Investigating multiple factors affecting the successful infection and transmission of arboviruses

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Abstract

Arboviruses are arthropod-borne viruses that cycle between a vertebrate host and an arthropod vector. This disparate life cycle involves adapting to two alternate animals and the differing tissues and systems within them. A range of factors influence the ability of a mosquito to transmit a virus, including internal factors such as mosquito immunity, the mosquito microbiota and the mutation rate of the virus itself, and external factors such as exposure to agricultural chemicals and warming global temperatures. I looked at several factors affecting the establishment of infection and transmission of arboviruses. Three low-fidelity mutants of Venezuelan Equine Encephalitis Virus (VEEV), which produce a higher mutation rate than the wild-type virus, were previously identified as unable to successfully infect mosquitoes. To understand why these low-fidelity mutants were attenuated, I serially passaged wild-type VEEV and the three low-fidelity mutants in a new cell culture model designed to replicate bottlenecks within arbovirus transmission. This would enable us to understand why the low-fidelity mutants were attenuated in previous *in vivo* mosquito and mouse infections. Furthermore, we wanted to see if the low-fidelity nature changed the RNAi response, a key antiviral immune response within mosquitoes. We looked to see if the RNAi response was different for TC83_3X (a low-fidelity mutant) compared to the parent TC83 VEEV-vaccine strain using *in vitro* cell culture experiments and RNA sequencing. Finally, I wanted to understand the impact of external factors on vector competence, given many factors will act on wild mosquito populations. I determined if two agricultural chemicals, glyphosate and triticonazole, or exposure to the insect-specific virus Negevirus (NEGV), altered the vector competence of *Aedes aegypti* for Chikungunya virus (CHIKV) or Zika virus (ZIKV). To summarise the results, we found that the previously seen attenuation of the three low-fidelity VEEV variants is likely due to their inability to traverse bottlenecks associated with mosquito infection, and that the RNAi response did not differ between parent TC83 and low-fidelity TC83_3X. Both Glyphosate and Triticonazole slightly reduced the vector competence of *A. aegypti* for both CHIKV and ZIKV whilst exposure to NEGV increased the percentage of the mosquito population infected with both viruses. However, NEGV exposure reduced the percentage of infected mosquitoes with dissemination, which may subsequently reduce the likelihood of transmission due to lower likelihood of infectious virus

reaching mosquito salivary glands. Our work covered a range of internal and external factors and identified their influence on vector competence. This has greater implications for our understanding of the establishment of arboviral infection and their transmission.

Acknowledgements

I am extremely grateful to have had to opportunity to carry out this research. I'd like to thank Keele University for providing the funding that has allowed me to do so. I'd like to than the Royal Society of Tropical Medicine and Hygiene for awarding me the Early Careers Grant that allowed me to carry out my research with Venezuelan Equine Encephalitis Virus.

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List of Abbreviations

3’- 3 Prime

3X- TC83_3X Virus

4X- TC83_4X Virus

5’- 5 Prime

6K- 6-Kilodalton

A- Adenine

A. aegypti- Aedes aegypti

A. albopictus- Aedes albopictus

A. gambiae- Anopheles gambiae

A. taeniorhynchus-

A. Vigilax- Aedes vigilax

AGO- Argonaute

AMP- Antimicrobial Peptide

ANOVA- Analysis of Variance

C- Cytosine

C. taenipeus- Culex taeniopeus

CACT- CACTUS

CDC- Centres for Disease Control

CFAV- Cell Fusing Agent Virus

CHIKV- Chikungunya Virus

CPE- Cytopathic Effect Assay

CTRL- Control

Cx. Annulirostris- Culex annulirostris

Cx. Tarsalis- Culex tarsalis

CxFV- Culex *Flavivirus*

DCR2- Dicer-2

DENV-2- Dengue Virus Type-2

DIP- Defective Interfering Particle

Dissem- Dissemination

DPI- Days Post Infection

dsRNA- Double Stranded RNA

dsSINV- Double Subgenomic Sindbis Virus

EIP- Extrinsic Incubation Period

FEC- Fold Expression Change

FMDV- Foot and Mouth Disease Virus

G- Guanine

G7R- G7R Virus

GLY- Glyphosate

H/L- Heads and Legs

HF- High-Fidelity

HPI- Hours Post Infection

IFN- Interferon

ISV- Insect-Specific Virus

LCMV- Lymphocytic Choriomeningitis Virus

LF- Low-Fidelity

miRNA- Micro RNA

MOI- Multiplicity of Infection

NEGV- Negevirus

NS/S- Nonsynonymous/Synonymous

nsP- Nonstructural Protein

NT- Nucleotide

NWV- New World Virus

ONNV- O'nyong'nyong Virus

ORF- Open Reading Frame

OWV- Old World Virus

P123- Polyprotein 123

PCV- Palm Creek Virus

PFU- Plaque Forming Units

PIAS- Protein Inhibitor of Activated STAT

piRNA- PIWI-Interacting RNA

QS- Quasispecies

RC- Replication Complex

RdRp- RNA-dependent RNA polymerase

RNA- Ribonucleic Acid

RNAi- RNA Interference

RVFV- Rift Valley Fever Virus

SI- Specific Infectivity

SINV- Sindbis Virus

siRNA- Small Interfering RNA

SNV- Single Nucleotide Variant

ssRNA- Single Stranded RNA

T- Thymidine

TRI- Triticonazole

U- Uridine

VEEV- Venezuelan Equine Encephalitis Virus

VSV- Vesicular Stomatitis Virus

WHO- World Health Organisation

WNV- West Nile Virus

WSLV3- Wenzhou Sobemo-like Virus 3

WT- Wild-type

ZIKV- Zika Virus

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Chapter 1 Introduction

1.1 Introduction to RNA viruses

For centuries RNA viruses have presented major threats to the human population. In 1918 an Influenza outbreak was estimated to have caused 50 million deaths worldwide (Taubenberger *et al*., 2006), and in recent years there have been large outbreaks of Zika Virus (ZIKV), Chikungunya Virus (CHIKV) and the ongoing 2019 Coronavirus pandemic. The last two decades have seen an increase in the number of infections from viruses that have spread to new regions due to increased travel, population density and transportation of goods (Gould *et al*., 2017, Musso *et al*., 2018, Paixo *et al*., 2017, Randolph *et al*.,10). Poor infrastructure and public education of disease in low-income countries, coupled with differing cultures, and a social stigma of disease that is still found in first world countries, erect barriers to efficient control. Additionally, increasing global temperatures broaden the range of the vectors capable of carrying and transmitting the viruses (Whitehorn *et al*., 2019), and lack of effective control measures against these lead to more countries facing these problems. It is therefore imperative to understand how viruses maintain their enzootic cycles within the environments, as understanding this will allow us to better predict how viral populations may spill over into human populations.

1.1.2 Arboviruses

Arboviruses are **a**rthropod-**b**orne viruses with endemic and epidemic cycles (Chamberlain *et al*., 1968). During an enzootic cycle they obligately cycle between an arthropod vector and a vertebrate host. Occasionally, a transmission event enables viruses to infect and replicate in an epizootic/epidemic host, including humans. Arboviruses are found in climates across the globe, with outbreaks occurring in endemic regions such as subtropical and tropical regions of South America, Asia and Africa, whilst nineteen countries in Europe reported 24,974 Tick-borne Encephalitis cases between 2012 and 2020 (Heuverswyn et al., 2023. Spatiotemporal spread of TBE in the EU/EEA, 2012 to 2020. Eurosurveillance).

