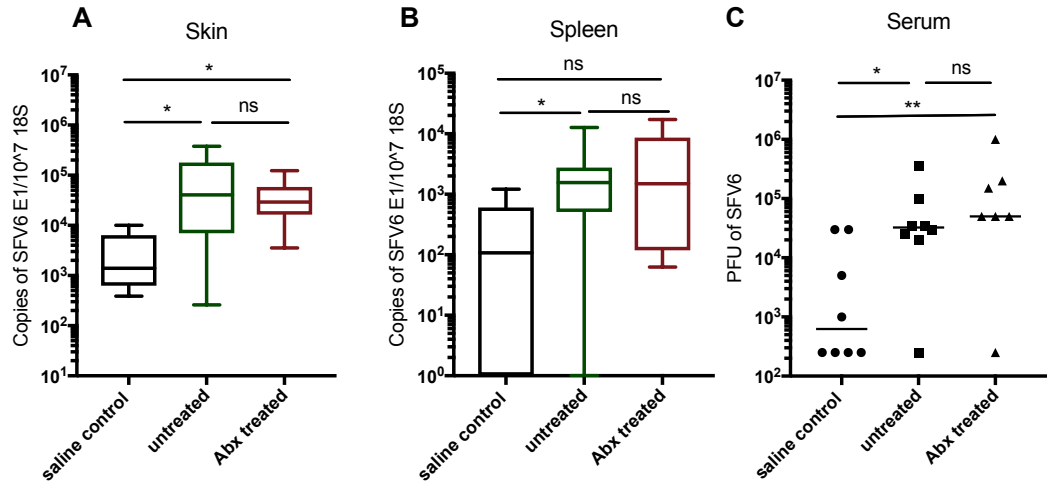


Figure 4.10. Salivary microbiota does not affect mosquito saliva's ability to enhance infection
In order to investigate whether salivary microbiota affects the modulation of arbovirus infection, mice were inoculated subcutaneously in the dorsal side of their left foot with 250 PFU SFV6 on its own (black), or alongside 5 mosquitoes worth of saliva from untreated (green) or Abx treated (red) *Ae.aegypti*. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data is presented as dot plots with each dot representing a separate biological sample. (n=8) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

4.9 Summary and Conclusions

In this chapter we developed an *in vitro* model system for the study of mosquito saliva dependent virus enhancement. However, utilising murine macrophages and dermal fibroblasts we demonstrated that, *in vitro*, mosquito saliva does not enhance virus infection; instead, mosquito saliva significantly inhibits infection of SFV *in vitro*. A similar observation was made using SGE by Conway et al. 2016 [467] for DENV infection of U937 cells. In this paper they attributed this inhibition to salivary protein D7. However, we demonstrated that saliva-induced inhibition occurs due to the presence of microbiota in the saliva, as saliva acquired from antibiotic treated mosquitoes did not inhibit virus infection. Pre-treatment of macrophages with an interferon blocking antibody also prevented salivary mediated inhibition of virus infection *in vitro*. This suggests that microbiota triggers a type I IFN response that makes cells more refractory to virus infection. In contrast, others have observed an enhanced virus infection in response to mosquito saliva in keratinocytes [448]. However, it is debatable how relevant these observations are as during mosquito probing, the mosquito probes through the keratinocyte layer and therefore these cells are unlikely to be important targets of virus infection *in vivo*.

Furthermore, as mosquito saliva, in the absence or presence of salivary microbiota, did not enhance virus infection *in vitro*, it suggests that saliva does not directly aid virus infection by directly aiding virus attachment to susceptible cells or by making cells more susceptible to infection. By utilising explanted mouse skin, we also demonstrate that mosquito saliva activates responses that only occur *in vivo* for viral enhancement to occur. This was because no enhancement of SFV infection was observed in mouse skin injected with virus/saliva, but then cultured *ex vivo* i.e. following the caseation of circulation and plasma-derived factors. **Together, this suggests that the mechanisms involved in facilitating arbovirus enhancement by mosquito saliva is dependent on an intact circulation and possibly by the influx of fluid or recruited immune cells [310] [95].**

Finally, it has previously been suggested that microbiota present in mosquito saliva could modulate arbovirus infection *in vivo* by eliciting inappropriate immune responses that inadvertently enhance infection. Therefore, we wanted to determine

whether mosquito salivary microbiota were responsible for the virus enhancing effect that mosquito saliva exhibits. Utilising our newly developed antibiotic treatment, we demonstrate for the first time, that microbiota present in mosquito saliva does not modulate virus infection *in vivo* as both microbiota depleted and untreated mosquito bites successfully enhanced infection to a similar extent. Ideally this experiment should be repeated utilising extracted mosquito saliva from Abx treated and untreated mosquitoes. However, we can conclude that the salivary microbiome do not make up the factors in mosquito saliva responsible for enhancing virus infection; instead other factors within mosquito saliva must be responsible for triggering virus enhancement.

CHAPTER 5: Mosquito salivary components responsible for arbovirus enhancement are not present in all mosquito species and associates with vector competence.

5.1 Introduction

In the previous chapter it was demonstrated that microbiota is not involved in modulating arbovirus infection *in vivo*; therefore another factor in mosquito saliva must be implicated in facilitating mosquito saliva dependent arbovirus enhancement. Blood feeding in mosquito species is a mechanism used exclusively by females of certain mosquito species to facilitate oviposition. Transcriptomic analysis of female and male *Ae.aegypti* mosquitoes have demonstrated that certain salivary transcripts appear to exhibit differential expression between males and females with 207 transcripts present in the salivary gland that are female specific [458]. This differential expression of transcripts could alter *Ae.aegypti* saliva's ability to enhance arbovirus infection. Differences in the ability of male, compared to female, saliva to modulate virus infection in mice might suggest that these more limited number of differentially expressed salivary genes are responsible. To further complicate the interpretation of our results, the act of blood feeding by a female mosquito leads to alteration in the levels of some salivary transcripts [460]. Therefore, blood-fed mosquitoes may salivate saliva that differs in its ability modulate arbovirus infection in mammals. This suggests that the act of blood feeding could alter the ability of saliva to facilitate arbovirus enhancement.

Furthermore, there are several species of blood feeding mosquitoes, many of which are of medical relevance due to the arboviruses they transmit. There have been several studies investigating whether different mosquito species can enhance the infection of a specific virus that they transmit, including *Ae.aegypti* enhancing SFV and RVFV [490], *Culex tarsalis* which has been demonstrated to enhance WNV [447] whilst Cache-Valley (CV) virus infection severity has been demonstrated to be increased by the bites of *Aedes triseriatus*, *Ae.aegypti*, and *Culex pipiens* [491]. Also, *Aedes triseriatus* mosquito bites/saliva have also been found to enhance vesicular stomatitis New Jersey virus (VSNJV) [492]. Importantly however, it remains unknown whether different blood feeding mosquito species enhance virus infection in a similar way. Nor is it known whether the ability of their saliva to enhance arbovirus infection is restricted to the specific arboviruses they transmit. Therefore, in this chapter we wanted to investigate whether extracted saliva acquired from different blood feeding mosquito species successfully enhance infection of SFV. For this purpose the

enhancing effect of *Ae.aegypti* saliva was compared to that of the closely related *Ae.albopictus* which belongs to the same genus, as well as *Cx.pipens* which is a mosquito species that commonly transmits arboviruses including WNV and JEV. In addition, two species from the *Anopheles* genus were selected, *An.gambiae* and *An.stephensi*, as *Anopheles* blood feeding mosquitoes have been demonstrated to be inefficient vectors of the majority of arboviruses. To date, no one has investigated whether *Anopheles* mosquito bites and/or saliva are capable at enhancing arbovirus infection.

Therefore the aims of this chapter were:

1. **To determine whether male *Ae.aegypti* saliva enhances arbovirus infection to the same extent as female saliva.**
2. **To investigate whether the act of prior blood feeding alters mosquito saliva in a way that impacts the modulation of arbovirus infection.**
3. **To determine if salivary factors responsible for arbovirus infection are proteins.**
4. **To determine if mosquito saliva from different mosquito species differ in their ability to enhance arbovirus (SFV and ZIKV) infection.**

5.2 Male mosquito saliva does not enhance SFV infection as efficiently as female mosquito saliva

As mentioned, male *Ae.aegypti* mosquitoes do not feed on blood whilst female mosquitoes do. Transcriptome analysis of female and male salivary glands have demonstrated differences in the expression of genes in the salivary glands. Therefore, we wanted to determine whether male mosquito saliva to enhances SFV infection. To do this, mice were injected subcutaneously with 5 mosquitoes worth of extracted mosquito saliva from either male or female *Ae.aegypti* mosquitoes alongside 10,000 PFU of SFV4. 24h post infection mice were culled and tissues collected. Quantity of SFV RNA at the inoculation site was significantly higher in mice injected with female saliva in comparison to mice injected with male saliva (see figure 5.1). Also, whilst

female *Ae.aegypti* saliva caused a significant increase in virus titres detected in the serum, compared to virus alone, male saliva did not.

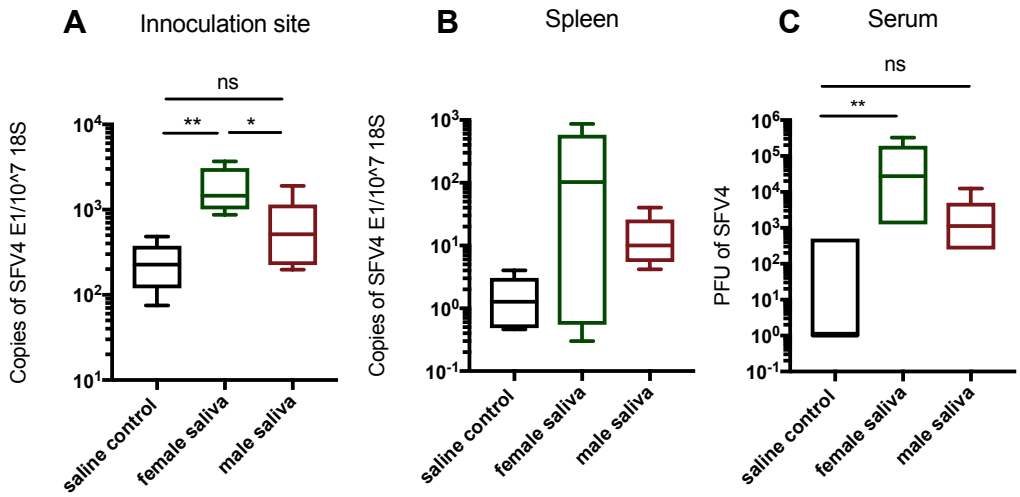


Figure 5.1. Male *Ae.aegypti* saliva does not enhance SFV infection *in vivo*

In order to investigate whether male *Ae.aegypti* saliva enhances arbovirus infection, mice were inoculated subcutaneously in the dorsal side of their left foot with 10,000 PFU SFV4 on its own (black), or alongside 5 mosquitoes worth of saliva from female (green) or male (red) *Ae.aegypti*. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

As there was some concern regarding the potential difference in the total quantity of mosquito saliva injected between male and female mosquitoes the experiment was repeated following a measurement of protein concentration via nanodrop. The quantity of protein injected was then controlled for. In this experiment quantity of viral RNA at the inoculation site and in the spleen as well as viral titres in the serum, were compared between mice inoculated with 10,000 PFU of SFV4 in combination with female or male *Ae.aegypti* saliva. Similarly to what was previously observed above, the quantity of viral RNA in the skin was significantly lower in mice inoculated with male mosquito saliva to those injected with female saliva (see figure 5.2). Similarly, there was significantly less infectious virus present in the serum of mice injected with male mosquito saliva in comparison to mice injected with female saliva. No significant difference in quantity of viral RNA was observed in the spleen. This could simply be due to the fact that spleens are more subject to variation than serum and therefore serum levels can be considered the key finding. These

experiments suggest a role for salivary factors associated with blood feeding in virus enhancement.

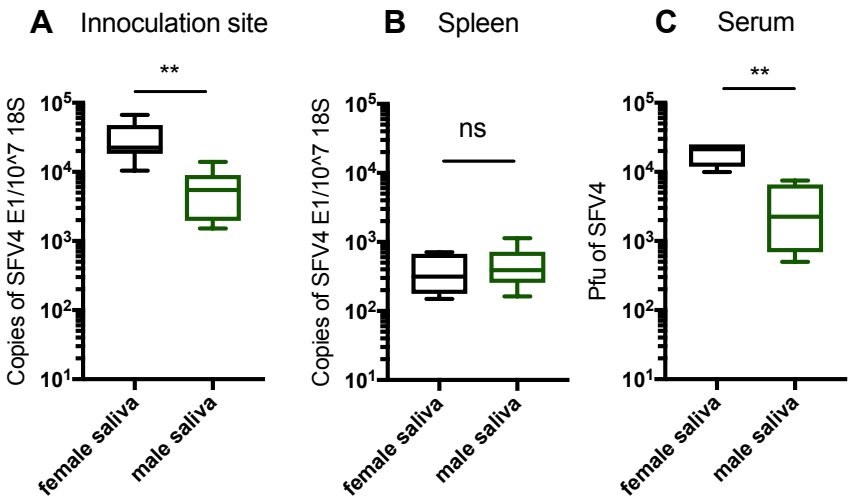


Figure 5.2. Significant difference in quantity of SFV infection following co-inoculation with female or male *Ae.aegypti* saliva

In order to control for the impact on virus infection by the quantity of male saliva, male and female saliva was quantified via nanodrop. The same quantity of salivary protein was then injected into both groups. Mice were infected subcutaneously with 10,000 PFU SFV4 alongside female (black), or male (green) saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Mann Whitney test * $p<0.05$, ** $p<0.01$).

5.3 Act of prior blood feeding does not alter mosquito saliva's ability to enhance SFV infection

As male mosquito saliva proved to be less efficient at enhancing arbovirus infection than female saliva, next we wanted to investigate whether the act of blood feeding in female mosquitoes has an impact on the ability of the saliva for modulating arbovirus infection in mammals. For this experiment, saliva was extracted from exclusively sugar fed mosquitoes as well as from mosquitoes that had received a blood meal twice previously at weekly intervals. 5 mosquitoes worth of either saliva was then injected alongside 10,000 PFU of SFV4 into mice. 24h later mice were sacrificed and tissues acquired. Interestingly, no significant difference in quantity of viral RNA or viral titres were detected between the mice inoculated with saliva from exclusively sugar fed or saliva from blood fed mosquitoes (see figure 5.3). Importantly, this suggests that the transcriptomic changes observed in the salivary glands of *Ae.aegypti*

mosquitoes following blood feeding has no impact on *Ae.aegypti* saliva's ability to enhance arbovirus infection.

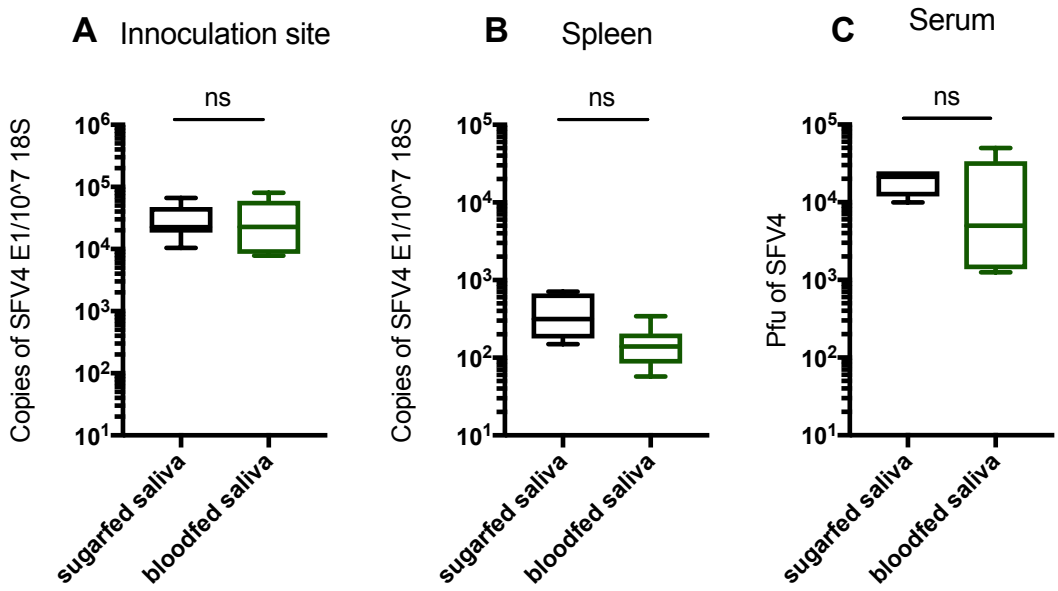


Figure 5.3. No difference in terms of modulation of infection between saliva from blood fed and non-blood fed mosquitoes

To investigate whether the act of blood feeding alters female *Ae.aegypti* saliva's virus modulatory effect, mice were infected subcutaneously with 10,000 PFU SFV4 alongside saliva acquired from exclusively sugar fed female *Ae.aegypti* mosquitoes (black), or from blood fed female *Ae.aegypti* mosquitoes (green). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Mann Whitney test).

5.4 Prior heat treatment of saliva suggests salivary factor is proteinaceous

Heat treatment of mosquito saliva prior to inoculation was conducted in order to denature any proteins present. Mice were co-inoculated with either natural/untreated or heat-treated *Ae.aegypti* saliva, and 10,000 PFU of SFV4. Analysis of tissues and serum acquired at 24hpi, demonstrate that heat treatment of *Ae.aegypti* saliva completely inhibits saliva mediated viral enhancement (see figure 5.4). This suggests that one or several proteins are implicated in facilitating enhancement of arbovirus infection.

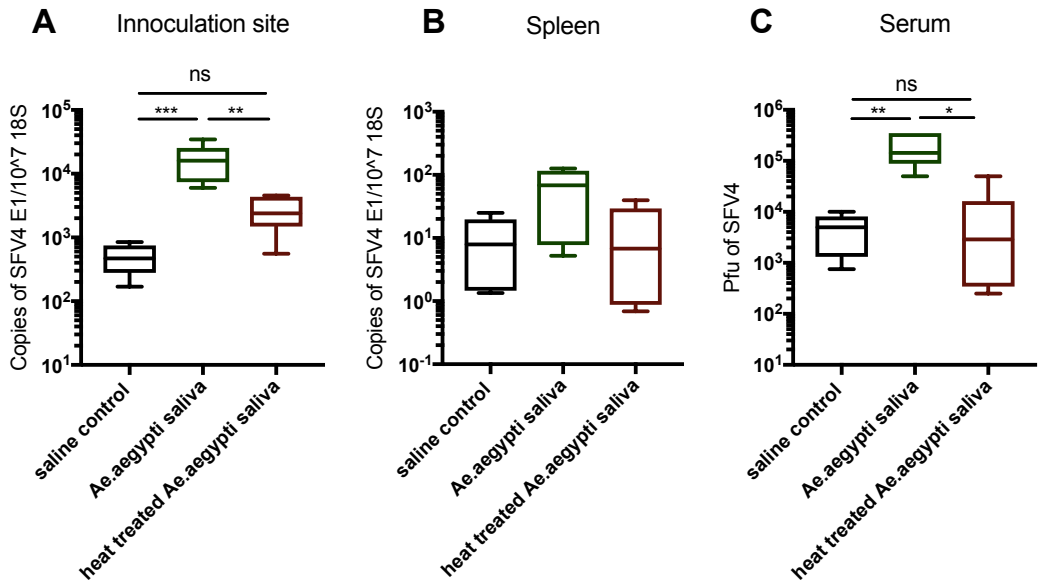


Figure 5.4. Heat treatment of mosquito saliva inactivates saliva's ability to enhance viral infection

To investigate whether the factor responsible for arbovirus enhancement in female *Ae.aegypti* saliva is heat sensitive, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of untreated (green) or heat treated (red) *Ae.aegypti* saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

5.5 Saliva from different mosquito species differ in its ability to enhance SFV infection

Following the discovery that the salivary factor responsible for facilitating saliva-dependent viral enhancement are likely proteins associated with female mosquito blood feeding, the next step was to determine whether these factors, and the ability to enhance viral infection, are present in other blood feeding mosquito species. Initially, the enhancing effect of *Ae.aegypti* saliva on SFV4 infection was compared to that of a related mosquito species within the same genus; *Ae.albopictus*. Both species are competent in transmitting alphavirus and flavivirus arboviruses and as such we hypothesised that both species' saliva would possess the ability to enhance arbovirus infection in mammals. In order to test this, 5 mosquitoes worth of saliva extracted from *Ae.aegypti* or *Ae.albopictus* mosquitoes were inoculated subcutaneously in the dorsal side of the left foot of the mouse alongside 10,000 PFU of SFV4 and samples were acquired 24 hours later. QPCR and plaque assay data demonstrated that both

species caused a significant and similar level increase in SFV4 infection in comparison to virus administered on its own in the absence of mosquito saliva (see figure 5.5). As there was no difference in quantity of viral RNA or viral titres between mice inoculated with *Ae.aegypti* or *Ae.albopictus* saliva, this confirmed our hypothesis that *Ae.albopictus* saliva possesses the same ability to enhance virus infection in mammals as *Ae.aegypti*.