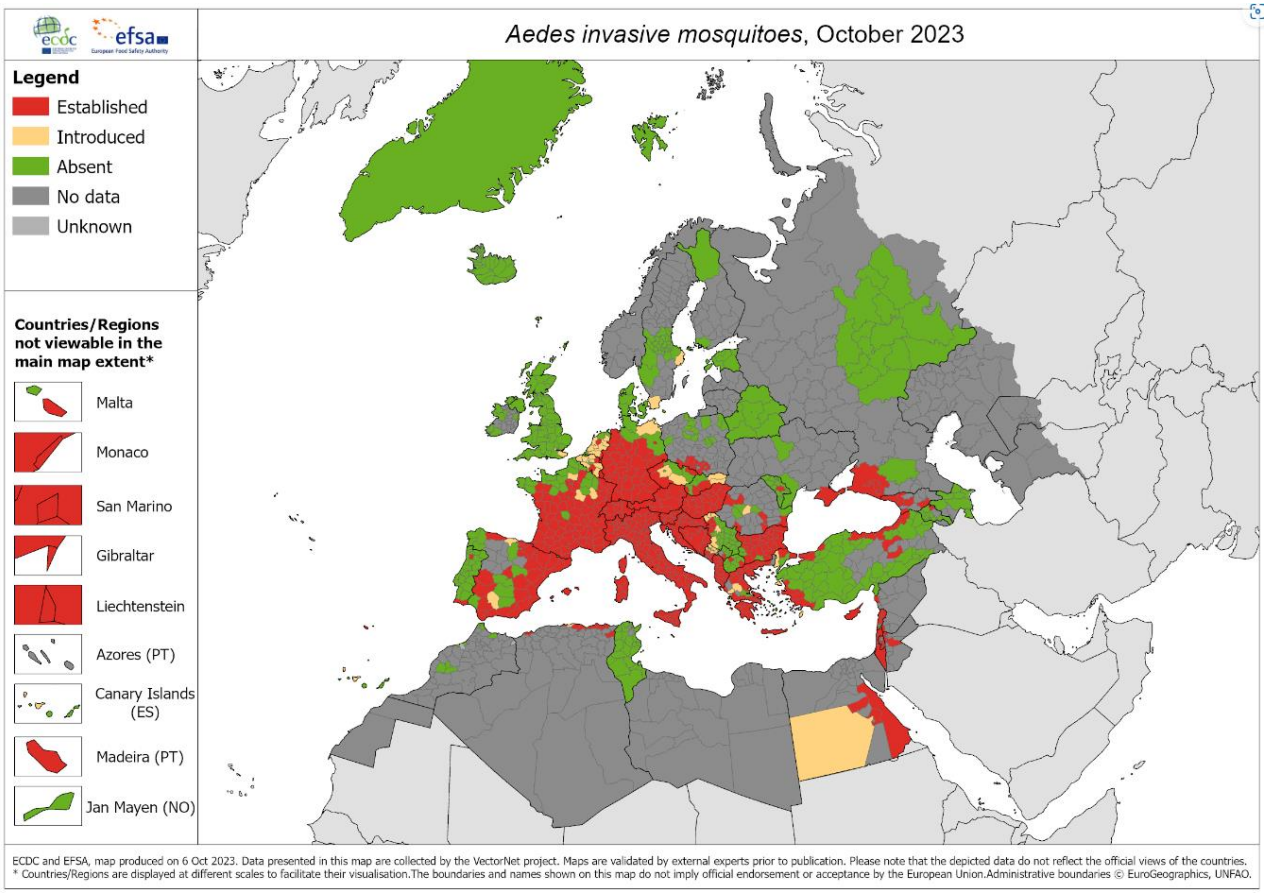


Figure 1.1 The distribution of *Aedes* invasive species (*A. aegypti*, *A. albopictus*, *A. atropalpus*, *A. japonicus* and *A. koreicus*) in Europe as of October 2023. Red areas indicate areas where one or more of these vectors are established, yellow indicates areas where one or more of these species have been introduced, green indicates areas where these vectors are absent as of 2023 and dark and light grey indicate areas where it is unknown if the vectors are there or there is currently no data. European Centre for Disease Prevention and Control and European Food Safety Authority. Mosquito maps [internet]. Stockholm: ECDC; 2023. Available from https://ecdc.europa.eu/en/disease-vectors/surveillance/surveillance-and-disease-data/mosquito-maps.

Increasing changes in global temperatures will have a significant impact on the vector range of these viruses. The climate of Europe is becoming increasingly suitable for an invasive species of mosquitoes; *Aedes albopictus*, with the mosquito expected to expand its geographic location from 2011 to 2040 (Fischer *et al*., 2014). As seen in figure 1.1, Europe is becoming increasingly suitable for invasive species of *Aedes*. TBE cases have increased in Sweden since the 1980s correlating with

shorter, warmer winters and in the Czech Republic warmer temperatures are allowing colonisation of higher altitudes, increasing the prevalence in new populations (Baylis *et al*., 2017, Zeman *et al*., 2004).

1.1.3 Arboviruses encompass six families

With the exception of African Swine Fever Virus, arboviruses are single stranded RNA viruses (Forrester *et al*., 2014). They encompass over 500 viruses in 6 families; *Togaviridae*, *Flaviviridae*, *Bunyaviriales, Orbiviridae*, *vesiculoviridae* and *thogotoviridae* (Beckham and Kenneth, 2015, Reynolds *et al*., 2017). Over 100 of these viruses will infect humans, either as part of their enzootic cycle or as they spill out into an epizootic cycle which can lead to large and often fatal outbreaks. Over the last decade, arboviruses such as ZIKV, Dengue Virus (DENV) and CHIKV have dominated headlines, with multiple large epidemics causing the deaths of millions of people (Weaver and Barratt, 2004, Wikan *et al*., 2016).

Two important families within arboviruses include the *Flaviviruses* and *Alphaviruses.* DENV and ZIKV are prominent members of the *Flavivirus* family. The number of cases of DENV reported to the WHO increased from 505,430 in 2000 to 5.2m in 2019 (WHO) and as of 2021, 89 countries and territories showed evidence of ZIKV infection with infections peaking in the Americas in 2016 (WHO Zika Epidemiology Update, Wikan *et al*., 2016). *Flavivirus*es cause diseases displaying a wide range of symptoms, ranging from headaches and arthralgia associated with most, to microcephaly in infants as seen with ZIKV (Wikan *et al*., 2016, Brasil *et al*., 2016). Furthermore, by 2004 Japanese Encephalitis Virus caused 50,000 cases per annum, 15,000 of which were fatal and 50% of survivors suffered complex neurological conditions (Weaver and Barratt, 2004).

*Alphavirus*es present a substantial threat to the modern world (Lwande *et al*., 2015). *Alphaviridae* consist of 40+ members including Venezuelan Equine Encephalitis Virus (VEEV) and can be split into old world viruses (OWV) and new world viruses (NWV), with OWV including CHIKV, Sindbis virus (SINV) and Ross River Virus that cause arthralgic infections (Garmashova *et al*., 2006). On the contrary, NWV include the Equine Encephalitis Viruses that have evolved separately over the last 1000 years

leading to encephalitic disease rather than the arthralgic disease of the OWV (Garamashova *et al*., 2006). With no readily available vaccines or treatments, the encephalitis viruses have caused large and often fatal epidemics.

1.1.4 Zika Virus

ZIKV is a positive single-stranded RNA *flavivirus* that was first identified in Uganda 1947 following isolated from a Rhesus macaque (Dick, Kitchen and Haddow, 1952). ZIKV has a genome of 10.7kb in length with one open reading frame containing the three main structural and seven non-structural proteins. During infection, ZIKV attaches to receptors on a cell membrane and enters the cell through endocytosis (Li *et al*., 2020). Replication occurs, and immature virions mature at the golgi before release into the extracellular space (Sirohi and Kuhn, 2017).

Whilst humans are often incidental hosts in the sylvatic cycle of ZIKV, in which the virus cycles between primates and mosquitoes, humans may serve as the primary/reservoir host in areas where there are no non-human primates (Aziz *et al*., 2016). ZIKV is predominantly transmitted to humans by the *Aedes aegypti* mosquito in the Americas though studies have implicated various *Aedes* species as vectors (Duffy *et al*., 2009, Ledermann *et al*., 2014). *Aedes* is an important vector in ZIKVs transmission to humans given its urban lifestyle, with breeding sites commonly found pools of water in human populated areas (Overgaard *et al*., 2017).