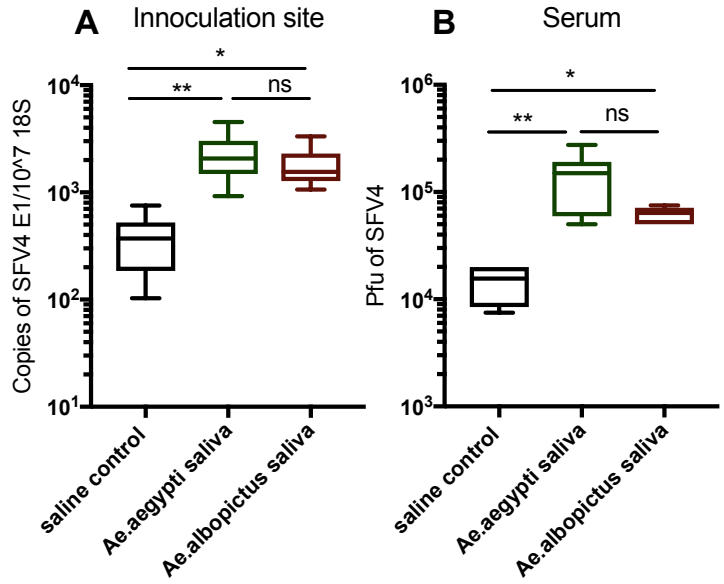


Figure 5.5. *Ae.albopictus* saliva enhances SFV infection

To investigate whether *Ae.albopictus* saliva enhances SFV infection, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* (green) or *Ae.albopictus* (red) saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (B). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Following this, we wanted to investigate whether mosquito saliva of a blood feeding mosquito of a different genus also has the ability to enhance arbovirus infection. For this purpose the mosquito species *Cx.pipiens* was selected. *Culex* mosquitoes are widespread across large portions of the globe and are competent to transmit arboviruses such as WNV, JEV and St. Louis encephalitis virus [226] [227]. Whilst studies have already confirmed that *Culex* mosquito bites can enhance infection of WNV [447], we wanted to determine if *Culex* mosquitoes can enhance infection of SFV4, even though *Culex* mosquitoes are not capable of transmitting alphaviruses such as the closely related CHIKV [493]. Importantly, by doing so it would enable us to establish whether *Culex* mosquito saliva enhances infection in a similar manner

to *Aedes* mosquitoes. Therefore, mice were inoculated with 5 mosquitoes worth of saliva of either *Ae.aegypti* or *Cx.pipiens* mosquitoes alongside 10,000 PFU of SFV4. At 24 hours post infection mice inoculated with *Cx.pipiens* saliva exhibited a significant enhancement of SFV4 infection to a similar extent as the mice inoculated with *Ae.aegypti* saliva (see figure 5.6).

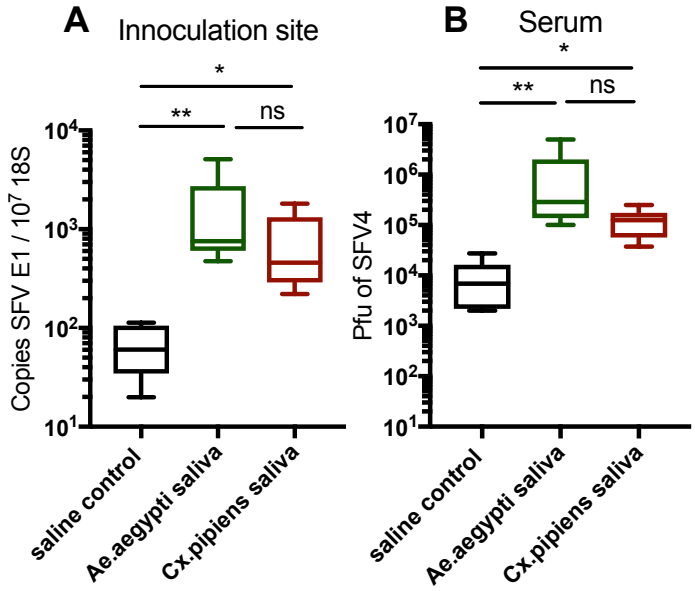


Figure 5.6. *Cx.pipiens* saliva enhances SFV infection

To investigate whether *Cx.pipiens* saliva enhances SFV infection, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* (green) or *Cx.pipiens* (red) saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (B). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

As both *Ae.albopictus* and *Cx.pipens* saliva were found to enhance SFV4 infection to a similar extent as *Ae.aegypti*, next, we wanted to look at a genus of a more distantly related blood feeding mosquito species that are known to be inefficient vectors of most arboviruses; the *Anopheles* mosquitoes. As mentioned in the introduction, *Anopheles* mosquitoes are mainly known for their ability to transmit malaria. In terms of arbovirus transmission though, they are known to be inefficient vectors of the majority of arboviruses, with the exception of one; ONNV which is predominantly spread by *An.gambiae*. It is not clear why *Anopheles* are such poor vectors of arbovirus, considering they routinely would sample viraemic individuals in arbovirus endemic areas. Indeed, some studies on the virome of *Anopheles* mosquitoes have observed that

Anopheles harbour a large number of different medically relevant arboviruses such as VEEV, WEEV, SFV, WNV and JEV. However, to date no studies have shown successful transmission of any of these viruses by *Anopheles* mosquitoes. This makes it clear that *Anopheles* species are incapable of transmitting and maintaining the circulation of arboviruses in the wild, with the interesting exception of ONNV [234].

Keeping this in mind, we wanted to investigate whether saliva from female, blood-feeding competent, *Anopheles* mosquitoes could enhance arbovirus infection in mammals. To achieve this, saliva acquired from 2 species of the *Anopheles* genus was used; *An.gambiae* and *An.stephensi*. The effect of 5 mosquitoes worth of mosquito saliva on SFV4 infection from either *An.gambiae* or *An.stephensi* mosquitoes was compared to that of *Ae.aegypti* saliva at 24 hours post infection. Interestingly, there was no significant increase in the level of viral RNA at either the inoculation site or in the spleen in mice co-inoculated with SFV4 and either of the *Anopheles* mosquitoes saliva, compare to injection with virus alone (see figure 5.7). Thus, the level of viral RNA was significantly lower in mice injected with *Anopheles* compared to *Aedes* mosquito saliva. The same pattern was reflected in the viral titres detected in the serum.

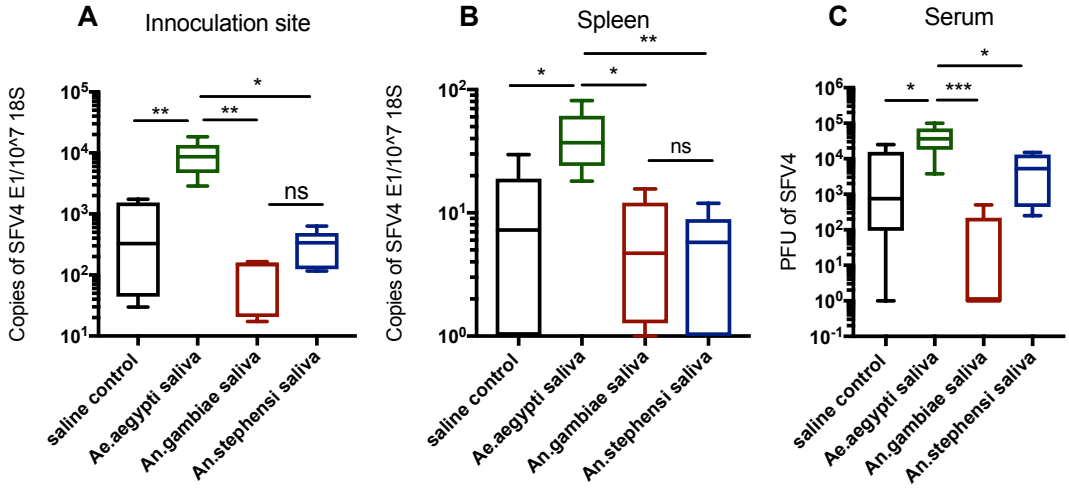


Figure 5.7. *Anopheles* saliva fails to enhance SFV infection

To investigate whether *Anopheles* saliva enhances SFV infection, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* (green), *An.gambiae* (red) or *An.stephensi* (blue) saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Following the discovery that *Anopheles* mosquito saliva is incapable of enhancing SFV infection *in vivo*, the next step was to confirm that the same applies for other arboviruses as well. As SFV is an alphavirus, we decided to utilise a medically relevant arbovirus from a different virus family, therefore, as a representative flavivirus, ZIKV was used. For this experiment, the ZIKV *in vivo* model described in section 3.6 was used, which relies on blocking expression of type I IFN via the use of an IFNAR blocking antibody. Mice were injected with the anti-mouse IFNAR-1 antibody 24 hours before the start of the experiment. These mice were then inoculated with 1000 PFU of ZIKV on its own or pre-mixed with 5 mosquitoes worth of either *Ae.aegypti* or *An.gambiae* saliva. Viral titres of ZIKV in the serum of mice 24 hours post infection demonstrated that whilst *Ae.aegypti* saliva significantly enhances ZIKV infection, *An.gambiae* saliva did not, with a similar trend observed with the quantity of viral RNA detected in the skin acquired from the site of inoculation (see figure 5.8).

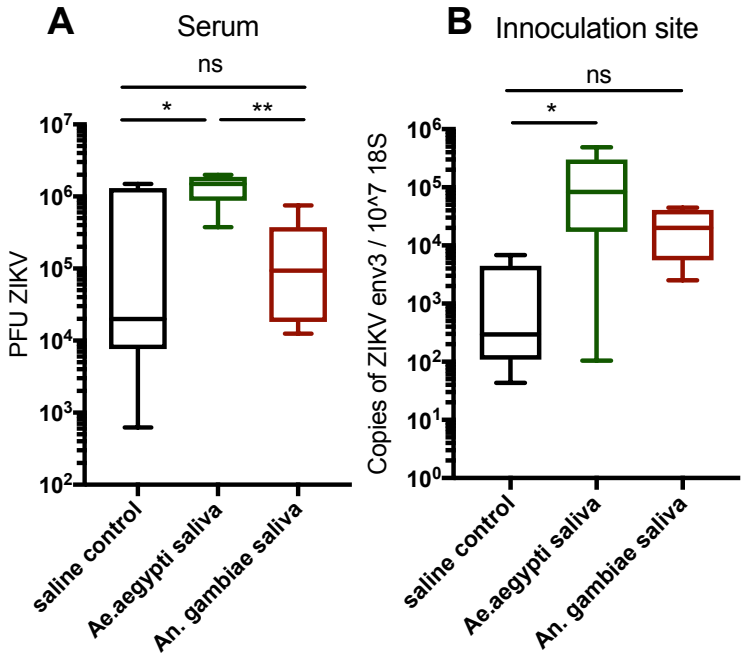


Figure 5.8. *Anopheles* saliva fails to enhance ZIKV infection

To investigate whether *Anopheles* saliva enhances ZIKV infection, mice were infected subcutaneously with 1000 PFU ZIKV on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* (green) or *An.gambiae* (red) saliva. Expression of viral ZIKV Env3 gene was measured using RT-qPCR in the skin (A) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (B). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$)

In order to eliminate the possibility that the mosquito bite of *An.gambiae* mosquitoes could somehow compensate for *An.gambiae* saliva's lack of ability to modulate arbovirus infection, the SFV and ZIKV experiments above were repeated with mice exposed to aproximately 2 mosquito bites on the dorsal side of their foot replacing the injections of mosquito saliva. Mosquitoes were left until fully engorged. Injection of 10,000 PFU of SFV4 or 1000 PFU of ZIKV was conducted at the bite site immediately following the completion of mosquito feeding. Similarly, as observed before, *Ae.aegypti* bites significantly enhanced SFV4 infection in the skin and serum whilst *An.gambiae* bites did not (see figure 5.9).

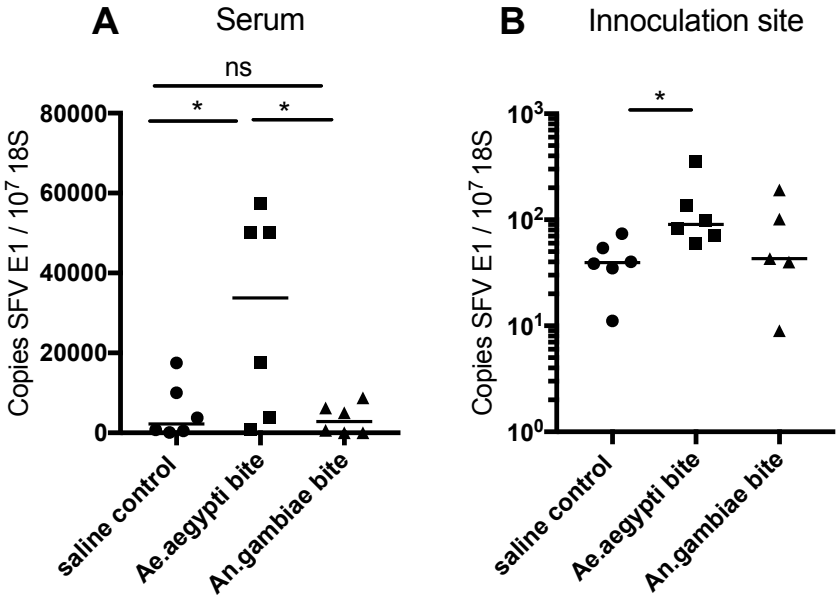


Figure 5.9. *An.gambiae* bites unable to enhance SFV4 infection

To investigate whether *An.gambiae* bites can compensate for their saliva's inability to enhance SFV4 infection, mice were infected subcutaneously with 10,000 PFU SFV4 on its own, or immediately following exposure to approximately 2 mosquito bites of *Ae.aegypti* or *An.gambiae* mosquitoes. Expression of viral SFV E1 gene was measured using RT-qPCR in the serum (A) and skin (B) 24 hours post infection. Data are presented as dot plots: with each dot representing a separate biological sample (n=6) (One way ANOVA (serum) and Kruskal Wallis test (skin) * $p<0.05$).

In the same way, whilst *Ae.aegypti* bites significantly enhanced ZIKV infection, *An.gambiae* bites did not (see figure 5.10). These experiments further support the hypothesis that *An.gambiae* mosquito bites do not enhance arbovirus infection.

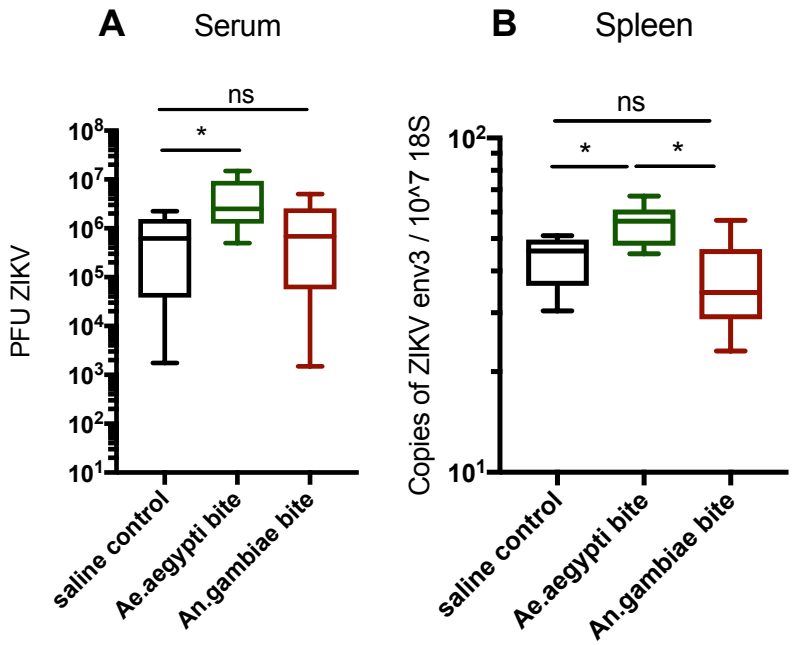


Figure 5.10. *An.gambiae* bites unable to enhance ZIKV infection

To investigate whether *An.gambiae* bites can compensate for their saliva's inability to enhance ZIKV infection, mice were infected subcutaneously with 1000 PFU ZIKV on its own (black), or immediately following exposure to approximately 2 mosquito bites of *Ae.aegypti* (green) or *An.gambiae* (red) mosquitoes. Expression of viral ZIKV Env3 gene was measured using RT-qPCR in the serum (A) and spleen (B) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

5.6 Different effects on mouse survival following virus co-injection with *Aedes* or *Anopheles* saliva

Following the discovery that *Anopheles* saliva does not enhance virus infection at 24 hours, we wanted to ensure that *An.gambiae* saliva did not enhance severity of infection at a later time point than was previously studied by ensuring that *An.gambiae* saliva does not have an impact on mouse survival following SFV4 infection.

For this purpose, mice were injected with 40.000 PFU of SFV4 with or without 5 mosquitoes worth of either *Ae.aegypti* or *An.gambiae* saliva. ("Virus alone" and "*Aedes* saliva" data presented previously in section 3.5). Mice were then left for up to 14 days and monitored for signs of illness. When mice reached a clinically defined and established endpoint (see chapter 2) they were euthanized in accordance with home office regulations. In this model system, there was a significant reduction in the

percentage of surviving mice when SFV4 was administered alongside *Ae.aegypti* saliva (see figure 5.11). However, no reduction in mouse survival was observed in the group inoculated with *An.gambiae* saliva. In fact, the groups that received SFV4 with or without *An.gambiae* saliva did not differ from each other in terms of mouse survival.

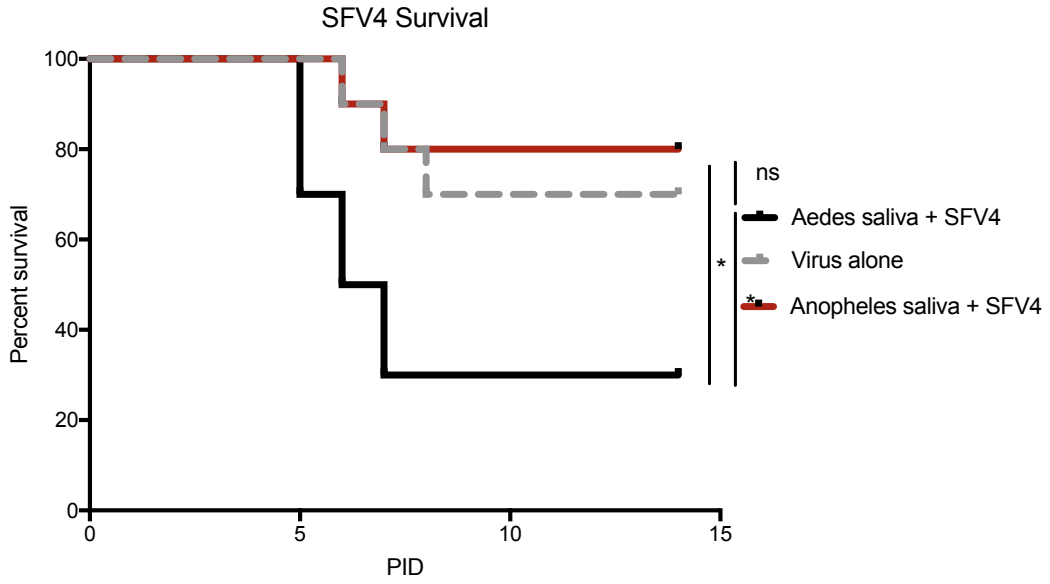


Figure 5.11. Survival to SFV challenge following inoculation with *Anopheles* or *Aedes* saliva

To compare impact on mouse survival following exposure to *Anopheles* or *Aedes* saliva, mice were infected with 40,000 PFU SFV4 subcutaneously on its own (grey), alongside 5 mosquitoes worth of *Ae.aegypti* saliva (black) or *An.gambiae* saliva (red). Mice were then monitored for signs of a clinically defined endpoint. Mice were left for a maximum of 14 days post infection. Graph demonstrates the survival percentages. Control groups presented previously in graph 3.6 (n=10) (Mantel Cox test * $p<0.05$).

Secondly, in order to investigate when a difference in virus quantity can successfully be detected at the site of inoculation following infection with SFV alongside *Ae.aegypti* or *An.gambiae* saliva, an experiment was conducted aiming to follow virus quantity progression at 5 hours, 10 hours and 24 hours post infection. By defining the time post inoculation that saliva modulates level of virus, it will help define whether its mechanism of action is direct/immediate or indirect/delayed in nature. For this purpose, mice were inoculated with SFV4 on its own or alongside *Ae.aegypti* or *An.gambiae* saliva. At 5 hours post infection, no difference in quantity of viral RNA at the inoculation site was detected between the 3 treatment groups. Differences were first detected 10 hours post infection where, mice inoculated with SFV4 pre mixed with *Ae.aegypti* saliva, had significantly higher quantities of viral RNA at the inoculation site than the groups treated with virus alone and virus pre mixed with

An.gambiae saliva (see figure 5.12). This supports the experiment in chapter 3 (figure 3.4) where *Ae.aegypti* saliva caused significant upregulation of virus in the skin at 10h and 24h post infection. In that experiment we concluded that *Ae.aegypti* saliva facilitates enhanced viral replication at the inoculation site whilst simultaneously increasing viral dissemination. Therefore, the differences observed in figure 5.12 in terms of quantity of viral RNA, are most likely due to differences in replication levels.

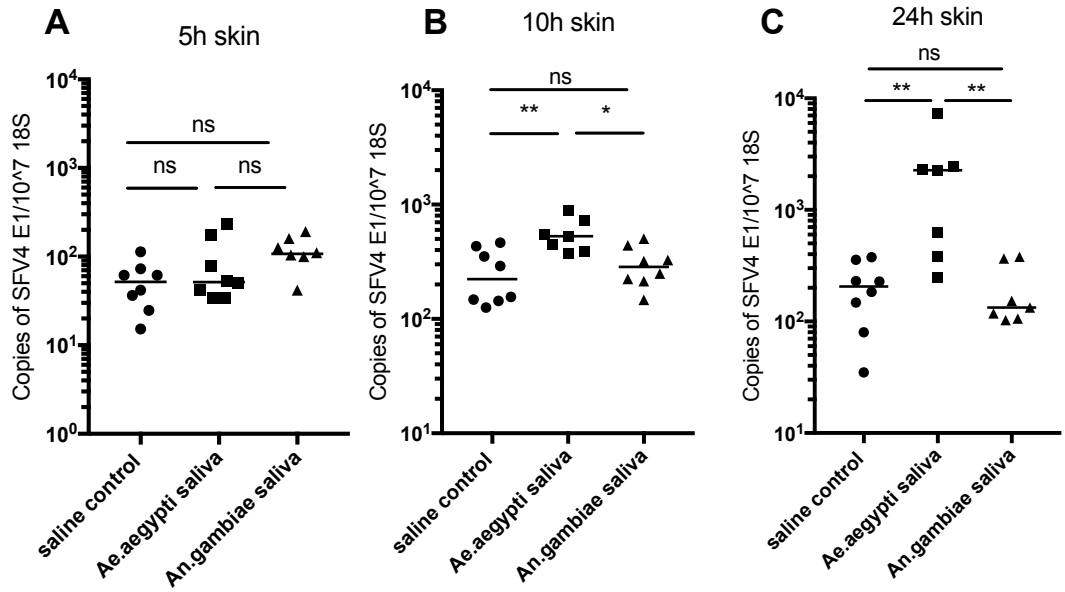


Figure 5.12. Time-course of SFV infection following inoculation with *An.gambiae* saliva

To investigate the kinetics of viral infection following inoculation with *An.gambiae*, mice were infected subcutaneously with 10,000 PFU SFV on its own, or alongside 5 mosquitoes worth of *Ae.aegypti* or *An.gambiae* saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin at 5 hours (A), 10 hours (B) or 24 hours (C) post infection. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=8) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

5.6 Summary and Conclusions

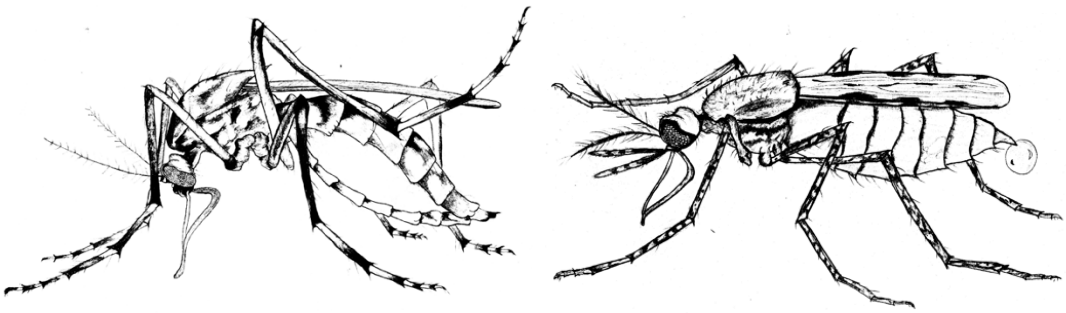
Responses of the mammalian host to the bite of blood feeding arthropods enhance infection of the viruses they transmit, and this is due to the injection of saliva during feeding. From our studies it appears as if the viral enhancing effect of arthropod saliva has co-evolved with the act of blood feeding. Within the mosquito insect group not all mosquito species feed on blood and out of the select mosquito species that do blood feed, only the female will ingest blood; never the males. Also, there is a huge variation in the virus vector competence between blood feeding mosquito species.

Transcriptome analyses on salivary glands of mosquitoes have discovered differences in the transcriptome of male and female *Ae.aegypti* mosquitoes. Transcriptomic differences have also been detected following the ingestion of a blood meal by the female *Ae.aegypti* mosquito. Therefore, in this chapter we sought to investigate whether the differences observed in the transcriptome of male and female *Ae.aegypti*, and the changes in gene expressions following the acquisition of a blood meal, affect the modulatory effect of saliva on virus infection. Our data demonstrate that female *Ae.aegypti* saliva causes a significant enhancement of SFV4 infection whilst saliva acquired from male *Ae.aegypti* mosquitoes does not. Because the majority of differentially expressed salivary genes in females are thought to be associated with blood-feeding, these data would suggest that those factors evolved to facilitate blood feeding are also responsible for enhancing infection with arbovirus in mammals. However, the act of blood feeding itself conducted by female *Ae.aegypti* mosquitoes does not appear to affect the mosquitoes saliva's ability to enhance infection during any subsequent feeds. This is important as presumably, unless the virus was transmitted via vertical transmission, a large portion of blood feeding mosquitoes will only become infected following a blood meal and will therefore only be able to transmit the arbovirus during a secondary blood meal.

After narrowing down that the salivary factors implicated in enhancement of arbovirus infection are only present in female mosquitoes which unlike the males, are capable of blood feeding behaviour, we sought to investigate whether the factors implicated are sensitive to heat treatment. In chapter 4, it was determined that microbiota present in mosquito saliva does not enhance arbovirus infection. A heat treatment of extracted mosquito saliva would kill bacteria present, but potential PAMPs including LPS and other lipids and carbohydrates should remain. However, a heat treatment would denature any proteins present and inhibit their function/activity. In this chapter, we demonstrate that heat treatment of mosquito saliva inactivates the salivary factors responsible for arbovirus enhancement as mice inoculated with virus alongside heat treated saliva exhibited no enhanced virus infection in comparison to when virus was inoculated on its own. This suggests that the factors implicated in saliva dependent arbovirus enhancement are proteins.

Finally, utilising mosquito saliva extracted from a range of blood feeding mosquito species including *Ae.albopictus*, *Cx.pipiens*, *An.gambiae* and *An.stephensi*, we demonstrate that the factors involved in mosquito saliva dependent viral enhancement, are not present in all blood feeding mosquito species. Our data suggests that *Anopheles* mosquito species such as *An.stephensi* and *An.gambiae* are incapable of enhancing arbovirus infections. We also ruled out the possibility that *An.gambiae* saliva enhanced virus infection severity at a later time point than *Ae.aegypti* by conducting a survival experiment with SFV4. The experiment, which compared the lethality of SFV4 infection in mice following virus inoculation on its own or when co-inoculated with either *Ae.aegypti* or *An.gambiae* saliva, demonstrated that whilst *Ae.aegypti* saliva significantly reduced the possibility of survival following infection, co-inoculation of *An.gambiae* saliva with SFV4 had no significant impact on mouse survival in comparison to virus inoculated on its own. This confirms the discovery that *An.gambiae* saliva does not enhance severity of arbovirus infection. This observation could help explain why the blood feeding *Anopheles* mosquitoes are inefficient vectors of the majority of arboviruses as a lack of a viral enhancing effect during virus transmission from the vector to the mammalian host, could prove detrimental for successful infection of mammalian host.

CHAPTER 6: Differences in host responses to *Aedes* and *Anopheles* saliva



6.1 Introduction

In the previous chapter it was established that the salivary factors implicated in causing enhancement of arboviral infection are heat sensitive which suggests that it is made up of one or several proteins. Furthermore, these factors do not appear to be prevalent in all blood feeding mosquito species saliva, as saliva from *Anopheles* mosquitoes failed to enhance both SFV and ZIKV infection in mice. We hypothesised that differences by which the host responds to *Aedes* and *Anopheles* saliva underlies their ability to modulate virus infection in mammals.

Therefore, as a next step, host responses elicited against *Aedes* and *Anopheles* were compared. This was done in order to determine which host responses are vital for the enhancement of arbovirus infection by *Aedes* saliva. Indeed, we suggest that any responses elicited by both types of saliva can be ruled out from being responsible for enhancing of arbovirus infection. In comparison, any potential differences observed in host responses triggered by *Aedes* and *Anopheles* mosquito saliva, may help us elucidate the mechanistic basis by which *Aedes* saliva enhances virus infection.

As part of this, based on the findings in chapter 3 were we demonstrate that *Ae.aegypti* saliva causes significant upregulation of pro-inflammatory genes at the site of inoculation, we wanted to compare the impact of *An.gambiae* saliva on pro-inflammatory genes in the skin to that of *Ae.aegypti.* This was done as any differences observed could potentially help explain lack of viral enhancement observed in response to *An.gambiae* saliva.

As discussed in section 1.6, mosquito bites can trigger early and late phase cutaneous inflammation accompanied by vascular leakage and the formation of oedema and pruritic lesion at the site of inflammation. Also, as has already been mentioned, mosquito bites and saliva induce vascular leakage, which can break down the extracellular matrix of fibroblasts which increases the permeability of endothelial cells [310] [444]. Enhanced permeability of blood vessels could allow for an increase in the extravasation of monocytes and neutrophils to the site of infection which can indirectly aid the virus [310].

Therefore, the aims for this chapter were to answer the following questions:

1. **Is there a difference in the upregulation of inflammatory genes caused by *Aedes* and *Anopheles* saliva?**
2. **Is there a difference in the amount of oedema formation following injection with either *Aedes* or *Anopheles* saliva?**

6.2 Inclusion of *Aedes* saliva compensates for the inability of *Anopheles* saliva to enhance infection

In order to gain a better understanding of the mechanisms involved in causing arboviral enhancement, we wanted to ask whether *Anopheles* saliva actively inhibited viral infection thereby cancelling out any enhancing effect that could have been triggered via a specific host response. To achieve this, *Ae.aegypti* and *An.gambiae* saliva were mixed together and their combined effect on SFV4 infection in mice compared. Therefore, mice were inoculated with 10,000 PFU of SFV4 on its own or in combination with 5 mosquitoes worth of *Ae.aegypti* and/or *An.gambiae* saliva. At 24 hours post infection relevant tissues were harvested and expression of viral gene E1 was measured via qPCR and viral titres in serum via plaque assays. Viral RNA expression in the skin and spleen of mice was similar in mice injected with *Aedes* saliva irrespective of inclusion of *Anopheles* saliva. As before, mice inoculated with only *Anopheles* saliva exhibited similar levels of viral E1 expression as the groups that had received virus alone (see figure 6.1). Similar patterns of virus levels between groups were measured in the serum.

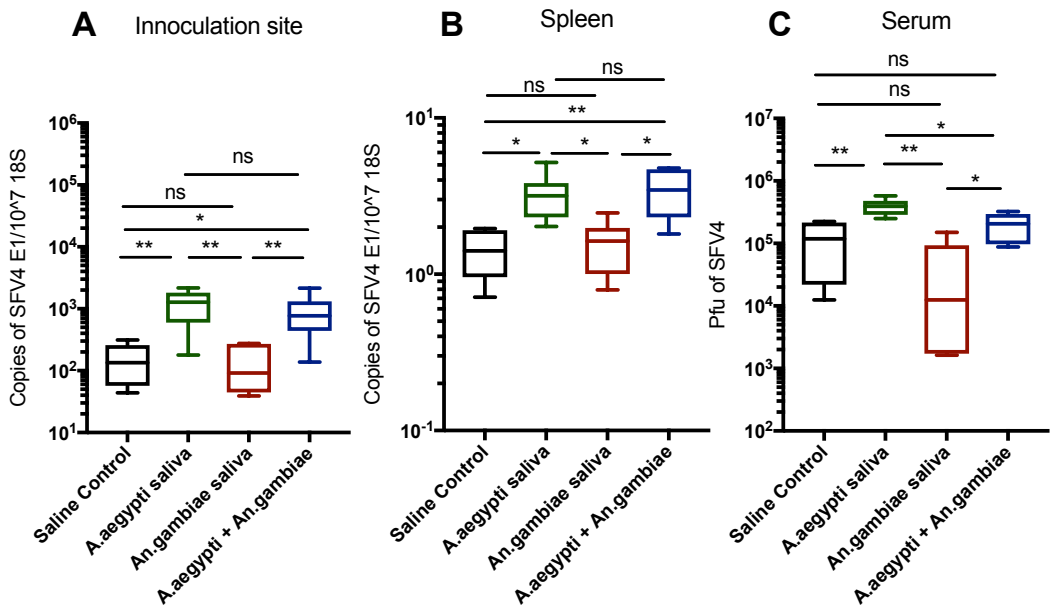


Figure 6.1. *Ae.aegypti* saliva compensates for *An.gambiae* saliva's inability to enhance infection

To investigate whether the addition of *Ae.aegypti* saliva to *An.gambiae* is sufficient cause viral enhancement, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of saliva from *Ae.aegypti* (green), *An.gambiae* (red) or a mixture of both (blue). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (A) and spleen (B) 24 hours post infection. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (C). Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$)

This suggests that *Anopheles* saliva does not actively inhibit virus infection. Instead, it appears as if it lacks one, or several factors, that are present in *Aedes* mosquito saliva that enhances infection with SFV.

6.3 *An.gambiae* saliva upregulates key inflammatory genes more than *Ae.aegypti* saliva.

After establishing that *Anopheles* saliva, unlike *Aedes*, does not modulate outcome of infection with SFV4 in immunocompetent mice, we wanted to compare host responses to either type of saliva. Differences in host response to each saliva could highlight the key mechanism responsible for enhancement of infection in mammals. As previous studies have looked at the influence of *Ae.aegypti* bites on the expression of inflammatory genes, and after us determining that *Ae.aegypti* saliva on its own in the absence of a bite mirrors the upregulation of genes observed by mosquito bites (as was discussed in section 3.4), we decided to investigate whether *Ae.aegypti* saliva

and *An.gambiae* saliva cause different modulation of pro-inflammatory gene responses.

For this purpose, the same genes that were looked at in section 3.4 were used (bite-associated IL1β, CCL2, CXCL2; and antiviral ISGs CCL5 and ISG15) as well as other key ISGs; CXCL10, viperin, IFIT2. It is noteworthy that these ISGs were previously shown not to be upregulated in the skin by *Ae.aegypti* mosquito bites in the absence of a virus. However, when SFV infection was accompanied by mosquito bites the expression of these genes was further enhanced in comparison to their expression in response to virus inoculated on its own [95]. Thus, there may be a poorly defined role for mosquito saliva in enhancing ISG expression in response to virus sensing. Even though *Aedes* mosquito bites have never been demonstrated to cause upregulation of IFNs in the absence of any virus, we also included IFNα, IFNβ and IFNγ in order to rule out the possibility that *An.gambiae* induces an interferon response.

For this experiment mice were inoculated subcutaneously with a total of 1µl of 5 mosquitoes worth of saliva of either *Ae.aegypti* saliva or *An.gambiae* saliva in PBSA on the upper part of their hind foot. Expression of pro-inflammatory genes were measured 6 hours post injection and expression was compared to skin inoculated with 1µl of PBSA as a control. This time point was selected as it has previously been demonstrated that expression of pro-inflammatory genes in response to *Ae.aegypti* bites peak at 6 hours post exposure [95]. In figure 6.2 (A) the expression of all genes looked at are listed. Overall, the expression of pro-inflammatory genes was higher in response to *An.gambiae* saliva in comparison to *Ae.aegypti* saliva. However the difference in expression of genes between *Ae.aegypti* saliva and *An.gambiae* was not always significant. Neither *Ae.aegypti* or *An.gambiae* saliva triggered a detectable IFN response. Figure 6.2 (B) provides a closer look at the genes which were found to be significantly more expressed in response to *An.gambiae* saliva than to *Ae.aegypti* saliva. As can be seen in the graphs in figure 6.2, *An.gambiae* saliva caused a significantly higher upregulation of IL1β, CXCL2, CXCL10 and CCL5. The gene most significantly upregulated by *Anopheles* saliva was CCL5.

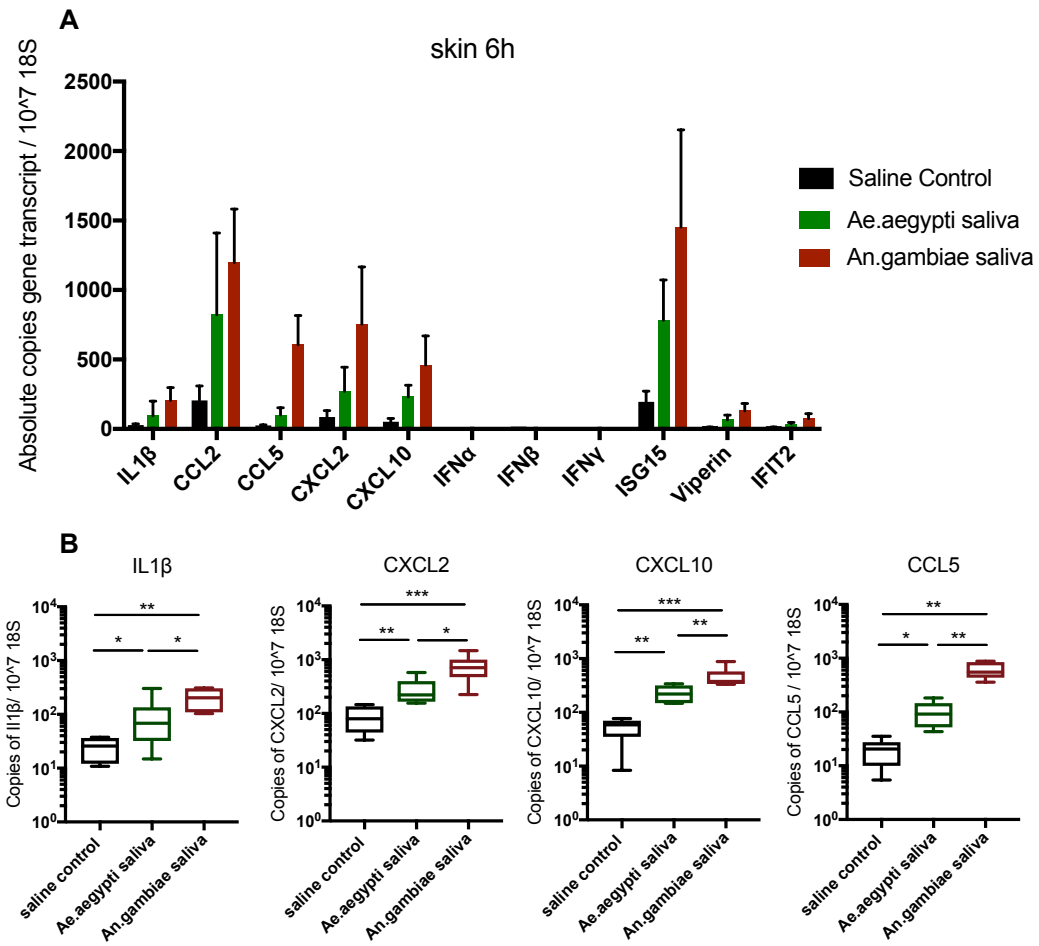


Figure 6.2. Differences in the upregulation of key inflammatory genes in the skin in response to *Aedes* and *Anopheles* saliva

To investigate *Aedes* and *Anopheles* cause differential modulation of the expression of key pro-inflammatory genes in the skin, mice were inoculated subcutaneously in the dorsal side of their left foot with 1µl PBSA to control for upregulation of genes in response to the trauma of inoculation (black), or with 5 mosquitoes worth of saliva from *Ae.aegypti* (green) or *An.gambiae* (red) mosquitoes in 1µl. Expression of IL1β, CCL2, CCL5, CXCL2, CXCL10, IFNα, IFNβ, IFNγ, ISG15, viperin and IFIT2 were measured using RT-qPCR in the skin 5 hours post inoculation. Data is presented via 2 separate methods; (A) All genes investigated are included and presented as median + range; (B) Data from genes where significant difference was observed between *Ae.aegypti* and *An.gambiae* groups are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

We also wanted to investigate whether *An.gambiae* saliva still significantly enhanced key inflammatory genes more than *Ae.aegypti* saliva when co-inoculated alongside virus. Therefore, 10,000 PFU of SFV4 was inoculated subcutaneously alongside either *Ae.aegypti* or *An.gambiae* saliva in the dorsal side of the foot of C57BL/6 mice. Skin samples were then acquired 10 hours and 24 hours post infection. qPCR analysis was conducted on the skin investigating the gene expression of the ISGs

CCL5, viperin as well as IFNα. Their expression was normalised against housekeeping gene 18S as well as against quantity of viral gene E1 in order to ensure differences in expression levels were not due to virus quantity. As can be observed in figure 6.3, significant difference was observed in the expression of CCL5, viperin and IFNα between the group inoculated with *Ae.aegypti* and *An.gambiae* saliva at 24 hours post infection, with *An.gambiae* saliva causing a significantly higher expression of all three genes investigated. No significant difference was observed at 10 hours post infection. Together, this demonstrates that *An.gambiae* saliva causes a more robust upregulation of some key anti-viral genes than *Ae.aegypti*; especially CCL5 and viperin.