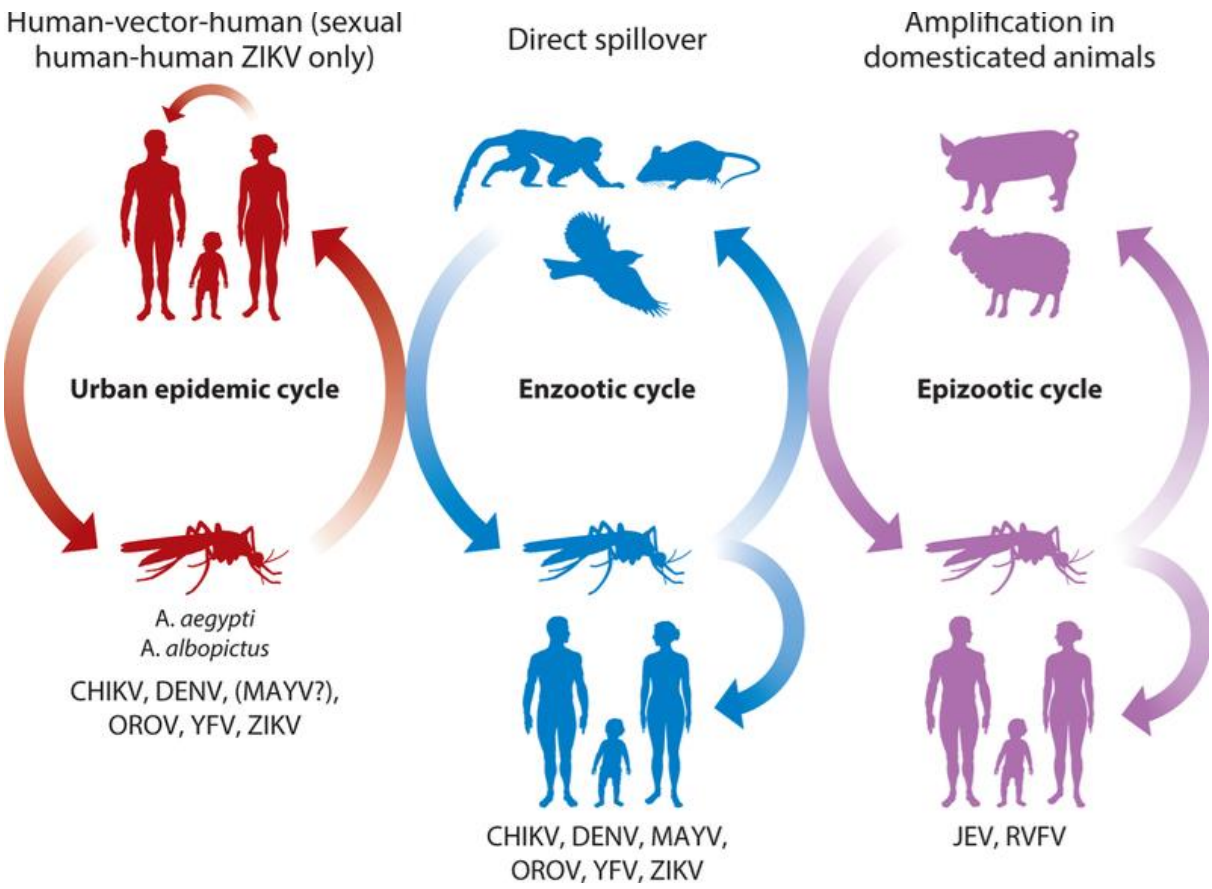
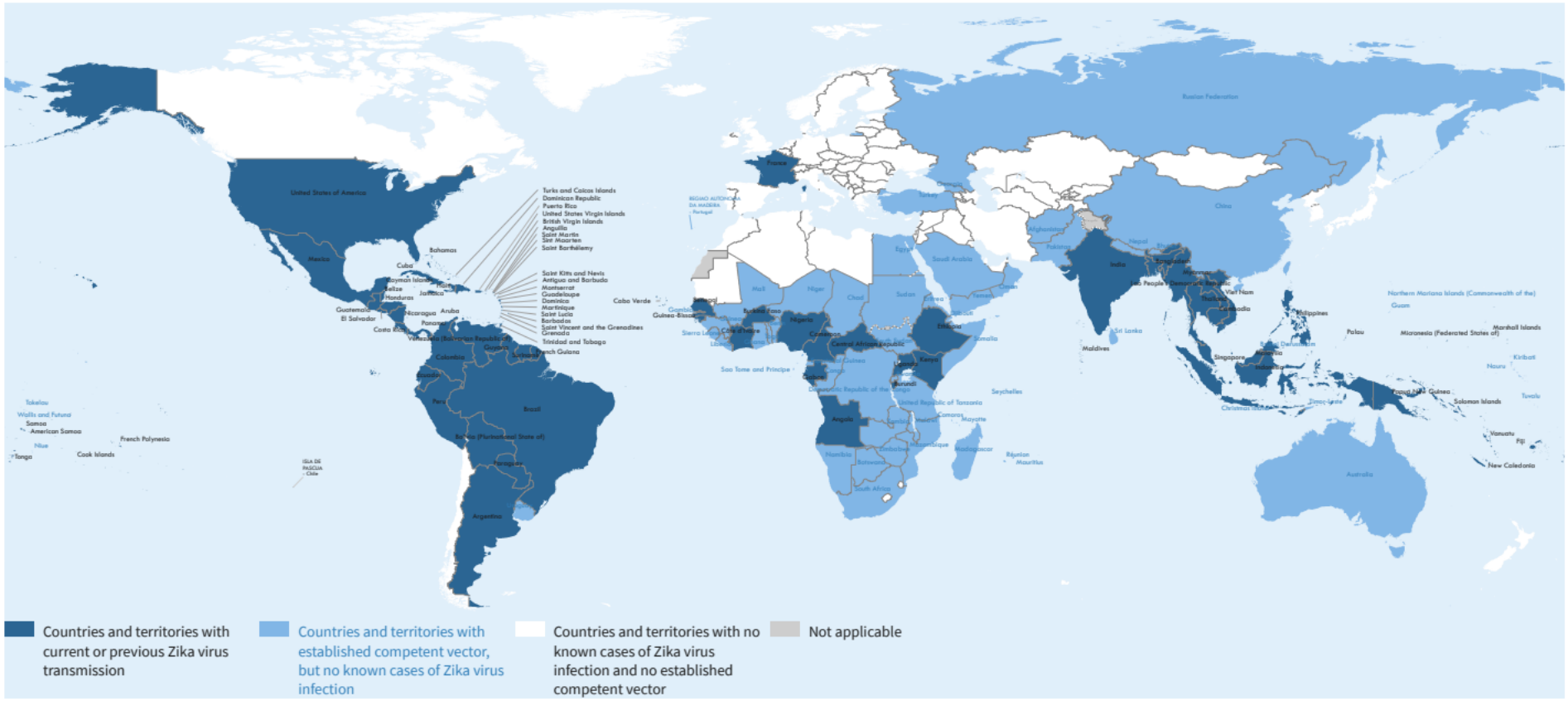


Figure 1.2 Transmission cycle of several arboviruses including ZIKV and CHIKV. Several *Alpha-* and *Flaviviruses* are transmitted within an urban cycle directly between mosquitoes and humans. Enzootic transmission cycles involve viruses circulating within wild animal populations with enzootic mosquito vectors that bite both humans and wild animals. Epizootic cycles occur when a virus is amplified in a second host, such as Japanese Encephalitis Virus (JEV) that amplifies within livestock (swine) within close proximity to humans. Additionally, mutations can arise allowing viruses to infect more invasive mosquito species, such as VEEV and CHIKV infecting *A. albopictus*, increasing the spread to humans. (Weaver *et al*., 2018).

ZIKV generally causes a range of symptoms including headaches, rash, fever and myalgia (Simpson *et al*., 1964). Whilst mosquito bites are the primary transmission, ZIKV can also be transmitted through sexual contact and importantly through breast milk and across the blood-brain barrier, infecting a foetus (Foy *et al*., 2011, Ittal *et al*., 2017). Following the September 2015 outbreak in Brazil ZIKV has been identified as a cause of microcephaly in infants and has a clear association with the development of

Guillain-Barre syndrome (Teixeira *et al*., 2016, Cao-Lormeau *et al*., 2016). Microcephaly is linked with a range of problems including seizures, developmental delay, vision problems and intellectual disability (CDC).

Countries and territories with current or previous Zika virus transmission



The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of WHO concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.

Map date: February 2022
Data Source: World Health Organization
Map Production: WHO Health Emergencies Programme

Zika virus country classification tables available at: https://www.who.int/emergencies/diseases/zika/countries-with-zika-and-vectors-table.pdf

World Health Organization

Figure 1.3 A WHO map of the epidemiology of ZIKV as of 2022, showing countries with current or previous outbreaks, countries with competent vectors but no known cases of ZIKV disease and countries with no known cases nor competent vectors. Dark blue areas show countries and territories that currently have or previously had ZIKV transmission. Light blue areas show countries/territories that do not currently or previously have ZIKV cases, but have established competent vectors. White areas show countries/territories with no cases of ZIKV or competent vectors. (https://cdn.who.int/media/docs/default-source/documents/emergencies/zika/map-of-countries_with_zika_transmission_feb2022.pdf?sfvrsn=802a352a_5

ZIKVs potential to be an epidemic virus was highlighted in several epidemics in 2013-15 following outbreaks in French Polynesia and Brazil which spread to most countries in South and Central America. As of 2022, 40,249 cases of ZIKV have been reported in the region of the Americas in 15/52 countries and territories, with the highest numbers in Brazil (84.9%) (PAHO Arbovirus Bulletin 2022). Though there are some currently showing promise (Essink *et al*., 2023), there are currently no vaccines, antivirals or prophylaxis available specifically for ZIKV and WHO has declared it a public health emergency worldwide.

1.1.5 Chikungunya Virus

Chikungunya is a positive ssRNA old-world *alphavirus* of the Togaviridae family first isolated from Tanzania in 1952. Similar to VEEV, the CHIKV genome is 11.7kb in length encoding 2 open reading frames with 5' and 3' untranslated regions. The 5' ORF is translated from genomic RNA resulting in the four nonstructural proteins whilst the 3' ORF is translated from subgenomic RNA to give the three major structural proteins of the capsid and the E1 and E2 glycoproteins. E2 interacts with cell receptors whilst E1 mediates fusion with endosomes, and then CHIKV is released into the cells via a pH-dependant mechanism that creates pores within the endosome to release the CHIKV nucleocapsid. *Alphavirus* replication is detailed in a later section.

There are three lineages of the virus, two African and one Asian. In Africa, CHIKV is maintained in a sylvatic cycle between non-human primates and forest dwelling mosquito species of the *Aedes* genus, with small outbreaks depending on environmental conditions such as increases in mosquito densities following heavy rainfall (Higgs and Vanlandingham, 2015). The Asian lineage is largely maintained by an urban cycle by the *Aedes aegypti* mosquito found near humans. *A. albopictus*, more aggressive in its feeding on humans, has spread globally within the last decade and is also an important vector capable of transmitting CHIKV to new territories.

In 2005-2006, a CHIKV East/South/Central African (ESCA) strain developed a mutation at position 226 of the E1 glycoprotein that increased the virus's fitness in *A. albopictus* allowing efficient replication and transmission. The resulting outbreak in the Indian Ocean Islands infected 775,000 with 237 deaths.

The outbreak was worsened by the immune naivety of the population, a result of the rapid spread of the promiscuous *A. albopictus*. CHIKV infection is normally characterised by fever and joint pain, however in 2005-2006 the outbreak evolved to include central nervous system involvement and severe illness. In 2006 CHIKV also emerged as an epidemic in India, causing an estimated 25,588 disability adjusted life years lost (Krishnamoorthy *et al*., 2009). This is a measure of disease burden, expressed as the number of years lost due to ill-health, disability or early death.