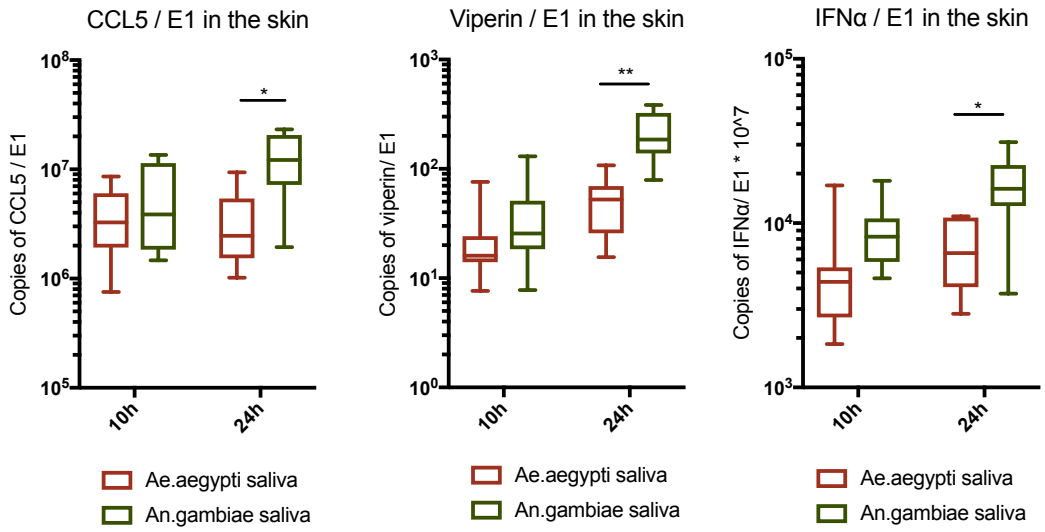


Figure 6.3. Differences in the upregulation of key inflammatory genes in the skin in response to virus inoculation alongside *Aedes* and *Anopheles* saliva

To investigate whether *Anopheles* saliva is more inflammatory than Ae.aegypti in presence of virus, mice were infected subcutaneously with 10,000 PFU SFV4 alongside 5 mosquitoes worth of saliva from *Ae.aegypti* (red) or *An.gambiae* (green) mosquitoes. Expression of CCL5, IFNα and viperin were measured using RT-qPCR in the skin 10 and 24 hours post inoculation. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Mann Whitney * $p<0.05$, ** $p<0.01$, ***$p<0.001$.)

6.4 *Ae.aegypti* saliva and bites cause significantly more oedema than *An.gambiae* saliva

As mosquito bites are known to trigger early and late phase cutaneous inflammation we wanted to investigate whether *An.gambiae* saliva and mosquito bites differed to *Ae.aegypti* saliva and bites, in terms of amount of the oedematous response they induce. To measure tissue oedema levels, mice were injected systemically with Evans

Blue dye, which binds covalently to serum albumin. Endothelial cell barriers lining blood vessels, under normal physiological conditions, prevent passage of albumin into tissues. However during inflammation, endothelial barrier function is disrupted, allowing macromolecules such as albumin to pass through the barrier, including Evans Blue bound serum albumin. By measuring the concentration of Evans Blue dye present at the site of inflammation, it is possible to measure amount of oedema at the site.

For the purpose of comparing oedema formation, mice were injected subcutaneously in the neck with 200µl of 1% Evans Blue. The dye was allowed to spread systemically in the blood for 1 hour, after which saliva was injected into the skin (subcutaneously in the dorsal side of their hind feet). Mice were injected with 5 mosquitoes worth of either *Ae.aegypti* or *An.gambiae* saliva and oedema levels was compared to resting skin controls. Samples were collected at 30min, 3 hours or 6 hours post inoculation of saliva. Immediately following cull, a blood sample was taken for normalisation purposes. Then, prior to the acquisition of tissue samples, whole body perfusions were conducted immediately in order to ensure the complete removal of any residual dye from the blood in the skin tissues. Skin samples of the saliva inculcation site were then left to soak in formamide overnight. Dye concentration was then measured normalised against the concentration of dye detected in the blood.

Interestingly, *Ae.aegypti* saliva caused significant amounts of oedema whilst *An.gambiae* saliva did not (see figure 6.4). These differences in oedema quantity were more substantial at earlier time points. This was also the case following mosquito biting; mice were exposed to 1 or 2 of either *An.gambiae* or *Ae.aegypti* mosquito bites 1 hour after the injection of 200µl of 1% Evans Blue. Skin and blood samples were acquired at the earlier time points, 30min and 3 hours post exposure (as informed by the studies with saliva alone). Interestingly, whilst *An.gambiae* mosquito bites did cause a oedema by 3 hours post exposure to bites, this was significantly lower than the oedema induced by *Ae.aegypti* bites (see figure 6.5). At 30 minutes post bite exposure, no significant formation of oedema was observed by *An.gambiae* mosquito bites, whilst *Ae.aegypti* bites caused significant amounts of oedema.

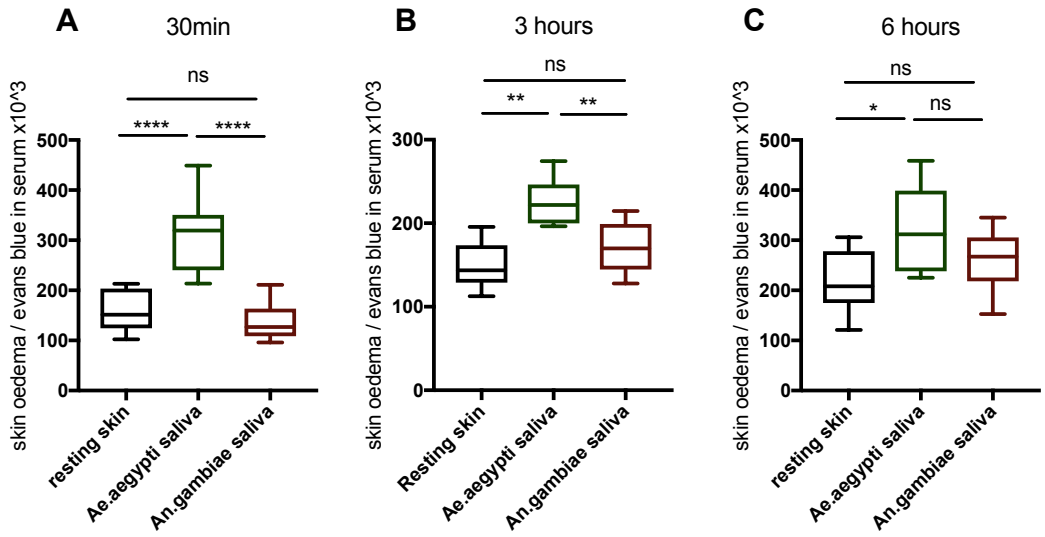


Figure 6.4. Quantity of oedema following *Aedes* and *Anopheles* saliva exposure

To investigate whether *Aedes* and *Anopheles* saliva have a different effect on oedema formation, mice were inoculate subcutaneously with 5 mosquitoes worth of saliva from *Ae.aegypti* (green) or *An.gambiae* (red). Quantity of oedema was compared to resting skin (black). Oedema was measured 30min (A), 3 hours (B) and 6 hours (C) post exposure. Oedema was measured via colorimetric assay. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test, * p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001).

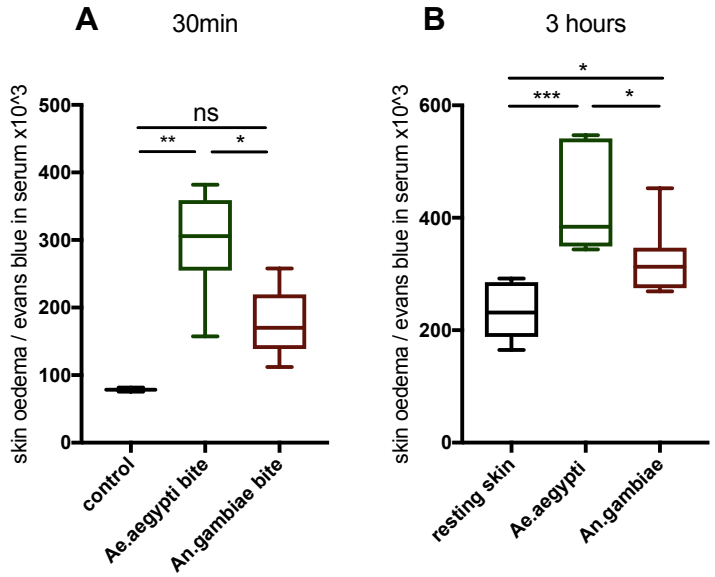


Figure 6.5. Quantity of oedema following *Aedes* and *Anopheles* bite exposure

To investigate whether *Aedes* and *Anopheles* bite have a different effect on oedema formation, mice were exposed to 1 or 2 bites from *Ae.aegypti* (green) or *An.gambiae* (red). Quantity of oedema was compared to resting skin (black). Oedema was measured 30min (A) and 3 hours (B) post exposure. Oedema was measured via colorimetric assay. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test, * p<0.05, ** p<0.01, ***p<0.001).

6.5 Summary and Conclusions

Following the discovery in the previous chapter, that *Anopheles* mosquitoes bite/saliva are incapable of enhancing SFV and ZIKV infection in mice, in this chapter we sought to compare host responses to *Ae.aegypti* and *An.gambiae* in an attempt to better understand the mechanisms involved in facilitating enhancement of arbovirus infection by *Ae.aegypti* saliva. As such, *Anopheles* saliva provides a naturally-occurring functional knockout for arbovirus enhancement. Although many factors present in both species saliva will have similar functions, *Anopheles* lacks the ability to induce the host response that enhances virus. We therefore decided to define if there were any key differences in host response to each species' saliva.

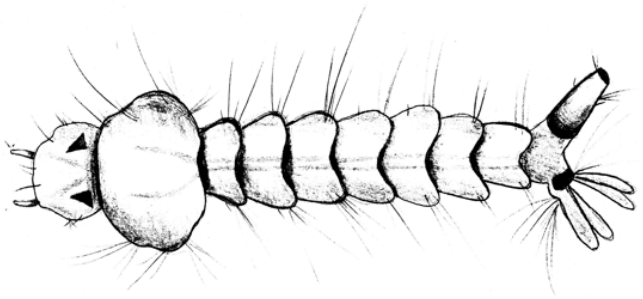
Host responses to *Aedes* mosquito bites have previously been shown to enhance arbovirus infection partly via its ability to recruit virus-susceptible myeloid cells to the bite site, which become infected and replicate virus. Therefore, the inability of *Anopheles* saliva to enhance virus infection may be a result of reduced leukocyte recruitment. However, instead of a reduced induction of chemokine expression, our studies demonstrate that *An.gambiae* saliva causes significantly higher upregulation of key pro-inflammatory genes in the skin at 6 hours post injection including IL1β, CCL5, CXCL2 and CXCL10. When saliva was co-inoculated with virus, *An.gambiae* saliva was still found to cause a significantly higher upregulation of inflammatory genes than *Ae.aegypti,* but only at 24 hours post infection, which is likely too late to affect host susceptibility to infection.

It should be noted that *Anopheles* saliva also induced higher expression of some ISGs, and this may partly explain the reduced level of virus levels in mice. Indeed, *An.gambiae* saliva caused a significantly higher upregulation of IFNα than *Ae.aegypti*. However, this cannot exclusively account for the phenotype, as virus infection (ZIKV) was enhanced in IFN-signalling deficient mice (Figure 5.8 and 5.10). In addition, as the mixing of *Ae.aegypti* saliva and *An.gambiae* saliva resulted in a similar enhancement of infection as observed by *Ae.aegypti* saliva inoculation on its own, this suggests that *An.gambiae* saliva does not actively inhibit virus infection (eg via IFN expression). Instead, it seems more likely that *An.gambiae* saliva simply lacks one, or several factors, that are necessary to cause enhancement of arbovirus infection.

Therefore it seems unlikely that the reason *An.gambiae* saliva does not exhibit a virus enhancing effect *in vivo* is due to an enhanced upregulation of an antiviral response, as this would have presumably inhibited viral infection when in the presence of *Ae.aegypti* saliva as well.

Instead, oedema formation by *Ae.aegypti* saliva and not by *An.gambiae* saliva is arguably more likely to be the mechanism responsible for causing enhancement of viral infection. Indeed, *Ae.aegypti* saliva/bites, unlike *Anopheles* saliva/bites, caused significant amounts of oedema in the skin, particularly at earlier time points. Mosquito biting by both mosquito species caused some limited oedema – although this is to be expected, as probing will puncture blood vessel walls. What this data implies, is that *Aedes* saliva has additional factors present that impairs endothelial barrier function. Together, this data suggests that oedema formation may be the key aspect of the mechanism by which *Aedes* saliva enhances arbovirus infection. Although previous work has suggested mosquito bite-induced oedema may affect virus dissemination to draining lymph nodes, it is not clear how or in what way this manifests. Hypothetically, oedema may retain virus in the skin for a longer period of time, thereby facilitating the infection of dermal cells [95]. In contrast, another study suggested oedema may enhance viral dissemination to the draining lymph nodes. Another possibility is that an increase in vascular permeability of blood vessels could allow for an increase in the extravasation of monocytes and neutrophils to the site of infection, which can indirectly aid virus by providing additional cellular targets for infection [310]. Thus, in this case, oedema may not directly enhance infection itself, but rather by indicative of endothelial barrier dysfunction that additionally leads to higher leukocyte recruitment that can become infected.

CHAPTER 7: Role of oedema in the modulation of arbovirus infection.



7.1 Introduction

In the previous chapter, differences in host responses against *Ae.aegypti* and *An.gambiae* saliva were investigated in an attempt to uncover potential differences that could help explain the host mechanism involved in facilitating arbovirus enhancement by *Ae.aegypti* mosquito saliva. One major difference that was observed between the two, was the significant oedema in the skin following inoculation with *Ae.aegypti* saliva, and the lack of oedema in response to inoculation of *An.gambiae* saliva in naïve C57BL/6 mice. A similar trend was observed in the skin in response to exposure to either *Ae.aegypti* or *An.gambiae* mosquito bites, where whilst both mosquito species triggered significant oedema formation, *Ae.aegypti* bites proved to be significantly more oedematous than *An.gambiae*. This observation indicated a possible role for oedema in the enhancement of arbovirus infection by mosquito bites.

In order to investigate whether oedema, and/or the endothelial barrier dysfunction that it represents, is responsible in facilitating arbovirus enhancement, we decided to approach the question in two separate ways. Firstly by investigating whether oedema triggered in the absence of any mosquito factors causes viral enhancement; and secondly, whether it is possible to inhibit saliva induced oedema and whether this is sufficient to block arbovirus infection. To successfully develop an oedema blocking treatment that would prevent the occurrence of saliva mediated arbovirus infection enhancement, it is vital to understand the mechanisms responsible for oedema formation in response to mosquito saliva. Whilst it is known that mosquito bites cause oedema formation, the exact mechanisms involved in facilitating this remain unclear. Mosquito bites could be triggering oedema in a number of different ways, which could either be; immune mediated; or via a direct pharmacological action affecting endothelial cell permeability. Considering what we know about host responses to mosquito bites, immune mediated mechanisms could include: histamine release from mast cells, neutrophil-mediated oedema, and also allergy enhanced responses in saliva-experienced individuals.

In summary, we wanted to determine which host responses are responsible for oedema in response to mosquito saliva. In order to investigate whether histamine is responsible for saliva-mediated oedema, mice were treated with antihistamines.

Secondly, to define any additional routes to oedema that are present in previously saliva-sensitised hosts, we wanted to investigate whether pre-exposure to *Ae.aegypti* saliva additional altered host susceptibility to infection, and whether pre-sensitization to saliva had any impact on oedema formation. This is important as whilst all previous experiments were conducted in mosquito bite naïve mice, it is important to note that the majority of individuals who are at risk of suffering from arbovirus infection (especially individuals residing in arbovirus endemic areas), will have had prior exposure to saliva. Due to this, it is important to establish whether previous exposure and sensitisation to *Ae.aegypti* mosquito saliva and the subsequent adaptive immune responses, alter the virus enhancing effect of saliva which is observed in naïve mice.

Therefore, in this chapter we wanted to determine whether:

1. **Oedema induced in the absence of any mosquito derived factors can enhance arbovirus infection.**
2. **Inhibition of mosquito bite oedema using anti-histamines can modulate saliva mediated arbovirus enhancement.**
3. **Oedema formation in response to mosquito saliva differs between naïve and sensitised hosts.**

7.2 Histamine induced oedema causes viral enhancement in the absence of any mosquito factors

In order to test whether oedema is sufficient for facilitating enhancement of arbovirus infection, we wanted to test whether oedema triggered in the absence of any mosquito factors enhanced arbovirus infection. For this purpose, purified histamine was used, as histamine is commonly used to trigger a local cutaneous oedema at the site of injection. The histamine preparation used was the same that is commonly used by health professionals as a positive control during a "skin prick test" for diagnosing allergies. In this way, experimentally introduced histamine acts directly to disrupts endothelia barrier function. Initially, in order to validate whether purified histamine causes oedema formation in the skin of mice, mice were injected with 1µl of 10mg/ml of histamine dihydrochloride one hour after a subcutaneous injection of

1% Evans Blue as done previously. 30 min post injection of histamine, skin samples were acquired. After leaving samples to soak in formamide overnight dye concentration was measured which confirmed that purified histamine causes oedema in mice (see figure 7.1).

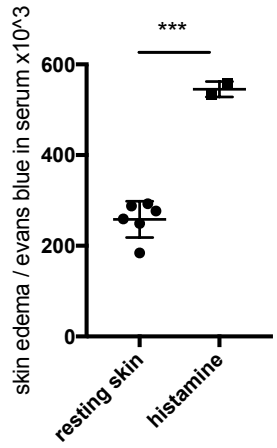


Figure 7.1. Histamine induces oedema in mice

To investigate whether purified histamine can successfully induce oedema in mice, mice were injected with 1µl of 10mg/ml of histamine dihydrochloride. Quantity of oedema was compared to resting skin. Oedema was measured 30min post exposure. Oedema was measured via colorimetric assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=2) (Mann Whitney test, ***$p<0.001$).

Following the confirmation that purified histamine triggers the formation of oedema in C57BL/6 mice, we wanted to determine whether histamine induced oedema could enhance infection of SFV. To define whether histamine alone is sufficient to induce arbovirus enactment, mice were infected with 10,000 PFU of SFV4 alone or with either *Ae.aegypti* saliva, histamine, *An.gambiae* saliva or a mixture of these. Mice were culled 24 hours post infection and skin, spleen and serum samples were collected. Viral titres in the serum and quantification of viral RNA via qPCR demonstrated that purified histamine alone significantly enhanced arbovirus infection in the absence of any mosquito factors. This occurred to a similar extent as *Ae.aegypti* saliva, indicating that endothelia barrier dysfunction is sufficient to result in enhanced arbovirus infection (see figure 7.2). Interestingly however, injection of a mixture of *An.gambiae* saliva and histamine did not enhance virus infection to the same extent as histamine alone. This suggests that *An.gambiae* saliva may contain factors that partially inhibit the level of virus enhancement, or more likely, oedema formation.

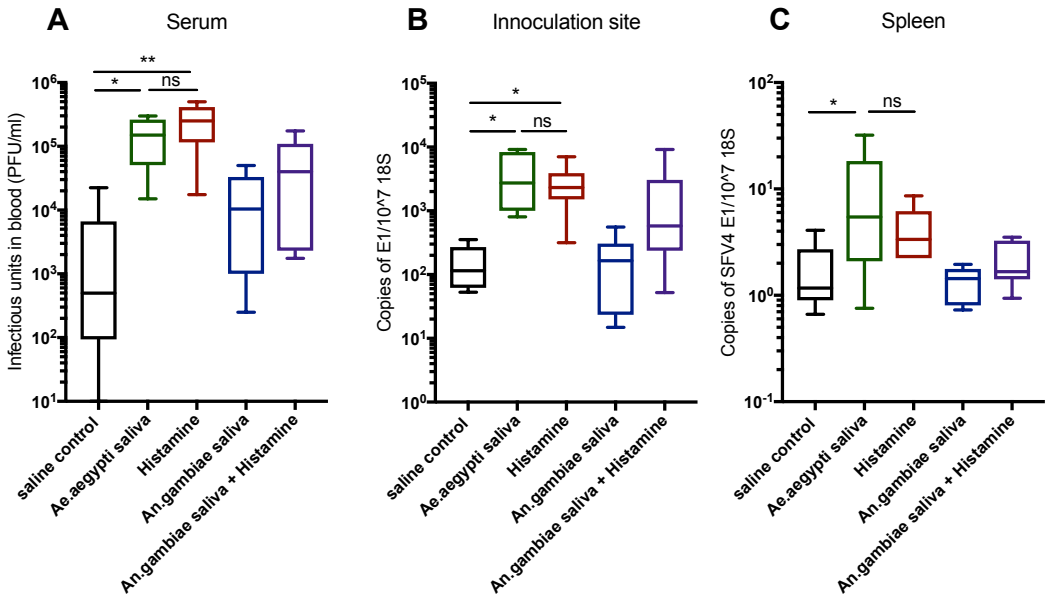


Figure 7.2. Histamine enhances SFV infection

To investigate whether histamine enhances SFV infection, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* saliva (green), 1µl of 10mg/ml of histamine dihydrochloride (red), or *An.gambiae* saliva (blue) or a combination of *An.gambiae* saliva and histamine (purple). Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (B) and spleen (C) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

7.3 Inhibition of saliva induced oedema with adrenaline not sufficient to prevent arbovirus enhancement

As histamine induced oedema was shown to enhance SFV4 infection as efficiently as *Ae.aegypti* saliva, this suggests that oedema plays a key role in facilitating saliva dependent arbovirus enhancement. Therefore, next we wanted to investigate whether inhibiting the formation of oedema could prevent the occurrence of viral enhancement. As a first step we wanted to investigate whether an injection of adrenaline inhibits *Ae.aegypti* bite and saliva mediated oedema. In order to do this, mice were injected with Evans Blue as done previously. Adrenaline treated mice were then injected with 4µl of Adrenaline (Epinephrine) Xylocaine 1%, containing lidocaine and adrenaline 1:200,000. Subcutaneous injection was made locally. Mice were then exposed to *Ae.aegypti* biting or injections of 5 mosquitoes worth of saliva. Mice were culled 30 min post exposure to mosquito bite or saliva injection. Samples were acquired following perfusion and left to soak in formamide overnight.

Measuring the concentration of Evans Blue demonstrated that adrenaline injections were efficiently inhibiting oedema formation caused by bites and saliva (see figure 7.3).

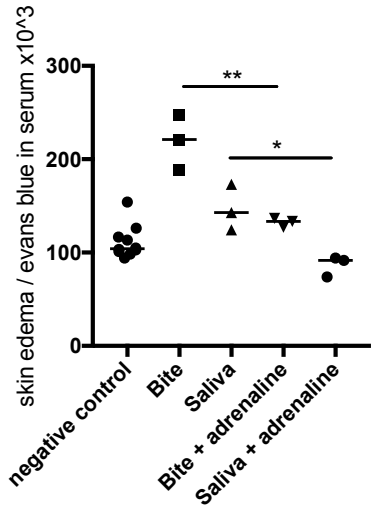


Figure 7.3. Adrenaline inhibits mosquito induced oedema

To investigate whether adrenaline can successfully inhibit mosquito induced oedema in mice, mice were exposed to up 5 mosquito bites or 5 mosquitoes worth of saliva injections in the absence or following of a 4µl adrenaline injection. Quantity of oedema was compared to resting skin. Oedema was measured 30min post exposure. Oedema was measured via colorimetric assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=2) (Mann Whitney test, *$p<0.05$, **$p<0.01$).

Next, in order to investigate whether the inhibition of salivary induced oedema from *Ae.aegypti* saliva by adrenaline injection is sufficient to prevent saliva dependent arbovirus enhancement, mice were inoculated with 10,000 PFU of SFV4 on its own or pre-mixed with 5 mosquitoes worth of *Ae.aegypti* saliva. One group of mice were pre-treated with 4µl of Adrenaline prior to saliva injections. Adrenaline was administered in the same dose again, 3 hours later. Mice were sacrificed 24 hours post infection. Viral titres in the serum and viral RNA quantified from the inoculation site and the spleen demonstrated that adrenaline injection does not prevent the occurrence of arbovirus enhancement following *Ae.aegypti* saliva injection (see figure 7.4). Strangely, saliva on its own did not significantly enhance quantity of viral RNA in the skin or spleen in this experiment, however, the experiment clearly demonstrates that adrenaline is not effective in preventing arboviral enhancement. This suggests that adrenaline activates other pathways that are pro-viral that makes oedema irrelevant in defining outcome to infection. Alternatively, and perhaps more likely, is that although adrenalin inhibits oedema itself (through direct restriction of

fluid flow in vessels), adrenalin may not directly prevent endothelial barrier dysfunction induced by *Aedes* saliva. This suggest again that oedema per-se is not involved in enhancing virus infection, but is instead marker of barrier dysfunction induced by *Aedes* saliva.

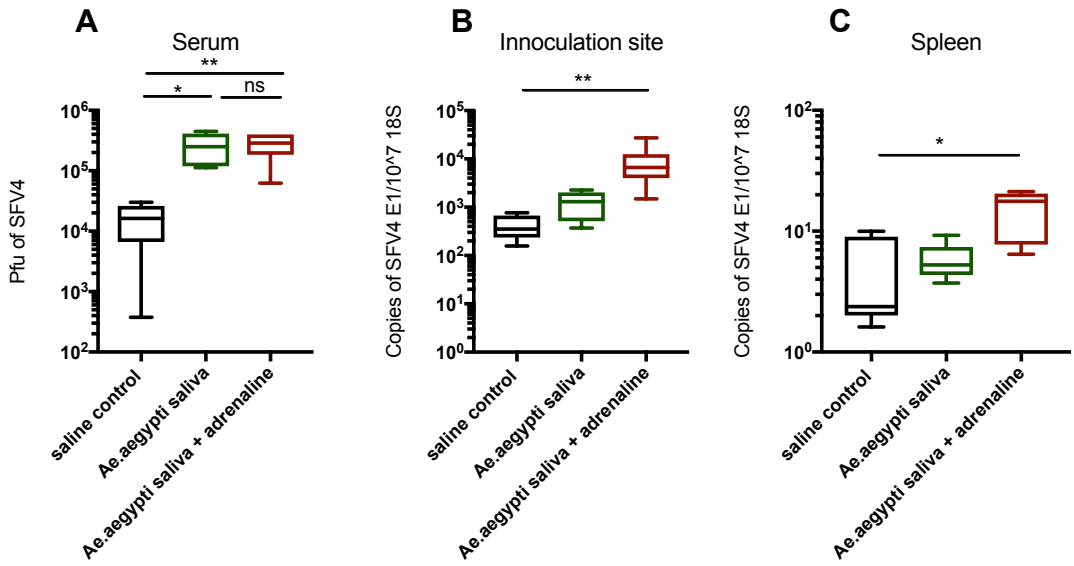


Figure 7.4. Adrenaline ineffective in prevention of arbovirus enhancement by mosquito saliva

To investigate whether adrenaline could prevent arbovirus enhancement, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* saliva in the absence (green) or following a 4µl adrenaline injection (red). Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (B) and spleen (C) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

7.4 Inhibition of oedema with antihistamines does not prevent arbovirus enhancement triggered by mosquito saliva

As adrenaline injections proved ineffective in preventing virus enhancement by *Aedes* saliva, the next step was to test the use of antihistamines. As was discussed in section 1.6.2.1, histamines are a known mediator of the allergic response and play a key role in instigating early phase immune reactions, especially in antigen-experienced individuals. As part of the cutaneous immune response, in the skin, histamine acts on H1 receptors of local blood vessels triggering an increase in vascular permeability and fluid extravasation. Histamine also acts on the H1 receptors of local nerve

endings resulting in vasodilation of local blood vessels which leads to reddening of the skin and potentially the formation of skin lesions as part of the wheal and flare reaction [426]. As some mosquito bites have been demonstrated to cause degranulation of mast cells and the subsequent release of histamine, we decided to investigate whether the use of antihistamines could prevent bite/saliva mediated enhancement of arbovirus infection. For this purpose a mixture of three different antihistamines were used; Cetirizine dihydrochloride, Loratadine and Fexofenadine hydrochloride which are all part of a family of second-generation antihistamines. Cetirizine is an antihistamine commonly used to treat hay fever which acts as an agonist of the histamine H1 receptor [494]. Cetirizine has been shown to bind to albumin with high affinity, allowing it to easily reach oedematous sites [495]. Loratadine and Fexofenadine, like Cetirizine, act as H1 receptor agonists. Final concentration of antihistamines injected were as follows: 0.5mg Cetirizine in 100µl, 0.02mg Loratadine in 100µl (aprox. 1mg/kg) [496] and 0.1mg of Fexofenadine in 200µl (aprox. 5mg/kg) [497]. Cetirizine and Fexofenadine were pre-mixed and given as a 300µl IP injection whilst Loratadine was given as a separate IP injection of 100µl. Infection with 10,000 PFU of SFV4 and saliva injections were made 1 hour post antihistamine treatment. Mice were culled 24 hours post infection.

Viral titres in the serum and quantity of viral E1 at the inoculation site demonstrate that antihistamine treated mice had equally high viral titres in the serum and quantity of viral E1 at the inoculation site suggesting that antihistamines are insufficient in preventing enhancement of SFV4 infection (see figure 7.5). Interestingly there was a significantly lower quantity of viral E1 detected in the spleen of mice treated with antihistamines. However, as the quantity of viral E1 detected in the spleen of all three groups is very low it is uncertain if the significance is of particular relevance.

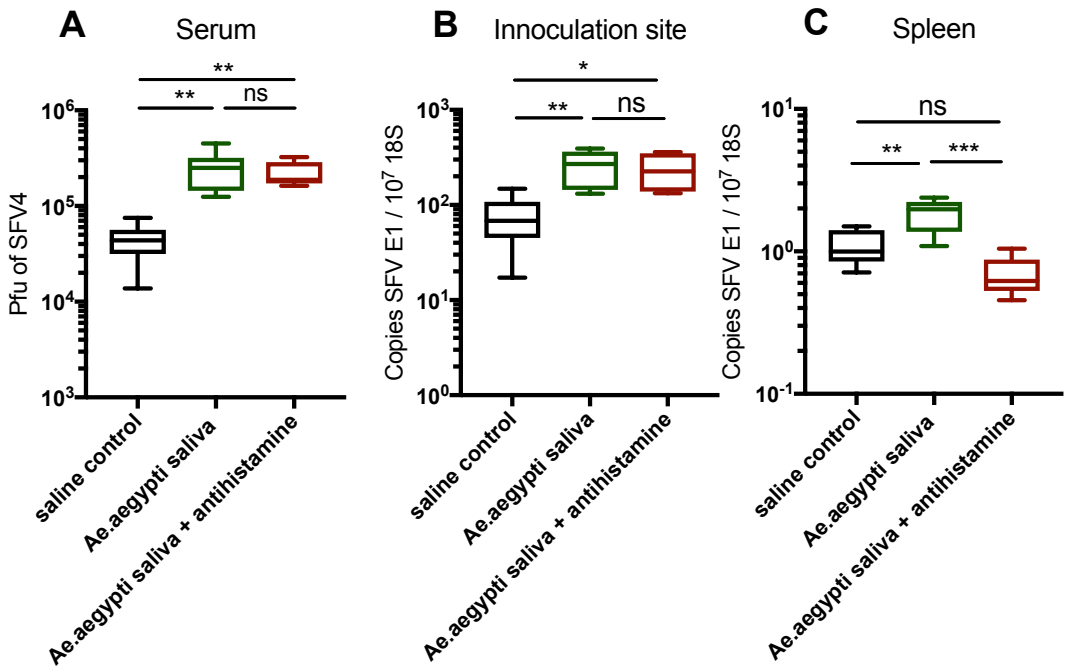


Figure 7.5. Antihistamines ineffective in prevention of arbovirus enhancement by mosquito saliva

To investigate whether antihistamines could prevent arbovirus enhancement, mice were infected subcutaneously with 10,000 PFU SFV4 on its own (black), alongside 5 mosquitoes worth of *Ae.aegypti* saliva in the absence (green) or 1 hour following antihistamine treatment (red). Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (B) and spleen (C) 24 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$).

As antihistamines proved inefficient at preventing arbovirus enhancement, we wanted to determine whether antihistamines successfully inhibited *Ae.aegypti* saliva mediated oedema. Mice were injected with Evans Blue and the same antihistamine treatment as previously. 1 hour later mice were injected with either saliva or histamine which was used as a control. Importantly, whilst antihistamines efficiently prevented the formation of histamine induced oedema, the administered antihistamine treatment only partially inhibited saliva-mediated oedema (see figure 7.6). This could explain why antihistamines are ineffective at preventing saliva mediated enhancement of arbovirus infection. This also suggests that saliva mediated oedema formation occurs predominantly independently of histamine release and another mechanism involved with oedema formation must be responsible instead.

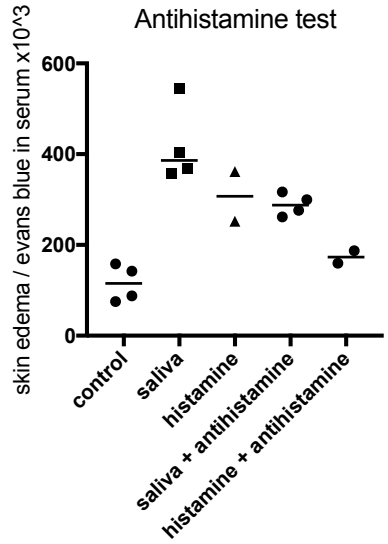


Figure 7.6. Antihistamines only partially inhibit saliva induced oedema

To investigate whether antihistamines can successfully inhibit mosquito induced oedema in mice, mice were injected with 5 mosquitoes worth of saliva or histamine injections in the absence or following antihistamine treatment. Quantity of oedema was compared to resting skin. Oedema was measured 30min post exposure. Oedema was measured via colorimetric assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=4) (n=2) (Mann Whitney test, *$p<0.05$, **$p<0.01$).

7.5 Pre-sensitization to *Ae.aegypti* saliva does not alter saliva dependent modulation of arbovirus infection

Whilst all previous experiments were conducted in mosquito bite naïve mice, it is important to note that most individuals at risk of suffering from arbovirus infection, will not be. The majority of people will have been exposed to mosquito bites during their life time, and people inhabiting countries were arboviruses are endemic will have been sensitised to local mosquito species. Therefore it is important to investigate whether previous exposure and sensitisation to mosquito saliva, and the subsequent adaptive immune responses, alter the virus enhancing effect of saliva which is observed in naïve mice. In addition, it is important to note that antibodies that are generated against saliva may also inhibit their function, in addition to activating hypersensitivity reactions. Therefore, it is hard to predict how prior immune sensitisation might affect the ability of saliva to induce oedema and/or virus enhancement.

As was briefly discussed in section 1.7.1.4 it remains unclear whether the adaptive immune response plays a role in mosquito bite dependent arboviral enhancement. A previous study investigating the pre-sensitization of mice to *Culex tarsalis* mosquito saliva in C57BL/6 mice and its subsequent effect on WNV infection by mosquito saliva, observed no significant difference between naïve and pre-sensitised mice [447]. Similarly, Pingen et al 2016, also demonstrated that pre exposure of C57BL/6 mice to mosquito bites lead to no change in arbovirus enhancement of SFV by mosquito bites. However, as C57BL/6 mice are largely considered incompetent at mounting a Th2 response, we wanted to investigate whether pre-sensitization to mosquito saliva of a mouse strain that are competent at mounting a successful Th2 response, leads to a difference in the severity of virus infection, compared to that of naïve mice [498]. As histamine release was determined not to be the cause of saliva mediated oedema, the next step was to determine whether oedema formation is aggravated by an allergic response.

For this purpose, BALB/C mice, which can readily activate Th2 immune responses, were used. Although, prior mosquito biting of these mice with *Aedes* mosquitoes did appear to worsen infection with WNV [499], the effect of pre-sensitization of BALB/c mice to mosquito saliva has not previously been investigated. Mice were pre-exposed to mosquito saliva for 4 consecutive weeks. On the 5th week mice were then re-challenged with saliva with or without virus in the inoculum. To validate that mice were primed to activate Th2 responses following repeated injection with saliva, cytokine expression of skin injection site was assessed by qPCR for IL-5 and IL-13 (see figure 7.7). In addition, the number of cells in draining lymph node were counted.

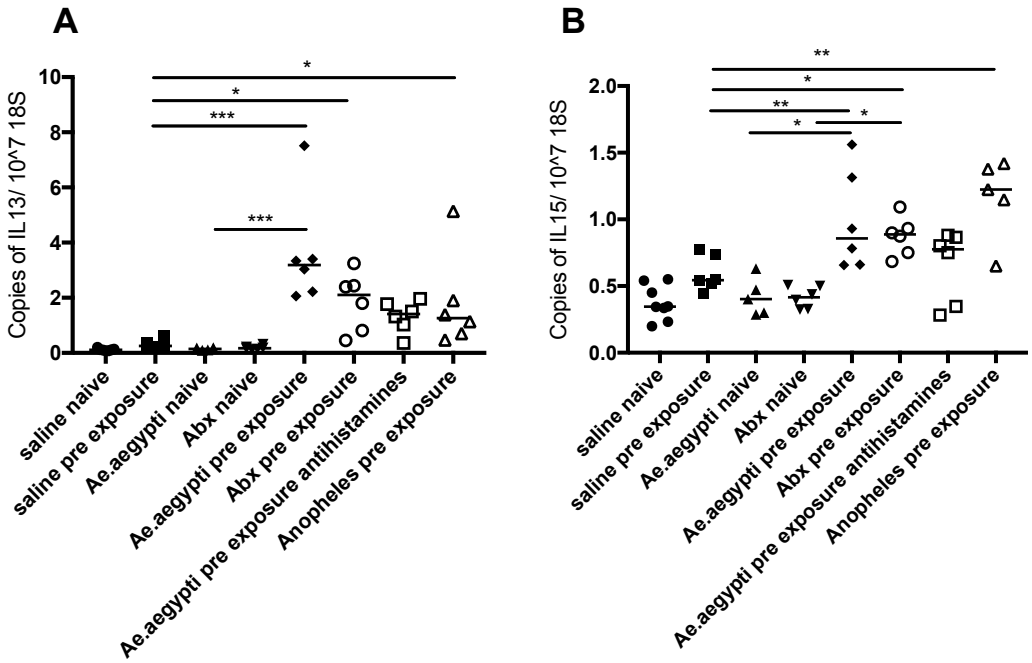


Figure 7.7. IL-13 and IL-5 expression in saliva sensitized mice

To validate that mice were primed to activate Th2 responses following repeated injection with saliva, cytokine expression of skin injection site was assessed by qPCR for IL-13 (A) and IL-5 (B). Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, *** $p<0.001$).

As can be observed in figure 7.7, mice sensitized to mosquito saliva exhibited a significantly enhanced expression of IL-13 and IL-5. No significant difference was observed between mice sensitised to *Aedes*, Abx treated *Aedes* or *Anopheles* saliva. In figure 7.8, mice inoculated with *Ae.aegypti* saliva alongside SFV4 which were pre-sensitised to *Ae.aegypti* saliva, exhibited a significantly higher quantity of cells present in the lymph node than non-sensitised mice co-inoculated with *Ae.aegypti* saliva and SFV4. No significant difference was observed in number of cells present in non-sensitised uninfected, virus only or virus co-inoculated alongside *Ae.aegypti* saliva. A slightly higher serum IgE response could be detected in sensitised mice as well in comparison to naïve. Interestingly, mice pre-sensitised to saliva acquired from antibiotic treated mosquitoes did not exhibit a detectable IgE response, suggesting the IgE responses against saliva are partly dependent on microbiota.

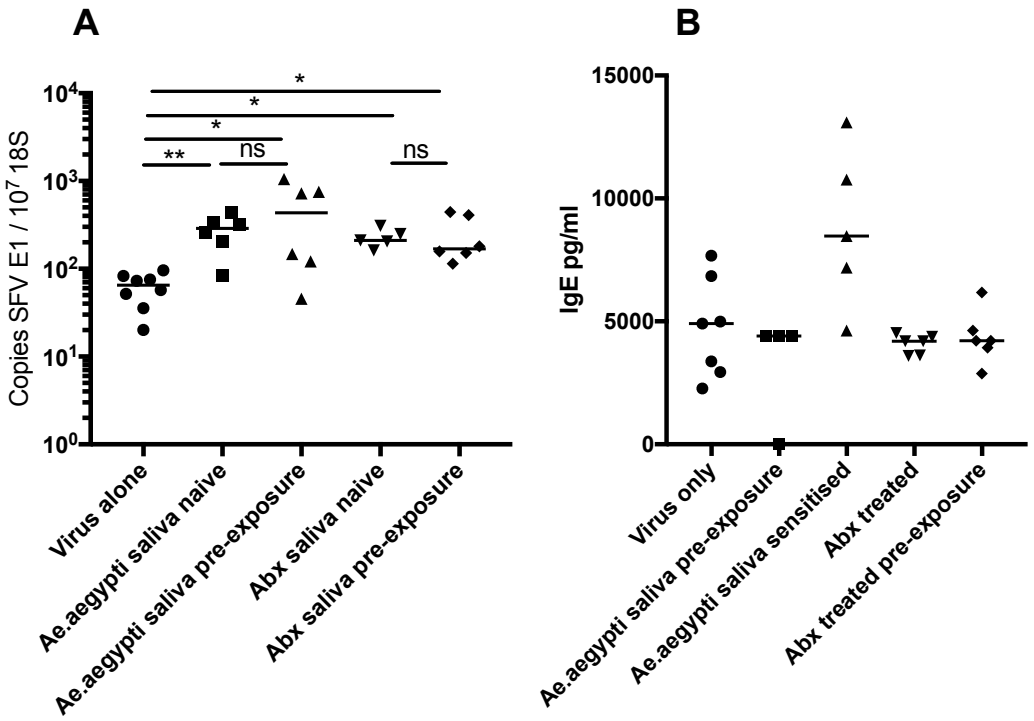


Figure 7.8. Adaptive immune responses to mosquito saliva

To investigate whether sensitisation of mice to mosquito saliva triggered a successful adaptive immune response, mice were infected with 10,000 PFU of SFV alone or alongside 5 mosquitoes worth of mosquito saliva. Mice were either naïve or pre-exposed to mosquito saliva weekly for 4 consecutive weeks prior to infection. Cellularity of lymph nodes of uninfected, virus infected, naïve infected alongside mosquito saliva and sensitised infected alongside saliva were compared 24 hours post infection (A). IgE concentration of serum was measured 24 hours pi via ELISA (B). Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=8) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

We next wanted to investigate whether pre-sensitization had an impact on oedema formation in BALB/c mice. Therefore, as done previously, mosquito naïve and pre-sensitised mice were injected with 200µl of 1% Evans Blue subcutaneously 1 hour prior to saliva inoculation. 5 mosquitoes worth of *Ae.aegypti* saliva was injected subcutaneously in to the skin of the dorsal side of their hind feet. Mice were culled at 30 min and 6 hours post inoculation and perfusion was conducted. Skin samples were acquired and placed in formamide overnight with dye concentration measured the following day.