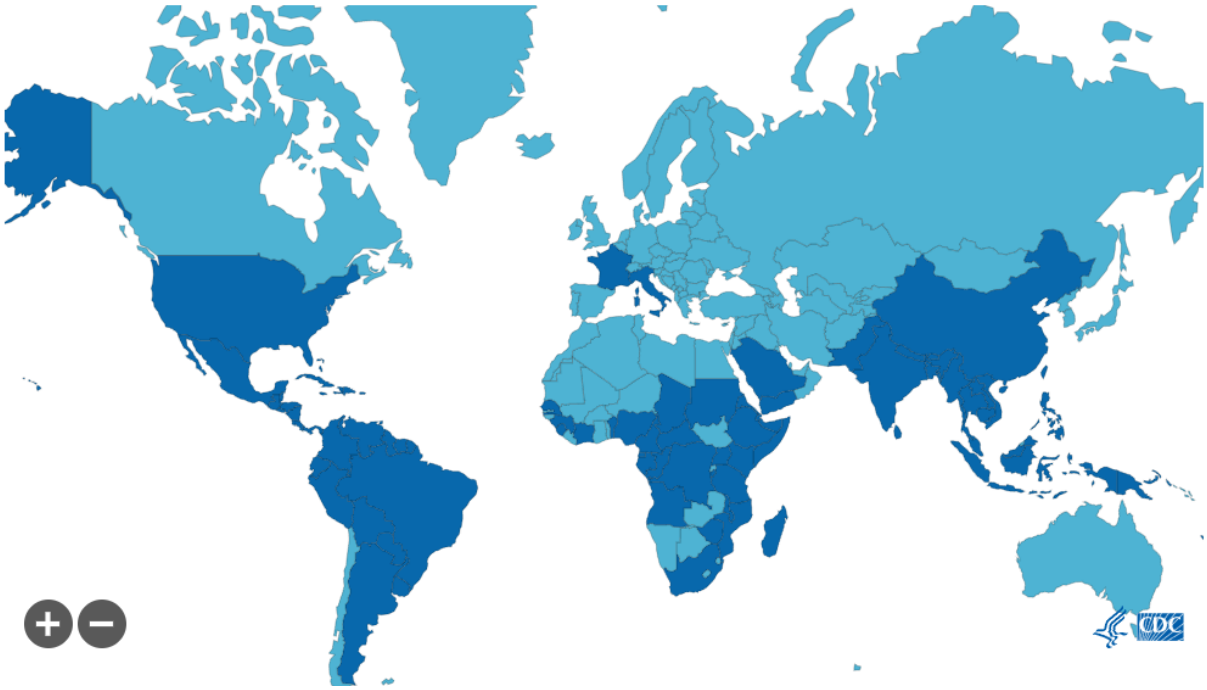


Figure 1.4 CDC epidemiology map showing countries with current or previous cases of CHIKV (dark blue) and countries with no reported cases of CHIKV (light blue) (https://www.cdc.gov/chikungunya/geo/index.html)

CHIKV has been reported in 105 countries and territories as of April 2023, with Fig 5 not included imported cases. Before 2005-6, most outbreaks were small and restricted to small African and Asian outbreaks including India, Kenya and the Comoros Islands (Charrel *et al*., chikv outbreaks 2007). The La Reunion outbreak led to CHIKV spread to other countries, including an outbreak in Italy of 205 cases, Malaysia and Bangladesh (Rezza *et al*., 2007, Cavalcanti, Pereira, Paula and Franca, 2022). In 2015, the Pan American Health Organisation received reports of 37,480 CHIKV cases which had

increased to 146,914 by 2016. Studies noted during a large outbreak of CHIKV in Brazil, several patients with severe CHIKV disease were suffering from coinfections with ZIKV or DENV (Brito *et al*., 2017, Mercado *et al*., 2016). In 2014 CHIKV reached America and by 2015 was a major health problem. There are currently no treatments or preventative vaccines currently licenced against CHIKV infections (Schmidt and Schnierle, 2022).

1.1.6 Venezuelan Equine Encephalitis Virus

A. B.

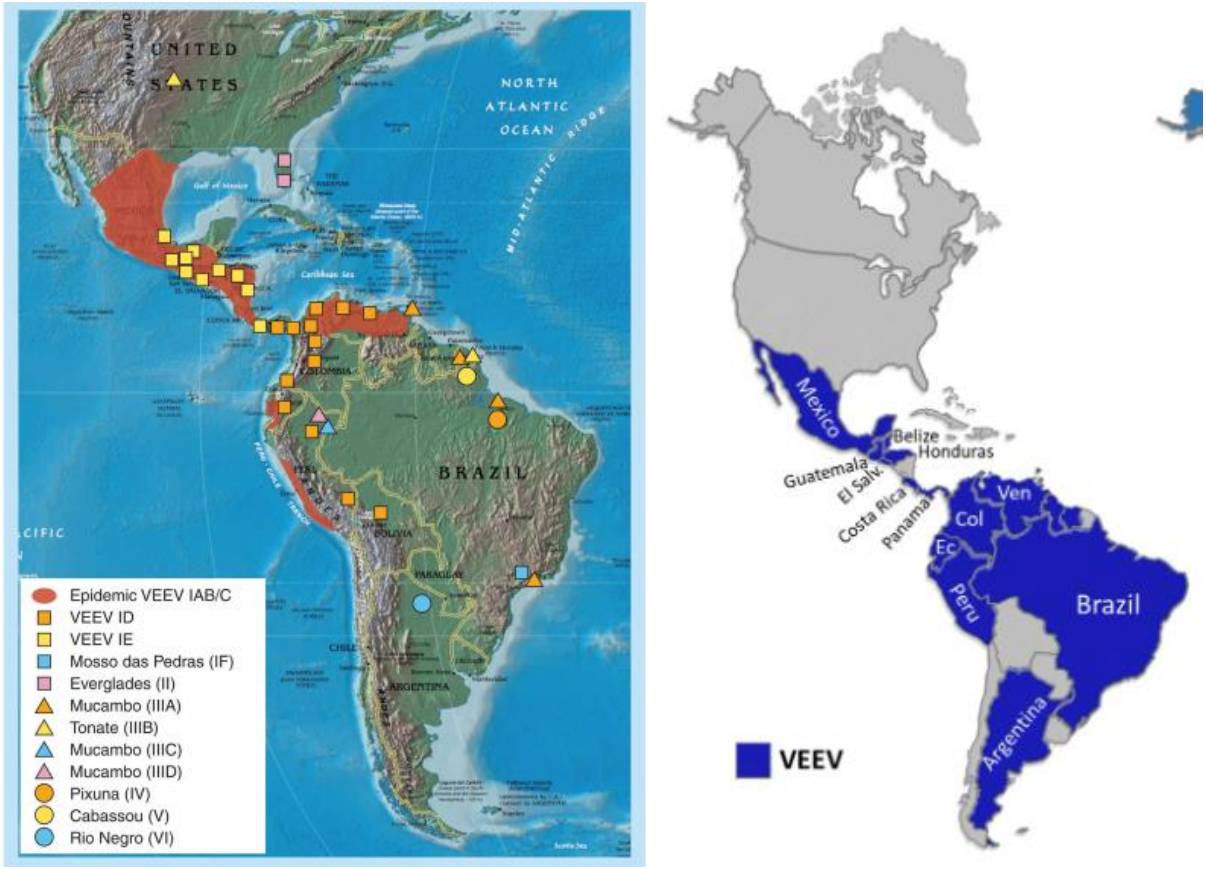


Figure 1.5 (A) Geographic distribution of epidemic/epizootic VEEV strains responsible for major outbreaks, and enzootic VEEV complex strains isolated principally in swamp or forest habitats as of 2011 (Aguilar *et al*., 2011) and (B) The geographical distribution of general VEEV in tropical America as of 2020 (Guzman-Teran *et al*., 2020).

VEEV has periodically emerged from its enzootic cycle since its isolation from an encephalitic horse in Venezuela in 1938 and is becoming increasingly important due to the possibility of the virus expanding

its geographical location into Europe. Multiple subtypes of the virus are categorized into the VEEV complex, all unique in their epidemiology, ecology and virulence in humans and equids (Aguilar *et al*., 2011). This complex comprises 6 subtypes of VEEV strains, with D, E and F of subtype I and II forming the enzootic strains of VEEV. Through their enzootic transmission, these endemic strains cycle between *Culex* (*Melanoconion*) mosquito vectors and rodents in forest and swamp habitats (Walton and Grayson, 1988), with subtypes ID and IE ranging from Bolivia to Mexico (Weaver *et al*., 2009). Recently, the IE strain has been discovered to cause encephalitis in humans, with outbreaks in 1993 and 1996 of IE VEEV strains in southern Mexico, whilst the IAB and IC epizootic subtypes have been the source of outbreaks for many years. These subtypes infect the invasive vectors *Aedes* and *Psorophora spp.* that transmit VEEV in agricultural settings, where the infected equids lead to frequent human infections (Weaver *et al*., 2009). In 1969 three VEEV epidemics originating in Ecuador, El Salvador, and Guatemala, resulted in 83,000 human cases and 403 deaths, followed by an estimated 70,000 equine deaths (Aguilar *et al*., 2011), when the latter two epidemics spread through Mexico. These epidemics tend to last months to several years before the virus exhausts the native population of equids or the remaining animals show immunity (Aguilar *et al*., 2011).

VEEV shows wide distribution across the Americas, particularly South America, with outbreaks in at least 12 countries (Figure 3). Infection from the VEEV complex result in symptoms similar to disease caused by DENV, leading to the number of cases likely to be underreported (Weaver *et al*., 2009).