Interestingly, no detectable oedema was observed in naïve BALB/c mice 30 min post inoculation with saliva whilst significant amounts of oedema was observed in pre-sensitised mice. This is in stark contrast to C57BL/6 mice that exhibited

significant oedema by this timepoint (as shown above). At 6 hours post injection both naïve and pre-sensitised BALB/c mice exhibited significant amounts of oedema, and no difference was observed between oedema quantified in naïve and pre-sensitised mice (see figure 7.9). This suggests that naïve BALB/c mice exhibit a delayed oedematous response following exposure to *Ae.aegypti* saliva in comparison to C57BL/6 mice, but that prior exposure to saliva enhances early oedema formation.

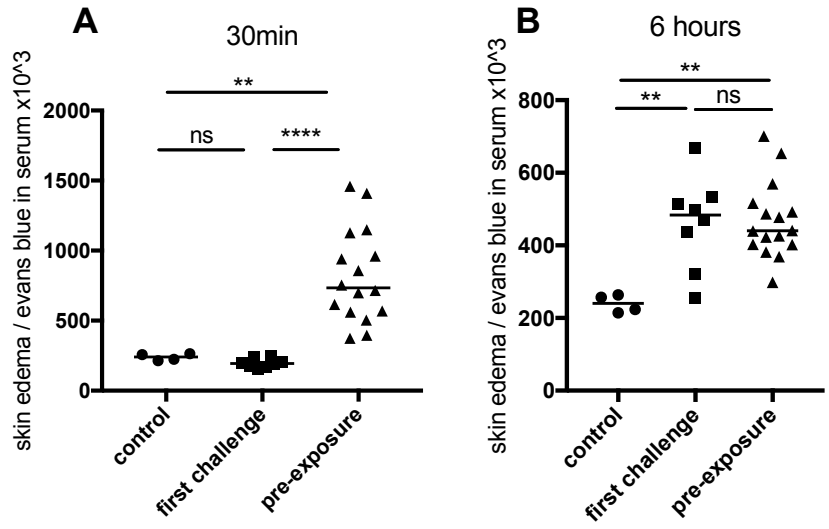


Figure 7.9. Impact of pre-exposure on oedema formation

To investigate whether pre-exposure to mosquito saliva alters oedema formation, sensitized or naïve mice were injected with 5 mosquitoes worth of saliva. Quantity of oedema was compared to resting skin. Oedema was measured 30min (A) or 6 hours (B) post exposure via colorimetric assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (control n=4) (first challenge n=8) (pre exposure n=16) (Kruskal Wallis test, *p<0.05, **p<0.01, ****p<0.0001).

In order to determine whether pre-sensitization to mosquito saliva is restricted locally to the site where pre-exposure had occurred or whether pre-sensitization was systemic, the samples from the pre-exposed mice acquired from the previous experiment were divided into two groups; left and right foot. All pre-exposure inoculations with mosquito saliva had been conducted previously into the dorsal side of their left foot. During the experiment, saliva was inoculated into the dorsal side of both hind feet 1 hour post Evans Blue injection. As can be observed in figure 7.10, no significant difference was observed in quantity of oedema detected at the site of pre-sensitisation in comparison to the distal foot. This demonstrates that sensitisation to mosquito saliva is systemic and not restricted locally to the original site of pre-exposure.

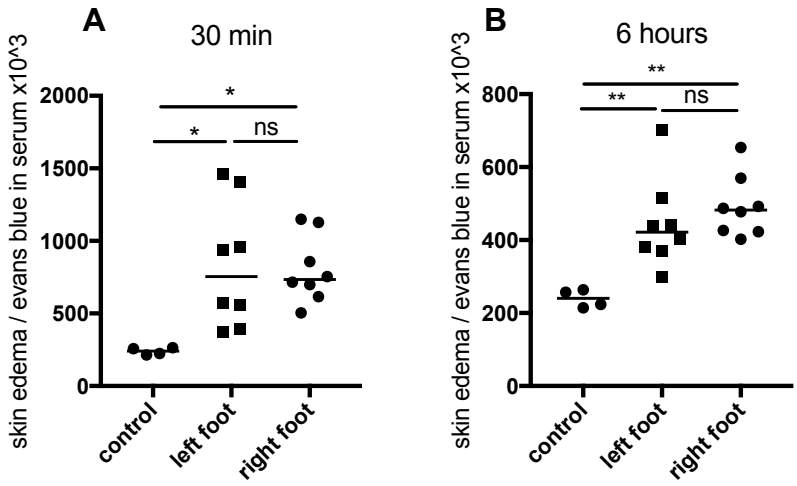


Figure 7.10. Sensitization to mosquito saliva is systemic

To investigate whether pre-exposure to mosquito saliva causes a systemic or local change in oedema formation, sensitized mice were injected with 5 mosquitoes worth of saliva in pre-exposed skin (left foot) or in naïve skin (right foot). Quantity of oedema was compared to resting skin. Oedema was measured 30min (A) or 6 hours (B) post exposure via colorimetric assay. Data present previously in graph 7.10. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (control n=4) (left and right n=8) (Kruskal Wallis test, *$p<0.05$, **$p<0.01$).

In order to investigate whether prior immune sensitisation affects the ability of saliva to induce virus enhancement naïve BALB/c mice and pre-exposed mice were injected with SFV4 on its own or in the presence of 5 mosquitoes worth of *Ae.aegypti* saliva. Mice were culled 24 hours post infection and serum, skin and spleen samples were acquired to define level of virus. Plaque assays conducted to titrate virus in serum demonstrated a significant increase in virus infection in mice co-inoculated with *Ae.aegypti* saliva in comparison to mice injected with virus on its own. No difference in viral titres was observed between naïve and pre-sensitised mice. A similar pattern of virus quantity was reflected at the inoculation site, where, there was a significantly higher quantity of viral E1 detected in mice where virus was inoculated alongside *Ae.aegypti* saliva, with no significant difference between naïve and pre-sensitised mice. No enhancement of infection was observed in the spleen (see figure 7.11). This suggests that pre-sensitisation to mosquito saliva does not alter outcome to virus infection.

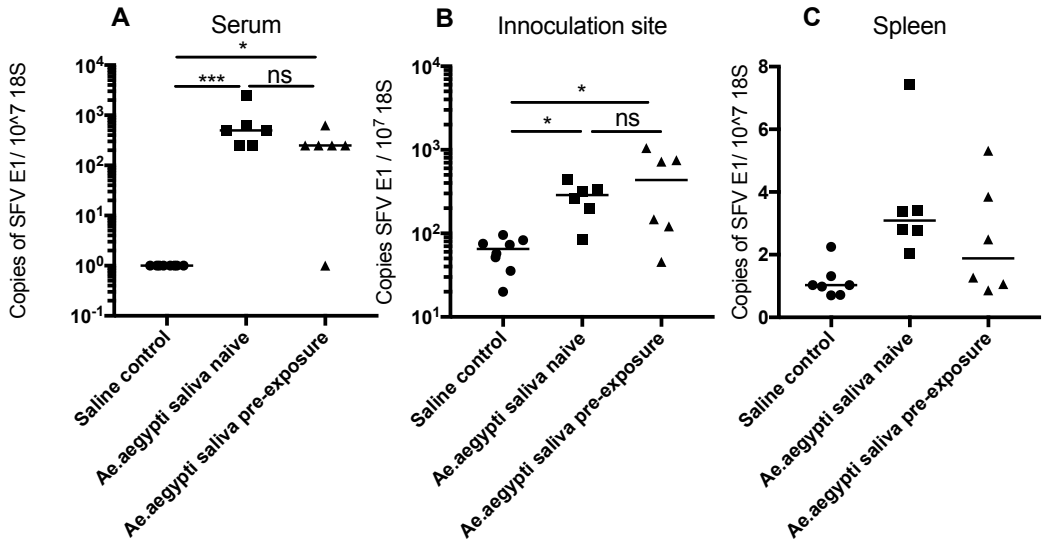


Figure 7.11. Pre-sensitisation to mosquito saliva does not alter viral enhancement by mosquito saliva

To investigate whether pre-sensitization to mosquito saliva alters mosquito saliva's ability to enhance infection, pre-sensitized mice were exposed to 5 mosquitoes worth of saliva injections once weekly for 4 weeks prior to infection. Non-sensitised mice were infected with 10,000 PFU SFV4 on its own or alongside 5 mosquitoes worth of saliva. Sensitised mice were inoculated with 10,000 PFU of SFV4 alongside 5 mosquitoes worth of saliva. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (B) and spleen (C) 24 hours post infection. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (Kruskal Wallis test * $p<0.05$, ** $p<0.01$, ***$p<0.001$).

In order to ensure that pre-sensitisation to *Anopheles* saliva did not cause *Anopheles* saliva to enhance infection, BALB/c mice were pre-sensitised as described previously, to either *Aedes* or *Anopheles* saliva once a week for 4 weeks prior to the experiment date. Mice were then injected with SFV4 on its own or alongside *Aedes* or *Anopheles* saliva. Similar to what was demonstrated in chapter 5 utilising naïve C57BL/6 mice, skin acquired from the inoculation site 24 hours post infection demonstrate *Anopheles* saliva did not cause an enhancement of virus infection in sensitised BALB/c mice (see figure 7.12). As was demonstrated in figure 7.7, sensitization to *Aedes* and *Anopheles* saliva both caused an enhanced expression of IL-5 and IL-13 suggesting that an enhanced Th2 response does not enhance virus infection.

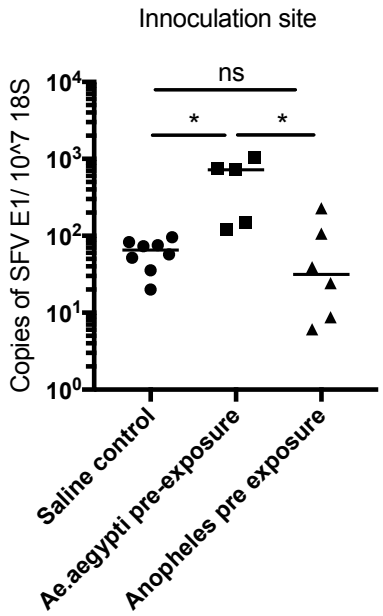


Figure 7.12. Pre-sensitization to *Anopheles* saliva does not alter lack of viral enhancement by *Anopheles* saliva

To investigate whether pre-sensitization to *Anopheles* saliva alters *Anopheles* saliva's inability to enhance infection, pre-sensitized mice were exposed to 5 mosquitoes worth of saliva of either *Aedes* or *Anopheles* injections once weekly for 4 weeks prior to infection. Mice were inoculated with 10,000 PFU of SFV4 on its own or alongside 5 mosquitoes worth of *Aedes* or *Anopheles* saliva. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin 24 hours post infection. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=6) (Kruskal Wallis test * $p<0.05$).

Whilst section 7.4 demonstrated that antihistamines in saliva-naïve mice were insufficient at preventing saliva mediated oedema or modulating virus infection. Here, we wanted to investigate whether antihistamine was more effective in pre-sensitised mice. This is because pre-sensitisation elicited an adaptive immune response, which could involve IgE primed mast cell degranulation and the subsequent release of histamine. To investigate the efficacy of antihistamines at inhibiting oedema formation in response to *Ae.aegypti* saliva in pre-sensitised BALB/c mice, Evans Blue and antihistamines were administered 1 hour prior to saliva inoculations. Skin samples acquired 30 min post inoculation demonstrated that antihistamines significantly reduced oedema formation in pre-sensitised mice exposed to *Ae.aegypti* saliva (see figure 7.13 B). Oedema formation was not completely inhibited however, suggesting that antihistamines are only partially effective at blocking saliva mediated oedema in pre-sensitised BALB/c mice.

We also investigated whether antihistamines were effective against virus enhancement. Therefore, using pre-sensitised BALB/c mice, mice were inoculated with virus on its own or in the presence of 5 mosquitoes worth of *Ae.aegypti* saliva. One group was also pre-treated with antihistamines, as described above, 1 hour prior to saliva inoculation and again at 6 hours post infection. Mice were culled 24 hours post infection with SFV. Quantification of viral RNA at the inoculation site demonstrated that mice treated with antihistamines exhibited no significant difference in virus levels, compared to untreated mice, or virus alone group (see figure 7.13). Thus, the variation in data makes it hard to state definite conclusions. However, as mentioned above, oedema formation does not appear to be completely inhibited as some oedema could still be detected in some mice. This pattern of incomplete oedema inhibition, where oedema is only completely inhibited in some mice, could explain why we only observe a slight reduction in virus infection in antihistamine treated mice comparison to untreated mice.

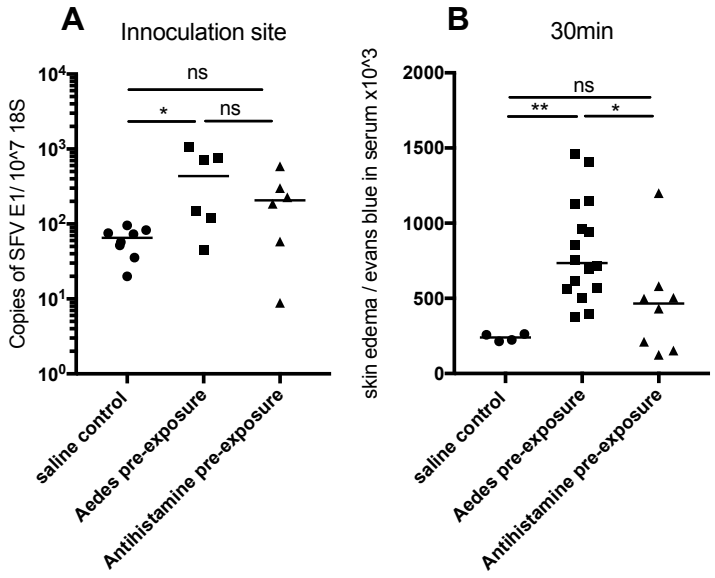


Figure 7.13. Antihistamines partially effective in prevention of oedema formation and arbovirus enhancement in pre-sensitized mice.

To investigate whether antihistamines are effective at preventing arbovirus enhancement in mice sensitised to mosquito saliva, sensitised mice were injected with 10,000 PFU SFV4 on its own or alongside 5 mosquitoes worth of Aedes saliva in the absence of, or following antihistamine treatment. Expression of viral SFV E1 gene was measured using RT-qPCR in the skin 24 hours post infection (A). To investigate the effectiveness of antihistamines in prevention of salivary mediated oedema in sensitised mice, mice were inoculated with 5 mosquitoes worth of saliva in in the absence of, or following antihistamine treatment. Quantity of oedema was compared to resting skin. Oedema was measured 30min post exposure (B). Oedema was measured via colorimetric assay. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (A n=6) (B n=8) (Kruskal Wallis test, *$p<0.05$, **$p<0.01$).

7.6 Summary and Conclusions

In the previous chapter we identified oedema as a potential instigator for viral enhancement caused by mosquito saliva as only *Ae.aegypti* saliva was discovered to cause detectable oedema in the skin whilst *An.gambiae* saliva did not. Therefore, as *Ae.aegypti* saliva has been determined to enhance virus infection whilst *An.gambiae* saliva appears to lack the factors responsible for causing viral enhancement, we decided to investigate whether oedema causes arbovirus enhancement. For this purpose, we sought to answer whether oedema induced in the absence of any mosquito derived factors could successfully enhance infection. Oedema triggered utilising purified histamine, significantly enhanced arbovirus infection to a similar extent as *Ae.aegypti* saliva, a phenomenon that supports our hypothesis that oedema and/or endothelial barrier dysfunction, is responsible for causing saliva-enhancement of arbovirus infection.

Whilst it is known that mosquito bites cause oedema, the exact mechanisms involved in facilitating this remain unclear. Mosquito bites could trigger oedema in a number of different ways, which could either be immune-mediated or via a direct pharmacological action that affect endothelial cell permeability. In order to successfully develop a potential treatment that would prevent enhancement of arbovirus infection from occurring, it is important to understand the mechanisms involved in oedema formation in response to mosquito saliva.

In an attempt to inhibit oedema in order to investigate whether inhibition/prevention of saliva induced oedema prevented the occurrence of arbovirus enhancement, two separate methods of oedema inhibition were tested; the use of adrenaline, and the use of antihistamines. However, neither proved to effectively modulate severity of virus infection. Whilst adrenaline proved successful at inhibiting histamine-induced oedema, the treatment of mice with adrenaline injection did not prevent arbovirus enhancement. In fact, some levels of virus were higher in adrenalin treated mice. Adrenaline has multiple effects on physiology and may act in ways that are hard to predict, including the enhancement of infection via an alternative mechanism. In terms of antihistamine, antihistamines were not found to significantly inhibit saliva induced oedema, which could explain why no reduction

in virus infection was observed in mice treated with antihistamines. This also suggests that *Ae.aegypti* saliva triggers oedema in a histamine-independent manner in saliva-naïve mice.

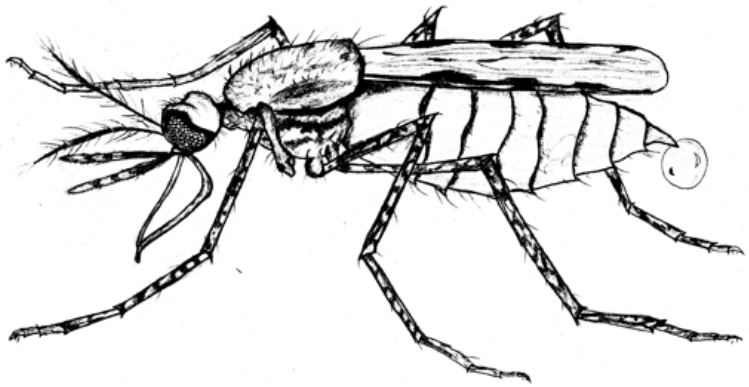
As all previous experiments had been conducted utilising naïve mice, we wanted to determine whether previous exposure to mosquito saliva had an impact on the outcome of infection. This is because, as was discussed in section 1.7.1.4, it remains uncertain whether the adaptive immune to saliva can further modulate the arbovirus infection enhancement effect elicited by blood feeding mosquito species saliva. A previous study investigating the pre-sensitization of mice to *Culex tarsalis* saliva and its subsequent effect on WNV infection by mosquito saliva in C57Bl/6 mice, observed no significant difference between naïve and pre-sensitised mice [447]. Similarly, pre exposing C57BL/6 mice to *Ae.aegypti* bites resulted in no change in arbovirus enhancement of SFV by mosquito bites, as pre-sensitised and naïve mice exhibited a similar level of enhanced infection when virus was inoculated in the presence of a mosquito bite [95]. However, both of these previous experiments were conducted utilising C57BL/6 mice and as C57BL/6 mice are largely considered incompetent at mounting a Th2 response, we wanted to investigate whether pre-sensitization to mosquito saliva in mice competent at mounting a successful Th2 response, leads to a difference in the severity of virus infection to that of naïve mice [498]. Therefore, we utilised BALB/c mice for this purpose.

In our experiments we demonstrated that there was no difference in the level of SFV infection following co-injection of virus alongside *Ae.aegypti* mosquito saliva, comparing naïve and pre-sensitised BALB/c mice. Oedema formation triggered in response to *Ae.aegypti* saliva in BALB/c mice differed than what has previously been observed in C57BL/6. Interestingly, as was demonstrated in previous chapters, significant oedema was detected by 30min post *Ae.aegypti* saliva exposure in naïve C57BL/6 mice, whilst no detectable oedema could be observed at 30min post exposure in naïve BALB/c. This suggests that naïve BALB/c mice respond differently to *Aedes* mosquito saliva, making comparison to our data generated previously with C57BL/6 mice difficult. Significant levels of oedema were exhibited at 30min post exposure in BALB/c mice previously exposed to *Ae.aegypti* saliva. However, levels of oedema were similar by 6 hours post exposure, compared to non-

primed mice. Importantly, both naïve and pre-sensitised BALB/c mice exhibited enhanced virus infection to the same level by 24 hours post infection, following co-inoculation of SFV4 alongside *Ae.aegypti*. This suggests that not all forms of oedema are pro-viral or that oedema formation following virus inoculation at time points later than 30 minutes post exposure are most important in defining outcome to infection.

The use of antihistamines in an attempt to prevent arboviral enhancement in sensitised mice proved to be ineffective. This likely reflects the inability of antihistamines to prevent saliva-induced oedema. The use of adrenaline, which acts by restriction of blood vessels, resulted in significantly decreased oedema. However, treating mice with adrenaline had no effect on modulating the success of virus infection. This may be via a separate mechanism of action however, as adrenaline appeared to worsen infection in some tissues. Importantly, adrenaline is effective at inhibiting oedema, presumably via the restriction of blood vessel dilation. Adrenalin may not, however, prevent other aspects of endothelial dysfunction. Therefore, mosquito saliva from *Ae.aegypti* mosquitoes may enhance infection via an increase in endothelial cell permeability, independent of fluid leakage per se. Further research is required to further define the mechanisms involved in saliva mediated virus enhancement.