1.1.7 Arbovirus life cycle

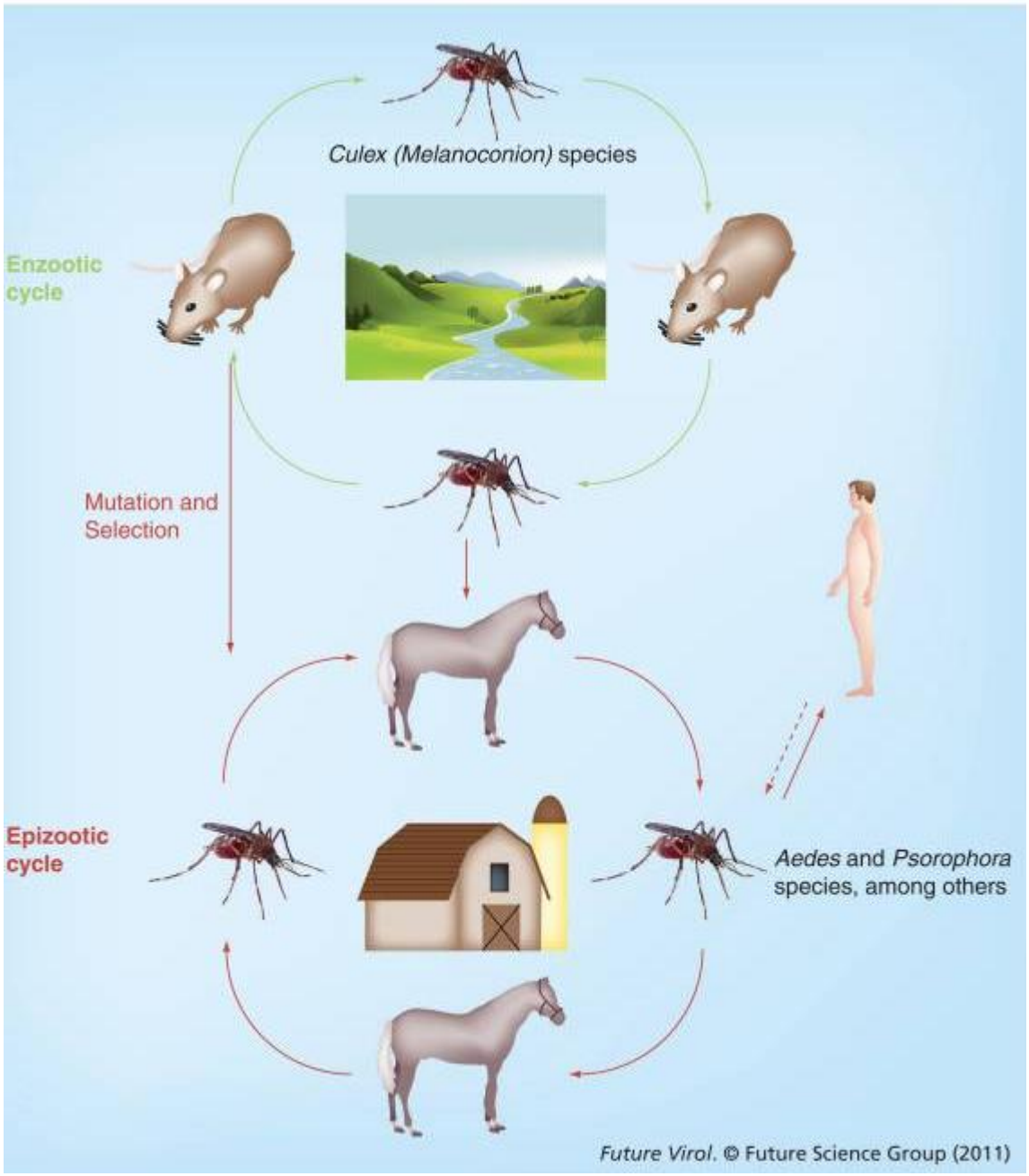


Figure 1.6 The lifecycle of endemic VEEV in the Americas (Aguilar *et al*., 2011). The *Culex* (*Melanoconion*) species of enzootic mosquito transmits VEEV between rodent populations. Mutations then occur in VEEV that allow it to infect equids and more invasive vectors, such as *A. albopictus* in an epizootic cycle. Proximity to equids and mosquitoes that bite both equids and humans allows for spill over into human populations.

Arthropod infection and transmission are key to the arbovirus lifecycle. The enzootic lifecycle of an arbovirus begins when an arthropod takes a blood meal from an infected vertebrate animal (Figure 1.7).

In the case of VEEV this would be a mosquito from the *Culex* (*Melanoconion*) genus and a small rodent. Once the mosquito takes an infected blood meal, the virus must infect the epithelial cells of the mosquito midgut to establish infection. The virus then disseminates from the midgut into the haemocoel, the circulatory system of the mosquito. From here, it infects all the other tissues, including salivary glands, ovaries, and the neural system. Once in the salivary glands, the virus can be transmitted to a vertebrate host the next time the mosquito takes a blood meal. As mosquitoes remain persistently infected with the virus, they can infect every vertebrate that they bite increasing the rate of transmission, assuming they take multiple blood meals (Franz *et al*., 2015). Mosquitoes are not the only vector, with four different orders of insects acting as vectors, whilst arbovirus hosts span a variety of animals including birds and mammals (Forrester *et al*., 2014).

1.1.8 The epidemic cycle of arboviruses

Occasionally, mutations occur that allow the virus to infect a different vector or host, resulting in an epidemic cycle of the virus. Single mutations in the E2 envelope protein of enzootic strains of VEEV increased its equine amplification viremia, leading to expansion of an epizootic strain capable of infecting humans (Anishchenko *et al*., 2006). Similar mutations allow the virus to infect invasive species of mosquito that act as a bridge vector to equids and humans (Brault *et al*., 2002). Often the *Culex* mosquito acts as an endemic vector for several arboviruses, including VEEV and West Nile Virus (WNV), maintaining a transmission cycle in the environment, whilst invasive mosquito species such as *Aedes* are more heavily involved in transmission of the virus to human populations.

Epidemic mutations have been recorded for several *alphavirus*es with CHIKV experiencing rapid geographic expansion since 2005 due to mutations that enhanced its infection of *A. albopictus* (Tsetsarkin *et al*., 2011, Tsetsarkin and Weaver, 2011). Arboviruses such as DENV and ZIKV are endemic in the Americas and infect humans at low continuous levels whilst others such as VEEV appear every 10-20 years and take thousands of casualties (Coffey *et al*., 2013). These viruses present a growing threat to populations across the world, and to understand how and when these epidemic outbreaks will occur, it is first necessary to understand how they maintain their enzootic cycles.

As will be discussed throughout the review, a multitude of mutations are generated throughout the replication cycles of these viruses. These enable the viruses to move into new vectors and hosts, establishing epidemic cycles and expanding geographical range. These mutations are key to the virus lifecycles and understanding how these enable the viruses to persist in nature is important to predict how and when they may outbreak into human populations.

1.1.9 *Alphavirus* intracellular lifecycle

When a mosquito ingests an infected blood meal, the virus enters the mosquito midgut. To be endocytosed into a host cell to initiate infection, the virus must first be recognised by a host cell receptor. The virus is surrounded by a host derived lipid bilayer, presenting 240 copies of two glycoproteins; E1 and E2 (Strauss *et al*., 1994). The E2 glycoprotein interacts with the host receptor for endocytosis, with the E1 glycoprotein potentially involved. Following this interaction and a conformational change of the E1 and E2 glycoproteins, endocytosis occurs, and the viral endosome experiences a drop in pH. This leads to conformational changes that form the late endosome (Kielian *et al*., 2010). During this process, the viral nucleocapsid (NC) is discarded into the cytoplasm. Within 5 minutes there is disassembly of the nucleocapsid and release of the genome ready for translation (Helenius *et al*., 1984).

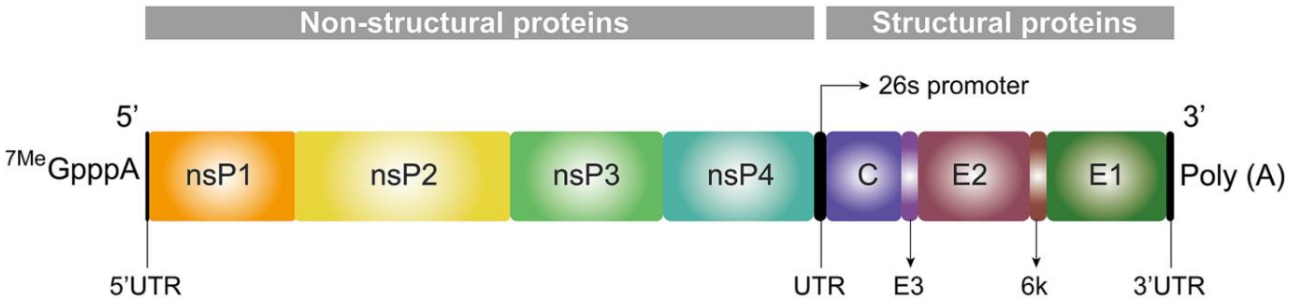


Figure 1.9 Venezuelan Equine Encephalitis Virus genome. The genome consists of the non-structural (nsP1-nsP2-nsP3-nsP4) and structural (Capsid-E3-E2-6K-E1) proteins in two open reading frames (indicated by the grey boxes for non-structural and structural proteins). The genome has a 5' untranslated region that is capped with a methyl residue at position 7 of the guanosine nucleotide. After nsP4, there is a UTR containing a promoter sequence for 26s subgenomic RNA. At the 3' end of the genome is a poly (A) tail (Han *et al*., 2023).

The *alphavirus* genome consists of two open reading frames (ORF). The first ORF encodes the four non-structural proteins: nsP1, nsP2, nsP3 and nsP4 (Figure 1.9). nsP1 initiates negative strand synthesis, has guanyltransferase activity involved in capping the mRNA and modulates the protease activity of nsP2. nsP2 amino-terminal region encodes the RNA helicase, and carboxyl-terminal encodes the protease previously mentioned. nsP3 is required for RNA synthesis, and nsP4 is the RNA-dependent RNA polymerase (RdRp) that carries out the actual replication. The second ORF encodes five structural proteins (Strauss *et al*., 1994). Upon nucleocapsid disassembly, the genome is released into the cytoplasm and the first ORF is translated by a host ribosome. This gives the nonstructural polyproteins P123 and P1234. nsP3 has an opal stop codon at its 3' end, readthrough of which occurs with 10-20% efficiency (Li *et al*., 1993), leading to predominance of P123 and lower levels of P1234. nsP2 is a viral protease responsible for processing these polyproteins and cleaves nsP4 from nsP3 leading to build up of P123 and nsP4, needed for synthesis of the negative strand genome (Jose *et al*., 2009). As the concentration of P123 increases, the nsP2 protease cleaves nsP1 and nsP2 from the P123 polyprotein. The individual nonstructural proteins nsP1, nsP2, nsP3 and nsP4, with the help of host factors, then form a complex called the replicase complex (RC) which remains bound to the endosomal membrane and is responsible for replicating the viral RNA to give both genomic and subgenomic strands of the whole viral genome (Kujala *et al*., 2001).

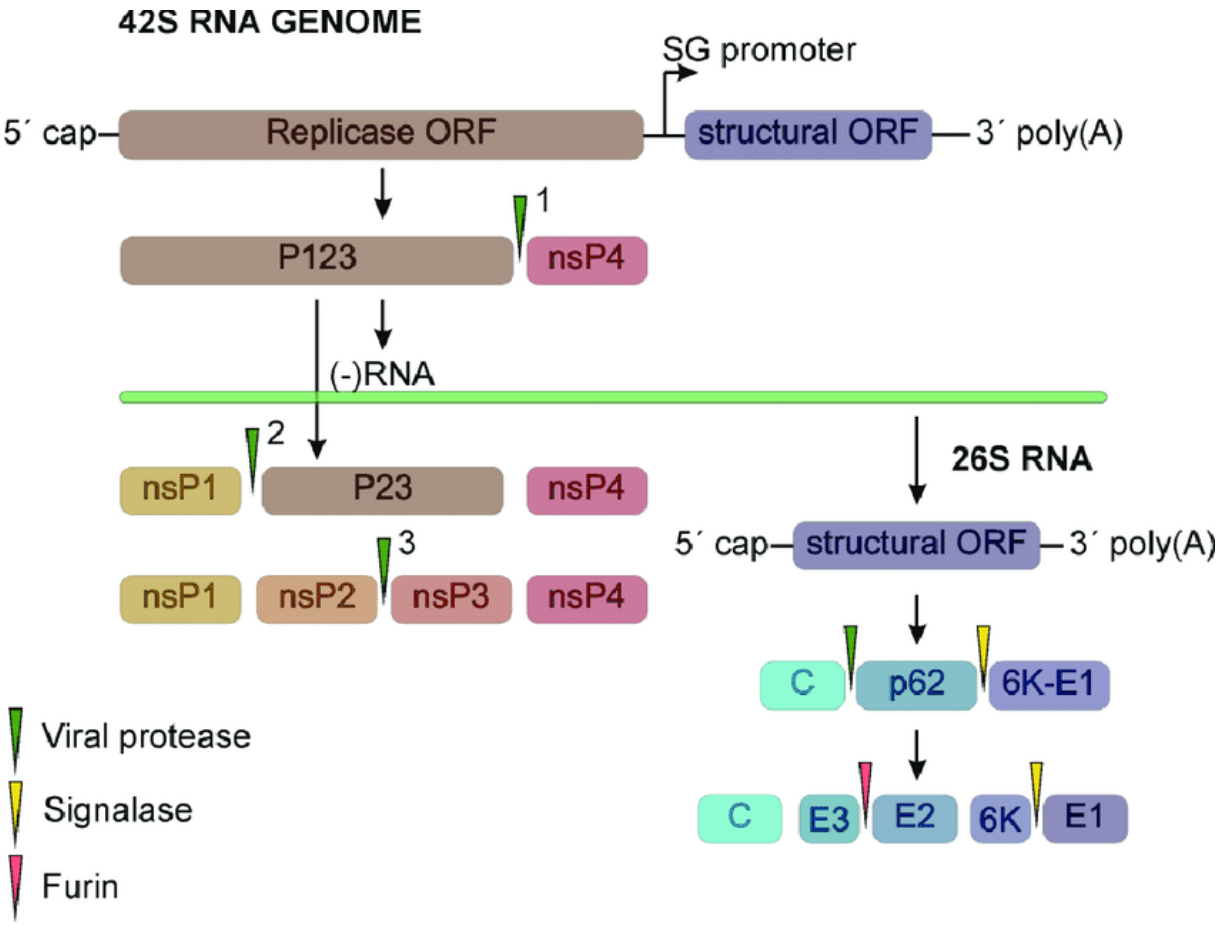


Figure 1.8 Alphavirus RNA genome organisation and polyprotein processing. The first open reading frame of the viral genome is translated into the P1234 polyprotein. The nsP2 viral protease cleaves the non-structural proteins within the polyprotein. The replication complex, now consisting of the individual non-structural proteins, replicates the genomic and subgenomic strands of the viral genome. The structural proteins are translated primarily from the subgenomic strands of the genome (Varghese *et al*., 2016).

Concurrently, translation of the structural proteins occurs primarily from the sub-genomic genome and this produces molar excess of the structural proteins, facilitating virion assembly. CP-E3-PE2-6K-E1 (Raju *et al*., 1991) which are processed and assembled at the surface of the host cell. The nucleocapsid contains one copy of genomic RNA and is formed of 240 copies of the capsid protein that mirrors the 240 copies of the E1 and E2 glycoproteins on the viral envelope. pE2 and E1 are matured in the endoplasmic reticulum, and pE2 is cleaved in the Golgi apparatus to give E2 and E3 (Jose *et al*., 2009). These glycoproteins then orient in the host cell plasma membrane. Nucleocapsids assembled in the

cytoplasm diffuse to the plasma membrane and bind cdE2 which provides the free energy to propel the capsid across the plasma membrane where they acquire the T = 4 lattice of the 240 copies of E1 and E2 (Strauss *et al*., 1994). The virus buds from the cell and is released to continue its infectious cycle in the next cell or tissue.

1.2 Role of the RNA-dependent RNA polymerase and the Quasispecies

RNA is more prone to mutations compared to its DNA counterpart due to several reasons; its single stranded state, use of uracil in place of thymidine, and cellular degradation enzymes such as exonucleases which cut the RNA. Importantly, the viral RdRp lacks a proofreading mechanism, harbouring a notoriously high error rate with 1 in 10,000 bases being mutated (Warmbrod *et al*., 2019). *Alphavirus*es such as VEEV are between 11-12kb, resulting in at least one mutation per genome. As a result, all progeny virions are different from the parental strain. Alongside rapid and exponential growth of the viral population, this leads to a vastly diverse cloud of variant genomes in a few generations (Forrester *et al*., 2014). Whilst there are many other factors involved in mutation, with elevated levels of reactive oxygen species and cellular metabolites in infection causing both the host cell and virus to mutate, the polymerase remains an important determinant of high mutation rate (Sanjuan *et al*. 2016).

Due to this low fidelity nature of the RdRp, the viruses are replicated into a quasispecies, a mutant swarm of diverse viral progeny all generated around the backbone of the consensus sequence (Lauring *et al*., 2010). This leads to thousands of distinct viral variants infecting an individual host (Lauring *et al*., 2010). The advantage of this generation of mutations is that some of the minority variants may be beneficial, allowing the virus to escape immune pressures or bind to different receptors in the next tissue. When encountering an immune defence, such as mosquito RNA interference (RNAi) that recognises and binds to secondary structures within the virus, a mutation in these sequences may allow the variant to evade recognition and move into the next cell and tissue and facilitate further replication.

1.2.1 The quasispecies consists of clouds of variants selected for as one

Importantly, variants within the quasispecies are not selected for individually. The consensus sequence of the virus represents the long-term dominant sequence of the virus, often isolated from nature, indicating little evolution in arboviruses over time. However, multiple mutations can be associated with a single gene. As many changes in the same gene would result in loss of genome viability, variants with the different individual beneficial mutations coexist synergistically within the viral swarm (Leeks *et al*., 2018). Quasispecies theory shows that clouds of these variants provide a phenotypic benefit to the entire quasispecies population, leading to selection of the whole swarm. Dominant sequences are surrounded by the cloud of mutant spectra and changes in selection pressures in the environment can bring forth a new cloud of dominant sequences more adept to survive specific pressures. This new cloud of dominant sequences is in turn surrounded by a cloud of mutants ready to become the dominant sequences, with the frequency of the consensus sequence diminished to suit environmental pressures (Domingo *et al*., 2019).

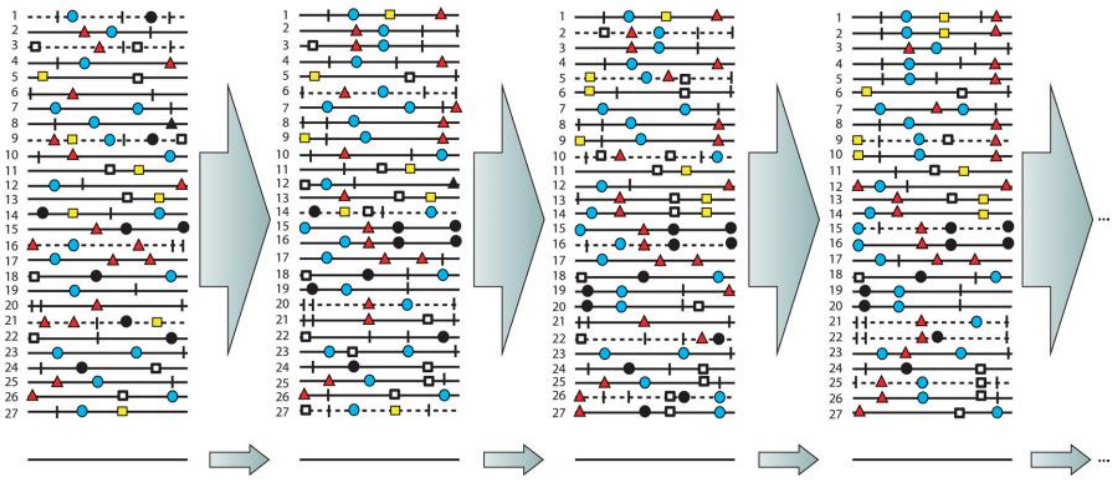


Figure 1.10 Schematic representation of the evolution (change in composition) of a viral quasispecies without modification of the consensus sequence (Domingo *et al*., 2012). Each of the four bars, moving left to right, represents a new generation of viral genomes. Mutations are represented as coloured symbols on the lines. Discontinuous lines show genomes that have acquired more than five mutations and cannot survive to the next generation. A constant evolution in the mutant spectrum can yield the same consensus sequence, shown as the line at the bottom of the figure with no mutations.