CHAPTER 8: ONNV virus infection is not modulated by saliva *in vivo*.



Anopheles gambiae

8.1 Introduction

In previous chapters we have demonstrated that the effect of mosquito saliva mediated arbovirus enhancement is not prevalent in all blood feeding mosquito species. We found that *Anopheles* mosquitoes were incapable of causing saliva/bite mediated enhancement of infection for both SFV4 and ZIKV in mice. Previous studies have demonstrated that *Anopheles* mosquitoes, which primarily transmit malaria, are very inefficient transmitters of the majority of arboviruses, with the exception of one virus; ONNV. ONNV is primarily transmitted by *An.gambiae* mosquitoes. Even though viral RNA of several arboviruses have been detected in *Anopheles* mosquitoes, no study has sufficiently proven that *Anopheles* mosquitoes can successfully transmit these viruses to a mammalian host. The reason why *Anopheles* mosquitoes are unsuccessful transmitters of the majority of arboviruses remains unclear. Several putative mechanisms have been hypothesised to explain these differences in vector competence including; difference in mosquito species tissue barriers; microflora; or molecular determinants required for viral replication [235]. The latter suggestion has some evidential support, as differences in the sequence of nsP3 gene is sufficient to confer infection of *An.gambiae* with CHIKV under laboratory conditions [130]. However, this alone is insufficient to explain the refractory nature of *Anopheles* to transmit the full gamut of genetically-distinct arboviruses, suggesting there exists another fundamental reason that prevents these mosquitoes from efficiently transmitting virus to the mammalian host.

In an attempt to understand why *An.gambiae* mosquitoes can successfully transmit ONNV virus we hypothesized that *An.gambiae* saliva could enhance the infection of ONNV. As was briefly discussed in section 1.3.3.5, ONNV is a neglected tropical disease, which means very little is known about this virus as very few studies have been conducted investigating ONNV. To the best of our knowledge, to date, only one previous study has investigated ONNVs ability to replicate in mice. This study determined that ONNV does not replicate in WT mice [136]. No study to date has investigated whether mosquito saliva has an impact on virus infection. We therefore had to develop a functional ONNV mouse model in order to work with this virus.

Therefore, the aims of this chapter are:

1. **To develop an ONNV virus mouse model.**
2. **To use the developed ONNV *in vivo* model in order to determine if *Aedes* or *Anopheles* saliva enhance ONNV infection.**

8.2 Development of ONNV mouse model

Due to limited studies previously conducted on ONNV, initially, an ONNV mouse model had to be developed. The groups tested were as follows:

- adult female C57BL/6 mice exposed to mosquito bites prior to infection
- adult female C57BL/6 mice pre-injected with anti-mouse IFNAR-1, exposed to mosquito bites
- 3 week old female C57BL/6 mice pre-injected with IFNAR-1, inoculated with *Ae.aegypti* saliva
- 3 week old male C57BL/6 mice pre-injected with IFNAR-1, inoculated with *Ae.aegypti* saliva
- 3 week old female CD1 mice pre-injected with IFNAR-1, inoculated with *Ae.aegypti* saliva

The specific groups were chosen as previous observations have suggested that infection may differ in male and female mice as well as between CD1 and C57BL/6 mice. Similarly, younger mice are sometimes found to be more susceptible to alphavirus infection. Also, the IFNAR-1 blocking antibody was used, as a previous study had determined lack of successful infection in WT mice, and because the closely related CHIKV replicate to far higher levels in the absence of IFN signalling.

All groups were inoculated with 200,000 PFU of ONNV on its own or pre mixed with 5 mosquitoes worth of *Ae.aegypti* saliva. IFNAR-1 was administered 24 hours prior to infection as an IP injection at a concentration of 1.5mg per mouse. The groups with adult female C57BL/6 mice were conducted as a separate experiment on a separate day from the rest and were culled 24 hours post infection. The remaining groups were culled 48 hours post infection. Quantification of viral ONNV E1 at the inoculation site and at the dLN showed that virus replicated most efficiently

in the group with 3-week-old female mice. In terms of viral titres in the serum, no virus was detected in the groups containing the adult mice, whilst the remaining three groups all exhibited high titres of virus within the serum. As 3-week-old female C57BL/6 mice previously injected with IFNAR-1 exhibited the highest viral titres of ONNV, this model system was selected for future experiments (see figure 8.1).

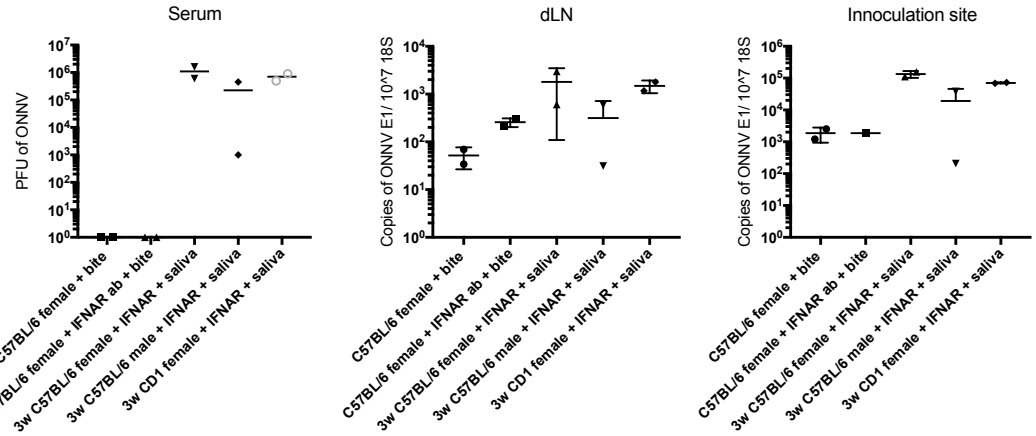


Figure 8.1. Establishing of ONNV *in vivo* model

In order to establish an ONNV *in vivo* model, mice were exposed to up to 5 *Ae.aegypti* bites or 5 mosquitoes worth of *Ae.aegypti* saliva alongside 200,000 PFU of ONNV. With the exception of the first group, all other mice were pre-treated with IFNAR-1 one day prior to infection. Infectious virus was quantified via plaque assay of serum at 24 hours (first 2 groups) or 48 hours (remaining groups) post infection (A). Expression of viral ONNV E1 gene was measured using RT-qPCR in the dLN (B) and skin (C) 24 hours post infection. Data is presented as dot plots with each dot representing a separate biological sample, with a line at the population median. (n=2).

Next, we wanted to determine in which tissues ONNV replicates. Using the parameters of the ONNV mouse model selected above, 3-week-old C57BL/6 mice, 24 hours post IP injection with IFNAR-1, were injected with 200,000 PFU of ONNV. 48 hours later mice were culled and a wide range of tissues were collected including skin taken from site of virus inoculation, foot joint, spleen, pancreas, draining lymph node, popliteal non-draining lymph node, as well as the inguinal non-draining lymph node. Virus level was then quantified via qPCR. Whilst high amounts of ONNV E1 expression was detected in the skin, foot joint, dLN as well as some expression in the spleen, no E1 was detected in the pancreas or either of the non-draining lymph nodes (see figure 8.2).

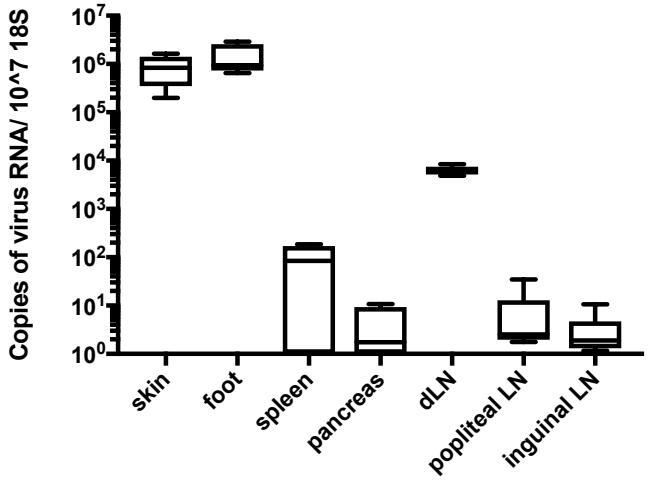


Figure 8.2 Sites of ONNV replication

To establish where ONNV replicates in the established *in vivo* model, mice were infected with 200,000 PFU of ONNV 1 day post injection with IFNAR-1. Expression of viral ONNV E1 gene was measured using RT-qPCR in the skin, foot (joint), spleen, pancreas, dLN, popliteal non dLN and inguinal non dLN at 48 hours post infection Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6).

8.3 ONNV infection not enhanced by mosquito saliva

After establishing a functional *in vivo* model utilising ONNV infection, next, we wanted to determine whether *Ae.aegypti* or *An.gambiae* bites enhanced ONNV infection. Therefore, 3 week old, IFNAR-1 treated C57BL/6 mice were inoculated with 200,000 PFU of ONNV in to resting skin, or following exposure to approximately 2 mosquito bites of either *Ae.aegypti* or *An.gambiae* bites. Mice were culled 48 hours later and serum and tissues were collected including skin taken from site of virus inoculation, foot joint, spleen, pancreas, draining lymph node, popliteal non-draining lymph node as well as inguinal non-draining lymph node. As can be observed in figure 8.3 there was no significant difference in viral titres detected in the serum or in the quantification of viral RNA in any of the tissues collected between any of the groups. This suggests that neither *Ae.aegypti* bites nor *An.gambiae* bites enhance infection of ONNV.

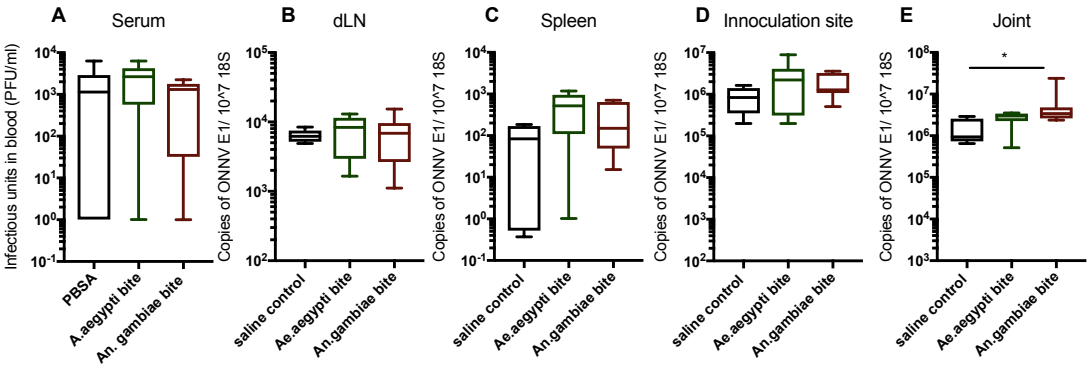


Figure 8.3. Mosquito bites from *Aedes* and *Anopheles* does not enhance ONNV infection *in vivo*

To investigate whether *Aedes* or *Anopheles* bites modulate ONNV infection, mice pre-treated with IFNAR-1 were infected with 200,000 PFU ONNV on its own (black) or following 2 mosquito bites from *Aedes* (green) or *Anopheles* (red). Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the dLN (B), spleen (C), skin (D) and joint (E) 48 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test).

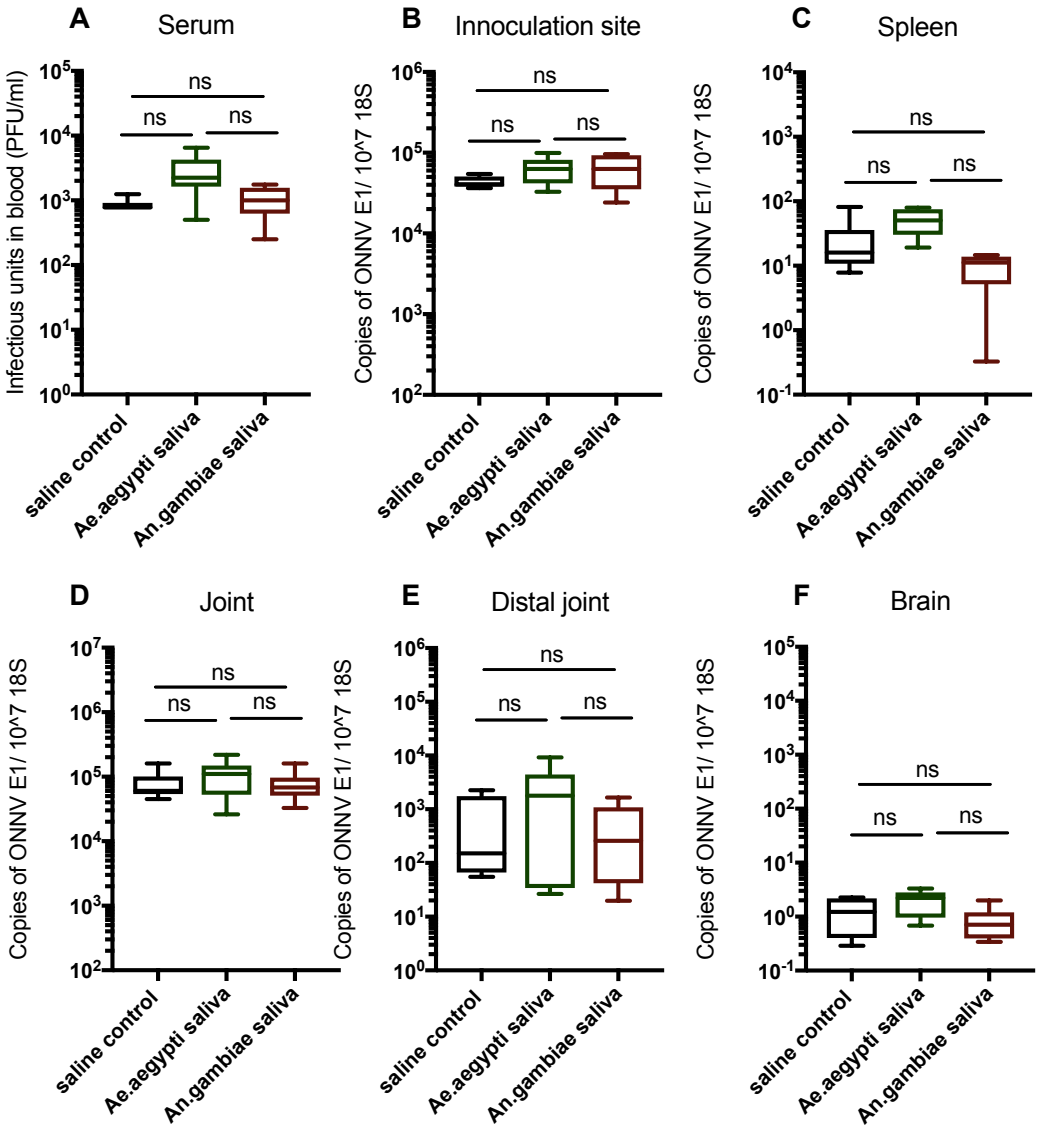


Figure 8.4. Mosquito saliva from *Aedes* and *Anopheles* does not enhance ONNV infection *in vivo*

To investigate whether *Aedes* or *Anopheles* saliva modulate ONNV infection, mice pre-treated with IFNAR-1 were infected with 200,000 PFU ONNV on its own (black) or alongside 5 mosquitoes worth of *Aedes* (green) or *Anopheles* (red) saliva. Infectious virus was quantified via plaque assay of serum at 24 hours post infection (A). Expression of viral SFV E1 gene was measured using RT-qPCR in the skin (B), spleen (C), joint (D), distal joint (E) and brain (F) 48 hours post infection. Data are presented as box and whisker plots: boxes extend from the 25th to the 75th percentile, with a line at the population median; whiskers extend to show the highest and lowest values. (n=6) (Kruskal Wallis test).

We next determined whether mosquito saliva, in the absence of a bite could modulate outcome to infection with SFV. Mice were inoculated with 5 mosquitoes worth of saliva from either *Ae.aegypti* or *An.gambiae* saliva. All other experimental parameters were kept the same as above. 48 hours post infection mice were culled and the following samples were collected: serum, spleen, skin from the inoculation site, joint, brain and distal joint. As limited studies have been conducted on ONNV, dissemination and replication of virus *in vivo* remains poorly understood. Therefore, as ONNV is closely related to CHIKV which is known to replicate well in joints, the distal joint was acquired as well in order to investigate whether viral RNA can be detected in the distal joint 48 hours post infection with ONNV. Brain samples were also collected to investigate whether ONNV crosses the blood brain barrier 48 hours post infection.

Similarly to the experiment above involving mosquito bites, neither *Ae.aegypti* nor *An.gambiae* saliva significantly enhanced infection of ONNV (see figure 8.3 and 8.4). Also, no viral RNA was detected in the brain, suggesting that ONNV either is incapable of affecting the brain, or it does so only after 48 hours post infection. Interestingly, viral RNA was detected in the distal joint at 48 hours post infection.

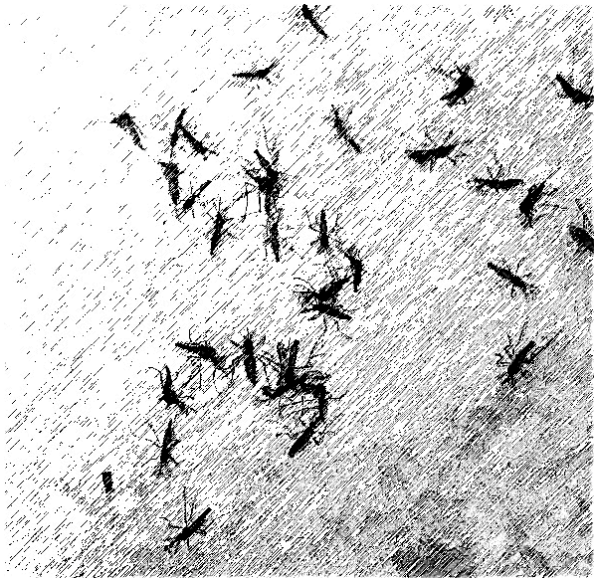
8.4 Summary and Conclusions

In this chapter we successfully developed an infectious ONNV *in vivo* model utilising C57BL/6 mice with the use of the IFNAR-1 antibody to temporally inhibit IFN signalling. We demonstrate for the first time that ONNV does not replicate in WT immunocompetent C57BL/6 mice, irrespective of the presence of *Ae.aegypti* mosquito bites/saliva. Instead, blocking of type I IFN responses are necessary for replication of virus to occur. We also show that we can detect ONNV at the inoculation site, spleen, joint, distal joint and dLN at 48 hours post infection.

Utilising the developed ONNV mouse model we demonstrate for the first time that ONNV infection is not enhanced by either *Ae.aegypti* saliva or bites, and therefore is quite distinct to the majority of other arboviruses. This is interesting considering the close sequence homology of ONNV to SFV and CHIKV, which have both been demonstrated previously to be enhanced by *Ae.aegypti* bites. We also demonstrate that

An.gambiae bites and/or saliva does not modulate the level of ONNV infection. This is interesting as majority of previously studied arboviruses appear to rely on mosquito saliva for effective transmission, infection and dissemination to occur. Together, this suggests that ONNV has evolved not to rely on mosquito saliva dependent arbovirus enhancement, and can therefore be more easily transmitted via other mosquito species that lack the salivary factors involved. This observation could also provide a further reason to account for the inability of *An.gambiae* mosquitoes to vector the majority of arboviruses with the exception for ONNV.

CHAPTER 9: DISCUSSION



9.1 Introduction

Emerging and re-emerging infectious arthropod borne viruses have a tremendous impact on global health as they constitute an ongoing threat due to limited availability of treatments and vaccines. Due to this, development of novel treatments are urgently required. The recent discovery that mosquito borne virus infections can be enhanced by mosquito bites, allows for the potential development of treatments that specifically target and inhibit this aspect of virus infection. Directly inhibiting virus enhancement has the potential to decrease the likelihood of serious disease development commonly associated with mosquito-borne virus infections. Research investigating the mechanistic principles responsible for virus enhancement by mosquito bites is therefore required to discover therapeutic targets.

The overall aims of this thesis was to increase our limited understanding of the mechanisms involved in arbovirus enhancement, as well as addressing key questions in the field. More specifically we were interested in investigating which components of mosquito saliva are responsible for saliva-dependent virus enhancement as well as which aspects of the host responses elicited against mosquito saliva that are required for virus enhancement to occur. Finally, we wanted to understand if all mosquito species saliva is capable of enhancing virus infection, and whether the host mechanisms and salivary components implicated are the same for all mosquito species. It is important to understand whether this is the case if we are to successfully develop a treatment targeting multiple arbovirus infections. It is possible that the enhancement of virus infections are highly species/virus specific. As a side, such factors could also have an impact on the determination of mosquito vector competence.

Therefore, the overall aims of this thesis were:

1. **To investigate which components of the mosquito saliva are important for facilitating saliva dependent virus enhancement.**

2. **To investigate which aspects of the host response against mosquito saliva are important in facilitating arbovirus enhancement.**

3. **To investigate whether the ability of mosquito saliva to modulate infection has an impact on mosquito vector competence**.