The generations of virus continue with each genome modified to produce one or several variant genomes, or unfit genomes are eliminated. Without a continuous elimination of unfit viral genomes, the range of genomic sequences would continue to expand, losing biological information and fitness (Domingo *et al*., 2012). This allows continued expansion of diversity whilst maintaining the mutant swarm around the consensus sequence. The quasispecies is fluid and over generations a vast range of variants are built during an infection. During a 6 week period of a CHIKV outbreak in the Americas, 25 CHIKV strains were taken from infected patients and deep sequenced. The study assembled the consensus sequences from each patient and identified a high-frequency of intra-host minority variants. Additionally, they found 5 synonymous consensus sequence changes compared to the strain reported at the start of the outbreak and a vast amount of high frequency minority variants scattered throughout the genome, unique to individual patients without affecting the consensus sequence (Stapleford et al., 2016). This corroborates the vast amount of variation found within the viral population during infection.

After mosquitoes ingested blood containing the WNV strain FtC-3699 isolated from wild-caught mosquitoes, unique populations of WNV with differing consensus sequences could be isolated from saliva, yet many of these novel consensus sequences did not correlate with known endemic or epidemic lineages (Grubaugh *et al*., 2017). This suggests that studies that focus on the consensus sequence in intra-host variation are limited, as the quasispecies is a constantly changing environment. Variant sequences retain dominance when an appropriate selection pressure arises. This is lost when further environmental pressures lead to the dominance of other more suitably adapted clouds of variants.

1.2.2 The consensus sequence remains dominant in long-term evolution

This is not to dispute the consensus sequence itself. Diversity from the consensus sequence is an inherent nature of the quasispecies that enables it to avoid extinction over temporal selection pressures. In the above WNV example, diversity in the saliva is important as it allows transmission into other species, yet it's rare to have populations of mutants found in enough frequency to displace the consensus sequence in the long term (Grubaugh *et al*., 2017). It's likely the study (Grubaugh *et al*., 2017) has documented individual clouds of mutants selected in that particular tissue and has named them

consensus sequences due to their dominance at the time of sampling. When three enzootic vectors; *Culex tarsalis*, *Culex quinquefasciatus* and *Culex pipiens* and the bridge vector *A. Aegypti* were infected with WNV, the majority of variants were not maintained across the tissues and the input haplotype remained dominant in most tissues, except for in *Cx. quinquefasciatus* (Grubaugh *et al*., 2016). The mutants within the quasispecies are generated with the long-term consensus sequence remaining the baseline sequence. This provides reasoning behind the lack of evolutionary adaptation seen in WNV and other arbovirus populations over time, despite the wealth of diversity generated. Nevertheless, the intra-host diversity generated through the quasispecies remains highly important in enabling viral survival in its lifecycle.

1.2.3 Variants within the quasispecies are fluid across infection of individual and separate hosts

Groups of minority variants can provide benefits for the quasispecies, collectively overcoming challenges faced in different tissues. Introduction of single nucleotide variants (SNVs) into the primary genome sequence may affect the ability of the virus to disseminate and transmit throughout the mosquito and other hosts (Vignuzzi *et al*., 2006). When individual SNVs were tracked from human plasma to mosquito abdomen and salivary glands, the human non-synonymous to synonymous (NS/S) SNV ratios were significantly higher than mosquito ratios for prM, E and NS1 genes, and the ratio for the C gene in humans was nearly double compared to mosquitoes, with hotspots identified in the envelope protein coding region in several isolates (Sim *et al*., 2015). Human antibody responses have previously been identified for these genes (Wahala *et al*., 2011), whilst the mosquito immune system lacks an antibody response. This suggests that the mutations generate and selected for within the quasispecies are specific for the selection pressures faced in different tissues, allowing the viral swarm to evade differing immune pressures between hosts and vectors. The NS/S ratio was significantly higher in NS3 in mosquitoes than for other viral genes. They further identified conserved spots in viruses isolated from mosquitoes in the prM and NS5 genes, which are functionally important. This may be where purifying selection is acting to target clouds of genomes with mutations that do not fall into this bracket of beneficial variants. These suggest that certain mutations are selected for within the quasispecies that enable the virus to traverse the differing selection pressures faced in the multiple tissues within their lifecycle. This highlights the need for diversity between the variants, showing how important the quasispecies is in terms of survival.

1.3 Viral fitness

1.3.1 A balanced fitness across the quasispecies prevents population extinction

Contrary to the description of Darwinian fitness, which refers to the ability of an organism to pass on its genes, the definition for viruses differs. Viruses, particularly RNA viruses, experience rapid generation times and a wide range of environments in the process. Therefore, viral fitness is considered their ability to produce infectious progeny within a given environment (Domingo *et al*., 1997). Viral fitness can be viewed as a mutational landscape, with hills and valleys representing variants of high and low fitness. This landscape is known as sequence space, and every point represents a viral sequence variant from the consensus sequence (Fig 1.11) (Kautz *et al*., 2018). This mutational landscape, mirroring the quasispecies, is constantly changing throughout the viral life cycle, with magnitude, location and frequency of peaks and valleys moving through time and space as the virus infects different tissues and hosts (Kautz *et al*., 2018). A landscape with steep hills and valleys, means dominant variants have drastically higher fitness resulting in a population more likely to become attenuated *in vivo*. Here, it is more likely that minority variants from the consensus sequence will have detrimental effects and prevents the quasispecies from maintaining overall fitness. A landscape with rolling hills is the optimal sequence space, named “survival of the flattest", as variants from the consensus sequence retain fitness, preventing detrimental effects on the mutant swarm. As arboviruses transmit between the cells and tissues in their insect and vertebrate host, their fitness can be visualised on two separate landscapes for arthropod vector and vertebrate host. Few fitness peaks on these two landscapes align given the drastic difference between the two animals (Coffey *et al*., 2013).

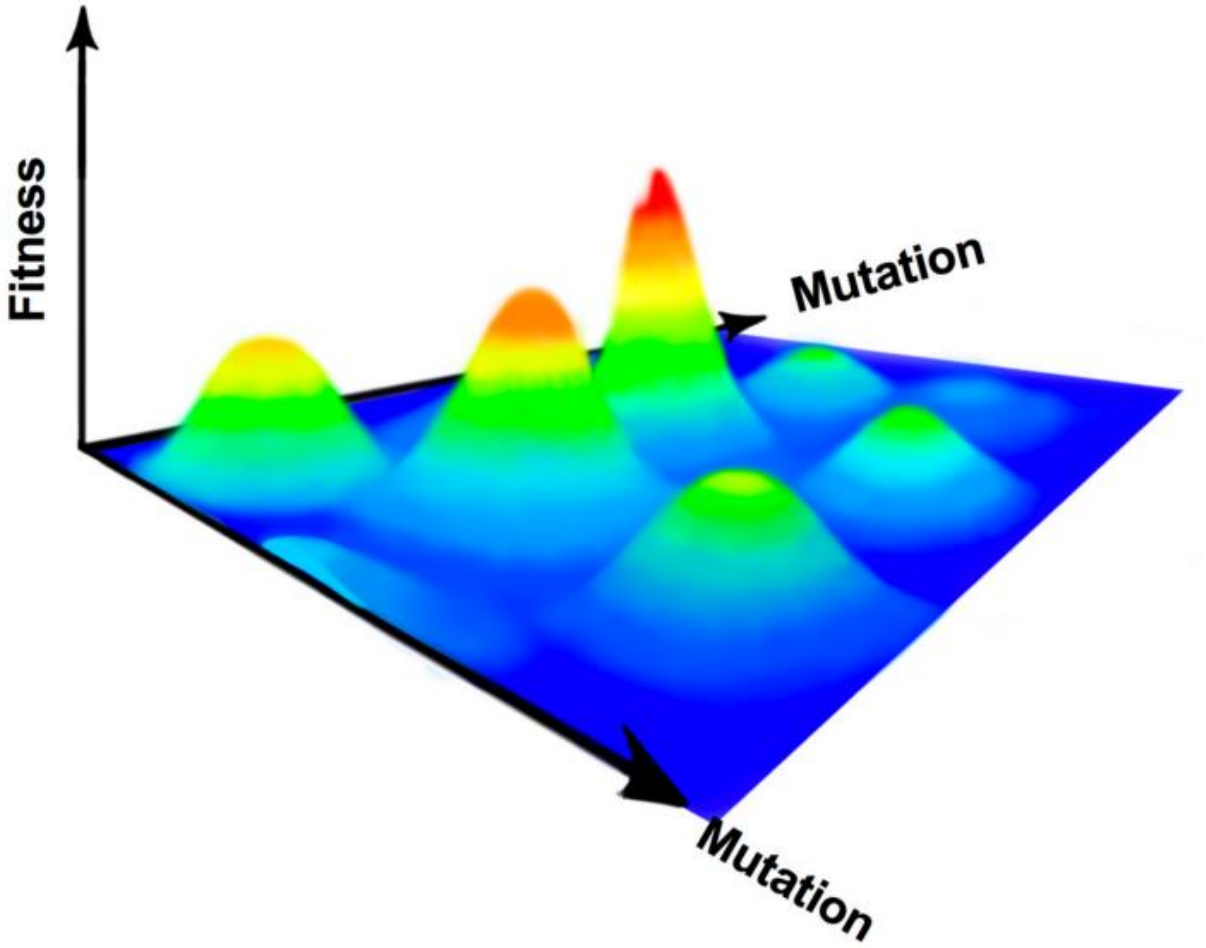


Figure 1.11 The RNA virus sequence space. As the viral population expands and mutates, the mutational landscape is explored to identify areas of higher fitness. It is hypothesised this sequence space is unstable and therefore optimal for RNA virus to exist in flatter areas of the space where fitness decreases will only be slight, hence, survival of the flattest (Kautz and Forrester, 2018).