9.2 *Ae.aegypti* saliva alone retains efficacy of mosquito-bite driven enhancement of virus infection

In this thesis we sought to determine whether mosquito saliva enhanced virus infection with the same efficacy as mosquito bites. Whilst previous works have demonstrated that mosquito bites and mosquito saliva enhance virus infection of several arboviruses, to date, no one has compared the efficacy of mosquito bites to that of saliva. Therefore, in chapter 3 our main aims were:

1. **To establish a mouse model that includes injection of mosquito saliva with virus. By determining the efficacy of mosquito saliva at enhancing arbovirus infection, this work aims to establish quantities of saliva required, method of salivary extraction and relevant time point post infection for assessing viral titres.**

2. **Utilising the model system developed, determine the effect of mosquito saliva on viral dissemination and animal survival in comparison to mice inoculated with virus at mosquito bites.**

In chapter 3 we hypothesized that mosquito saliva alone, was sufficient to enhance arbovirus infection with the same efficacy in the absence of a bite. During mosquito probing, the skin suffers from tissue trauma in addition to the deposition of saliva, and it remained unclear what role each had in the modulation of host susceptibility to virus infection. To investigate this, we established a mouse model utilising SFV and C57BL/6 mice and mosquito saliva, which was then used to determine the effect of saliva on dissemination of virus as well as on animal survival in comparison to

mosquito bites. Whilst previous studies have investigated the effect of mosquito bites, mosquito saliva or SGE on arboviral infection, to date no one has directly compared the efficacy of each in modulating arbovirus infection. By comparing the ability of SGE, mosquito saliva derived via forced salivation and mosquito bites, we demonstrate that mosquito saliva enhances SFV infection to a similar extent as mosquito bites. Importantly, this allowed us to utilise mosquito saliva, derived by forced salivation, to study mosquitoes saliva's effect on virus infection. We hypothesised that, even though the exact composition of the extracted mosquito saliva may differ slightly from that of the saliva voluntarily injected by mosquitoes during probing, the functional factor responsible for viral enhancement are present in our saliva preparation.

The quantity of saliva, and time post infection, required to reach peak viraemia of SFV4 in this model system were determined. The highest SFV4 titre was detected at 24hpi with 5 mosquitoes worth of saliva. Co-injection of mosquito saliva with SFV4 resulted in enhanced virus replication in the skin as well as simultaneous enhanced dissemination to the dLN. Also, injection of *Ae.aegypti* saliva causes upregulation of key inflammatory genes previously determined to be upregulated by *Ae.aegypti* mosquito bites including CXCL2, CCL2 and IL1β. In addition, a survival experiment concluded that mosquito saliva reduced mouse survival of SFV4 infection to a similar extent as 5 mosquito bites. Also, mosquito saliva was found to significantly enhance infection of mice with ZIKV. Finally, we investigated whether skin trauma induced by repeated needle tick injury modulated virus infection and concluded that tissue trauma on its own does not enhance virus infection.

In conclusion, in chapter 3, we established an *in vivo* mouse model utilising mosquito saliva acquired via forced salivation used for the study of the mechanisms of arbovirus infection enhancement by mosquito saliva. With this model system we have determined that *Ae.aegypti* mosquito saliva is responsible for the arbovirus infection enhancing mechanism displayed by *Ae.aegypti* mosquito bites, as mosquito saliva on its own in the absence of the bite is sufficient for the modulation of virus infection. This model allows for a more experimentally-controlled/tractable conditions for the studying of arbovirus enhancement, as quantity of mosquito saliva

and virus inoculated can be carefully controlled e.g. in comparison to transmitting virus to mice by way of infected mosquitoes

The main conclusions from chapter 3 are:

- Mosquito saliva alone is sufficient at enhancing virus infection in the absence of bite trauma.
- Tissue trauma in the absence of mosquito saliva does not modulate virus infection.

9.3 Virus infection enhancing salivary proteins are most likely those that have evolved to facilitate blood feeding and are not present in all mosquito species.

To date, it remains unknown which factors within mosquito saliva are responsible for causing enhancement of virus infection. Whilst several studies have suggested the implication of different specific components, as salivary components are numerous and highly diverse, the proteins that have been suggested to be implicated in salivary dependent virus enhancement, sometimes appear to counter act each other; with some factors enhancing or inhibiting immune responses. This suggests that it is unlikely that one single salivary factor is responsible for viral enhancement. Instead it is more likely that several factors and the subsequent total effect they have on the host immune response are important in facilitating virus enhancement. Due to this, in this study, we decided not to focus on specific components but rather on the nature of components implicated in virus enhancement.

For this purpose, initially it was important to determine whether the factors responsible for virus enhancement are salivary components or microbiota present within the mosquito saliva. Utilising a developed antibiotic treatment, we demonstrate for the first time that antibiotic sensitive microbiota present in injected mosquito saliva, does not modulate virus infection as exposure of mice to bites from untreated and antibiotic treated *Ae.aegypti* bites showed no difference in virus enhancement. Interestingly, heat treatment of extracted mosquito saliva inactivated saliva's modulatory effect on virus infection. As a heat treatment results in the lysing of microbiota present in saliva whilst simultaneously releasing any microbiota

associated PAMPs, such as LPS, and other immune agonists, a heat treatment would effectively denature and inactivate any proteins present. We demonstrate that heat treatment of mosquito saliva successfully inactivates the mosquito saliva factors implicated in enhancement of virus, as mice inoculated with virus alongside heat treated saliva exhibited no enhanced virus infection. These observations demonstrate that microbiota within mosquito saliva are not responsible for virus enhancement; instead the factors implicated in saliva dependent virus enhancement are most likely heat sensitive-proteinaceous factors.

As the majority of female salivary components have a role in hematophagy in blood feeding mosquito species, we hypothesised that salivary components implicated in virus enhancement have co-evolved to facilitate blood feeding. To investigate this, we compared the effect of female and male *Ae.aegypti* saliva on virus infection. We demonstrate that female *Ae.aegypti* saliva causes significant enhancement of SFV infection whilst male mosquito saliva does not. This is of importance as only female *Aedes* mosquitoes are hematophagous whilst males are exclusively sugar feeding. This suggests that the virus infection enhancing components within female mosquito saliva are those that have evolved to facilitate blood feeding. As a side, it was also important to clarify whether the act of blood feeding by female *Ae.aegypti* mosquitoes modulated the ability of female *Ae.aegypti* mosquitoes saliva to enhance infection. We were able to conclude this as we observe no difference in modulation of virus infection by saliva acquired from blood fed or exclusively sugar fed mosquitoes.

Finally, we demonstrate for the first time that the salivary components responsible for virus enhancement are not present in all blood feeding mosquito vectors. By comparing the ability of several blood feeding mosquito vectors to enhance virus infection we observe that whilst *Aedes* and *Culex* mosquito species successfully enhance infection, *Anopheles* does not. This provides us with a new tool for studying the mechanisms of saliva-dependent virus enhancement, as *Anopheles* can be used as an important comparator for our studies with *Aedes* saliva that does enhance infection.

The main conclusions from chapter 4 and 5 are:

- *Ae.aegypti* saliva inhibits SFV infection *in vitro* in a microbiota dependent manner.

- Modulation of virus infection by *Ae.aegypti* saliva does not occur in explanted skin.
- Antibiotic sensitive microbiota not implicated in enhancement of virus infection.
- *Aedes* saliva factor implicated with virus enhancement is associated with blood feeding behaviour and is proteinaceous.
- Virus infection enhancing saliva factor is not present in all blood feeding mosquito species.

9.4 Oedema formation in response to mosquito saliva is likely a key response required for virus enhancement

Based on the discovery that saliva from blood feeding *Aedes* mosquitoes enhance infection, whilst saliva from blood feeding *Anopheles* mosquitoes do not, and based on the assumption that host responses elicited against saliva are important in facilitating virus enhancement; we decided to compare host responses against *Aedes* and *Anopheles* saliva. This was done in order to discover differences in host responses which could be responsible for modulation of virus infection. The identification of specific host responses (oedema) were then studied further.

Therefore the aims of chapters 6 were to determine whether:

1. **There is a difference in the upregulation of inflammatory genes caused by *Aedes* and *Anopheles* saliva.**
2. **There is a difference in the amount of oedema formation following injection with either *Aedes* or *Anopheles* saliva.**

Initially we sought to compare the upregulation of pro-inflammatory cytokines in the skin in response to *An.gambiae* and *Ae.aegypti* saliva. Hypothetically, *Anopheles* saliva could be less inflammatory. This is key as some host inflammatory responses to mosquitoes bites enhance arbovirus infection. However, the studies here demonstrate for the first time that *An.gambiae* saliva causes higher upregulation of key pro-inflammatory cytokines in the skin at 6 hours post injection than *Ae.aegypti* with a significantly higher expression of IL1β, CCL5, CXCL2 and CXCL10. Co-

inoculation of virus with *An.gambiae* saliva caused a significantly higher upregulation of ISGs such as Viperin and CCL5, as well as IFNα itself, compared to *Ae.aegypti* at 24 hours post infection. This might suggest that differential type I IFN responses by the host may underlie the differences observed in the ability of saliva to enhance infection.

However, as we observe that mixing of *Ae.aegypti* saliva and *An.gambiae* saliva resulted in a similar enhancement of infection as observed by *Ae.aegypti* saliva inoculation on its own, this suggests that *An.gambiae* saliva does not actively inhibit virus infection (e.g. via IFN expression). Therefore it is unlikely that *An.gambiae* saliva enhances virus infection due to an upregulated antiviral IFN response, as this would have inhibited virus infection when *Anopheles* was co-inoculated with *Ae.aegypti* saliva. We also found that *Aedes* saliva could enhance ZIKV and SFV infection in the absence of type I IFN signalling. Instead, it seems likely that *An.gambiae* saliva simply lacks one, or several factors, that are necessary to cause enhancement of arbovirus infection.

Next, we studied differences in oedema formation in response to *Aedes* and *Anopheles* saliva/bites. We demonstrate that *Ae.aegypti* saliva (and bites) cause significant amounts of oedema in the skin at 30min, 3h and 6h post exposure with differences more pronounced at earlier time points. In contrast, *An.gambiae* saliva caused no detectable formation of oedema at the inoculation site at any time point investigated, and *An.gambiae* bites only triggered significant formation of oedema 30min post exposure. Oedema formation by *An.gambiae* bites was significantly less than that caused by *Ae.aegypti* bites. This suggests that oedema formation may be implicated in facilitating arbovirus enhancement. Based on these observations, oedema in response to *Ae.aegypti* saliva and the lack of oedema exhibited in response to *An.gambiae* saliva is a more likely candidate for causing enhancement of viral infection.

Therefore, in chapter 7 we wanted to further investigate whether:

1. **Oedema induced in the absence of any mosquito derived factors can enhance arbovirus infection.**
2. **Inhibition of mosquito bite oedema using anti-histamines can modulate saliva mediated arbovirus enhancement.**

3. Oedema in response to mosquito saliva differs between naïve and sensitised hosts.

Following the discovery of differences in oedema by *Aedes* and *Anopheles* saliva, we further wanted to investigate whether oedema is implicated in modulation of virus infection. For this purpose, the impact of oedema, triggered experimentally in the absence of saliva factors, on virus infection, was investigated. Utilising purified histamine, we demonstrate that histamine induced oedema is sufficient in enhancing virus infection *in vivo*. This suggests that oedema in response to mosquito bite/saliva is important in virus infection modulation.

Although oedema in response to *Ae.aegypti* saliva appears to be key in facilitating virus enhancement, the mechanisms involved in triggering oedema formation in response to *Ae.aegypti* saliva remain unknown. Saliva mediated oedema could be immune mediated; either via acute histamine release by resident cells; or via histamine release mediated by recruited cells (e.g. Leukocytes). Alternatively, saliva mediated oedema could be a direct result from an enhanced permeability of the endothelial cells lining the blood vessels. Utilising a combination of antihistamines to investigate whether oedema formation is instigated via histamine release, we demonstrate that antihistamines are insufficient at inhibiting saliva mediated oedema and consequently incapable of preventing salivary mediated virus enhancement. This suggests that oedema formation in response to *Ae.aegypti* mosquito saliva occurs independently of histamine release. Instead we hypothesised that *Ae.aegypti* saliva causes enhanced permeability of endothelial cells lining blood vessels resulting in oedema. This hypothesis is supported anecdotally by the fact that we cannot observe virus enhancement *in vitro* in macrophages, fibroblasts and skin explants as, these lack a functional blood vasculature system. Further studies are required to determine the mechanisms involved in *Ae.aegypti* saliva mediated oedema to allow for the identification of potential treatment targets.

In terms of the mechanisms by which oedema enhances virus infection, a previous study speculate that oedema formation may help retain virus in the skin [95]. Retention of virus in the skin could have two main advantages to the virus; 1.) by infecting skin cells that support high levels of replication; and 2.) by delaying innate

immune activation in the draining lymph node. By studying the kinetics and dissemination of virus when accompanied by mosquito bites and mosquito saliva, we demonstrate that whilst *Ae.aegypti* bites demonstrate delayed dissemination of virus to the dLN, *Ae.aegypti* saliva does not. In fact, mice inoculated with virus alongside extracted mosquito saliva exhibited enhanced dissemination of virus to the dLN whilst simultaneously exhibiting enhanced virus replication in the skin. As we have previously demonstrated that mosquito saliva enhances virus infection with the same efficacy as bites, we can conclude that oedema does not enhance infection by retaining virus in the skin e.g. where it can replicate in susceptible dermal cells and simultaneously delaying immune activation in draining lymph nodes.

As all our experiments had been conducted using naïve mice, we wanted to also investigate whether pre-sensitization to mosquito saliva altered oedema formation and subsequently the modulation of virus infection by saliva. In our experiments we demonstrated that there was no difference in the level of SFV infection following co-injection of virus alongside *Ae.aegypti* mosquito saliva, comparing naïve and pre-sensitised BALB/c mice. Oedema formation in response to *Ae.aegypti* saliva in BALB/c mice differed from what we previously observed in C57BL/6. As was demonstrated in previous chapters, *Ae.aegypti* saliva exposure in naïve C57BL/6 mice results in significant oedema formation by 30min post exposure, whilst in naïve BALB/c mice no detectable oedema could be observed at 30min post exposure. This suggests an alternative host response in naïve BALB/c mice in response to *Aedes* mosquito saliva, making comparison to our data generated previously with C57BL/6 mice difficult. However, significant levels of oedema were exhibited at 30min post exposure in BALB/c mice previously exposed to *Ae.aegypti* saliva, although levels of oedema were similar by 6 hours post exposure, compared to non-primed mice. Importantly, both naïve and pre-sensitised BALB/c mice exhibited enhanced virus infection to the same level by 24 hours post infection, following co-inoculation of SFV4 alongside *Ae.aegypti*. This suggests that not all forms of oedema are pro-viral or that oedema formation following virus inoculation at time points later than 30 minutes post exposure are most important in defining outcome to infection. It may also be that it is not oedema per se that enhances infection, but rather that this leakage of fluid it reflects a general state of endothelial barrier dysfunction, and that some aspect of this dysfunction supports higher levels of virus infection.

Main conclusions from chapter 6 and 7:

- *An.gambiae* saliva causes significantly higher upregulation of key pro-inflammatory genes in the skin at 6 hours post injection than *Ae.aegypti* saliva.
- *Ae.aegypti* saliva triggers significant oedema formation but *An.gambiae* saliva does not.
- Histamine induced oedema is sufficient to enhance virus infection.
- Saliva mediated oedema is histamine independent as antihistamines are insufficient at blocking saliva mediated oedema.
- Pre-sensitization to mosquito saliva does not further modulate saliva mediated virus enhancement.

9.5 Lack of modulation of ONNV infection by mosquito saliva may explain ONNV mosquito vector competence

Discovering that *Anopheles* mosquito saliva/bites are incapable of enhancing SFV4 and ZIKV in mice, and as it has been established that *Anopheles* mosquitoes are very inefficient transmitters of the majority of arboviruses, with the exception of ONNV; we next wanted to investigate whether *Anopheles* could modulate infection ONNV. We hypothesized that the saliva factor implicated in virus enhancement affects mosquito vector competence and may explain why this mosquito species is such a poor vector for most arboviruses.

Therefore, the aims of chapter 8 were:

1. **To develop an ONNV virus mouse model.**
2. **To use the developed ONNV *in vivo* model in order to determine if *Aedes* or *Anopheles* saliva enhance ONNV infection.**

In chapter 8, an infectious ONNV *in vivo* model utilising C57BL/6 mice with the use of the IFNAR-1 antibody to temporally inhibit IFN signalling was developed. We demonstrate for the first time that ONNV does not replicate in WT immunocompetent C57BL/6 mice, irrespective of the presence of *Ae.aegypti* mosquito bites/saliva. Instead, blocking of type I IFN responses are necessary for

replication of virus to occur. We also show that we can detect ONNV at the inoculation site, spleen, proximal joint, distal joint and dLN at 48 hours post infection.

Utilising the developed ONNV mouse model we demonstrate for the first time that ONNV infection is not enhanced by either *Ae.aegypti* saliva or bites, unlike other mosquito borne viruses. This is very interesting, considering the close sequence homology of ONNV to SFV and CHIKV, which have both been demonstrated previously to be enhanced by *Ae.aegypti* bites. Furthermore we also demonstrate that *An.gambiae* bites/saliva do not modulate ONNV infection. Together, these observations suggest that ONNV has evolved not to rely on mosquito saliva dependent arbovirus enhancement, and can therefore be transmitted via mosquito species that lack the salivary factors necessary for virus enhancement. This observation could provide a further reason for the inability of *An.gambiae* mosquitoes to vector the majority of arboviruses with the exception for ONNV.

9.6 Conclusions and future directions

In this thesis we have demonstrated for the first time that mosquito saliva is sufficient for the phenomenon of mosquito-bite mediated enhancement of virus infection. The saliva factor implicated in the modulation of virus enhancement is a proteinaceous factor implicated in blood feeding. This factor is not present in all blood feeding mosquitoes as we find that *Anopheles* species saliva does not enhance virus infections. Via a comparative study of host responses towards *Ae.aegypti* and *An.gambiae* mosquito saliva we demonstrate the importance of oedema formation in the modulation of virus enhancement. We also demonstrate that saliva mediated oedema is histamine independent.

Further work is required to further determine the mechanisms involved in oedema formation by *Ae.aegypti* saliva. Specifically, we need to study the impact of *Aedes* and *Anopheles* saliva on endothelial cell permeability. By identifying the specific mechanisms involved we can develop treatments that specifically target and block saliva mediated oedema and subsequently virus enhancement from occurring. Therefore further work is required that specifically investigates the impact of

mosquito saliva mediated oedema inhibitors and their efficacy in prevention of virus enhancement and disease severity.

In terms of saliva factors implicated in virus enhancement we demonstrated that this factor is not saliva microbiota, but instead a proteinaceous factor. Comparative transcriptomic analyses of salivary glands of mosquito species that enhance infection to mosquito species that do not (*Anopheles*), could highlight potential factors responsible for virus enhancement. Subsequent gene knockout *Ae.aegypti* mosquitoes, were potential candidate genes have been knocked out, could then be used to investigate impact of specific genes on saliva mediated enhancement.

In addition, we also demonstrate for the first time that saliva factors implicated in modulation of virus infection may be key for defining mosquito vector competence. Further studies should be conducted investigating the genetic differences between ONNV and other closely related arboviruses such as CHIKV and SFV in order to determine which genetic differences allow ONNV to replicate (and presumably transmit) independently of saliva mediated enhancement.

The understanding of the mechanisms implicated in saliva mediated virus enhancement allows for the development of a treatment that prevents oedema, virus enhancement and subsequently prevention of severe disease. To conclude, I would argue that this concept could be used for the development of a treatment and/or vaccine that targets the key mosquito saliva factor involved. Potentially, this could be useful against the vast majority of arbovirus infections, to be used in combination with infection limiting strategies such as vector control and mosquito repellents.

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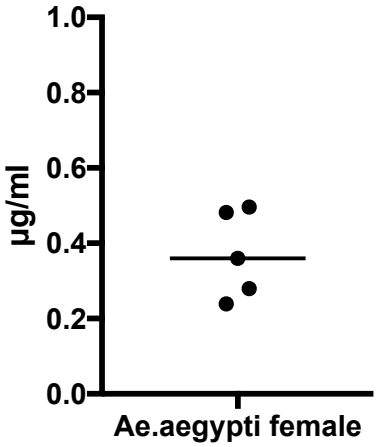
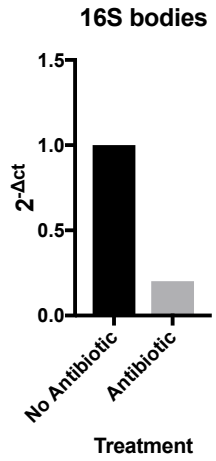
Appendix



Appendix 1: Protein concentration of female *Ae.aegypti* saliva.
Dot plot illustrating the protein concentration of female Ae.aegypti saliva acquired via forced salivation of mosquitoes. Each dot represents the average quantity of 1 mosquitoes worth of saliva estimated from the concentration measured from saliva pooled from a group of mosquitoes. Saliva protein concentration was measured via nanodrop. Concentrations range between 0.24µg/ml to 0.49µ/ml.



Appendix 2: Antibiotic treatment validation.
Quantity of 16S RNA was measured in whole mosquitoes of untreated (black) and antibiotic treated mosquitoes (grey).