When passaged repeatedly in a single host, VEEV showed significant increases in fitness, with no genome wide consensus sequence changes (Coffey *et al*., 2008), however CHIKV showed restricted fitness and diversity increases when alternatively passaged between different cell types. These experiments fit the trends seen in viral fitness, with gains of fitness after serial passage, and reduced fitness in hosts not previously passaged. After alternating passage, viruses show increases in fitness in the passaged hosts. Following alternate passage, Coffey (2011) found that variants that were beneficial

or neutral for CHIKV in both host cells and vector cells were maintained across the passages, indicating the virus can explore the sequence space through genetic drift, shifting dominance across the landscape of variants as selection pressures arise. After the initial fitness decrease in the new tissues, the fluid and cooperative nature of the quasispecies enables it to adopt survival of the flattest, ensuring long term sequences adapt to new hosts and tissues quickly.

1.3.2 Interactions within the quasispecies help maintain balanced fitness

Processes of complementation, cooperation, competition, and interference occur between clouds of variants. Much like bacterial biofilms, complementation occurs when clouds of variants produce a useful product that other variants are unable to produce (Arbiza *et al*., 2010). A poliovirus high fidelity mutant showed cooperation between subpopulations in the quasispecies allowed variants to infect the brain, whereas these same variants showed restricted diversity and inability to enter the brain and spinal cord when infected alone (Vignuzzi *et al*., 2006). Furthermore, coinfection can rescue mutants that are unable to succeed in infection alone (Turner *et al*., 2003), and it has been demonstrated that viral clones with very low fitness rarely become extinct, with compensatory mutations and cooperation rescuing unfit genomes (Escarmis *et al*., 2002, Lazaro *et al*., 2002, Lazaro *et al*., 2003). However, small amounts of cooperation can reduce the biodiversity by drastically increasing the fitness of the few variants involved in the cooperative processes. This can alter the balance of fitness, presumably focusing it onto a select few minority groups, making it less likely the quasispecies will have the wealth of fit variants to traverse the selection pressures of different tissues. When this cooperation involves most of the mutant swarm, both the balance of fitness and diversity are increased (Vignuzzi *et al*., 2006).

Due to the increased fitness of a beneficial variant, the variant can tolerate a greater number of deleterious mutations without becoming uncompetitive. Therefore, mutational load has a greater deleterious effect upon viruses with the beneficial variation than on those without, reducing the selective advantage of the individual groups of variants (Zhao *et al*., 2019). This feature may be a reason why beneficial variants can coexist with variants of reduced fitness with the overall fitness being maintained at a steady level. Controversial to this, in virus passaged in HeLa and A549 cells a frequent mutation

within the capsid region, VP3-E76G, was identified (Borderia *et al*., 2015). This mutation was the first to emerge by passage 11 and became the most abundant mutation found in the quasispecies. This increase in frequency was associated with increased viral fitness. Whilst this suggests that a single mutation led to a great increase in fitness, it's possible that *in vivo* other selection pressures would have suppressed this mutation leaving it to remain in minority variants and maintain the overall even fitness across the quasispecies.

1.3.3 Variants within quasispecies face competition and direct interference

Heterogenous viral progeny from single cells will move on to invade neighbouring cells leading to a level of competition among viral particles and genomes (Domingo *et al*., 2012). Viruses originating from different replicative units compete for invasion of tissues and organs during infection. Viral genomes unable to replicate themselves, such as defective interfering particles (DIPs), compete with the fully competent viral genomes. This competition can be for the ribosome binding site needed for translation or replicative proteins, that if used by the defective viruses reduce the number of competent viral genomes being transcribed. DIPs may also compete for wild type (WT) produced viral capsids resulting in fewer infectious WT viruses.

DIPs can also directly impede the replication and transmission of fit viral particles. Populations within the Vesicular Stomatitis Virus (VSV) quasispecies suppressed genomes with higher fitness than the general population when in isolation (De la Torre and Holland, 1990). Variants within Foot-and-Mouth-Disease virus (FMDV) antibody escape populations suppressed high fitness antigenic variants (Borrego *et al*., 1993), and pathogenic lymphocytic choriomeningitis virus (LCMV) was suppressed by non-pathogenic LCMV variants (Teng *et al*., 1996). This shows that the mutant spectrum controls the behaviour of different groups of mutants, allowing genomes with a lower fitness to gain a selective advantage if it maintains the overall fitness of the quasispecies. This prevents individual clouds of variants from gaining drastically higher fitness than the rest of the population, highlighting the fine balance of fitness throughout the quasispecies maintained by interactions between variants and mutational load.

1.4 Diversity and population bottlenecks

1.4.1 A bottleneck leads to the reduction of a viral population size

A viral population bottleneck is a transient reduction in the number of viral genomes within the population (Gutiérrez *et al*., 2012). This reduces the number of genotypes able to be replicated, whilst also impacting the viral gene copy number. The viruses also reduce the number of closely related genomes traversing the bottleneck by superinfection exclusion, where molecular mechanisms prevent infection by incoming viruses to save resources (Lee *et al*., 2005, Nethe *et al*., 2005, Ziebell *et al*., 2010). To summarise, a bottleneck during infection greatly reduces the size of the viral population.

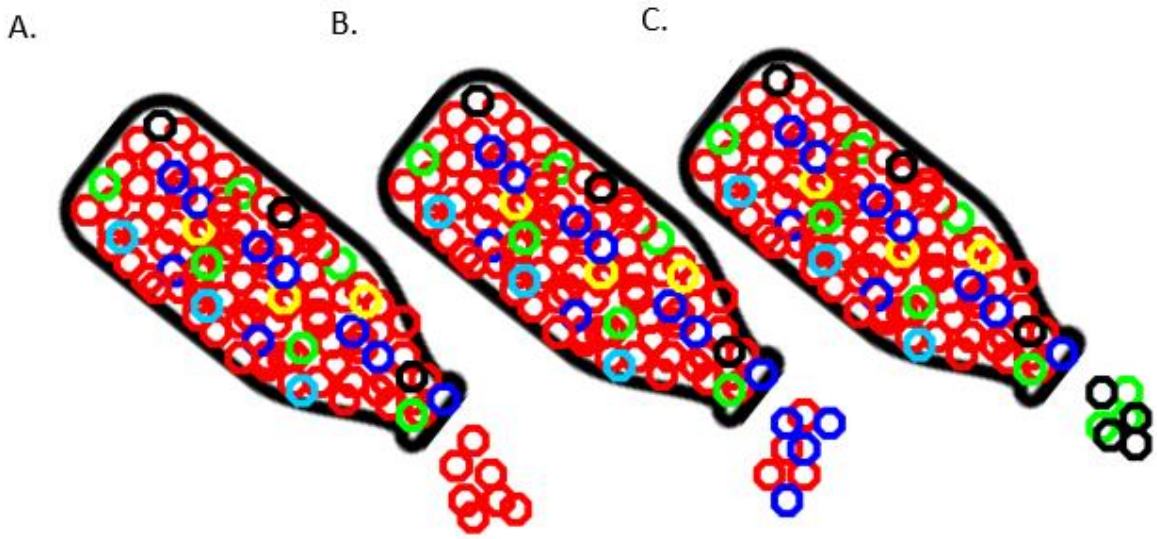


Figure 1.12 The effects of a bottleneck on the viral population (Forrester *et al*., 2014). A. Only the largest subpopulation of variants is maintained after the bottleneck, decreasing population diversity, B. variability decreases but a small amount of viral diversity is maintained, and C. The population diversity changes significantly when small subpopulations are randomly selected and the dominant sequence is lost.

There can be multiple outcomes of a genetic bottleneck on a viral population, including a decrease in viral variation whilst the largest subpopulations remains or a decrease in variation whilst a small amount

of variation remains (Figure 1.12). Genetic bottlenecks can also lead to dramatic changes in diversity due to random selection and extinction of the dominant sequence (Forrester *et al*., 2014). All can occur during infection and there's strong documentation of bottlenecks throughout studies of viral evolution. Arboviruses face bottlenecks during host-to-host transmission, when dealing with host defence mechanisms, in diverse cellular environments and anatomic restrictions such as the blood brain barrier (Vignuzzi *et al*., 2006). This reduces the size and diversity of the quasispecies, which is key for survival in the multi-host environment of arboviruses.

Viruses replicate in multiple cells and tissues within host and vector, which have been separated by millions of years of evolution. The mosquito vector is an important stage within the viral life cycle, and before the arbovirus establishes persistent infection, it must successfully infect the tissues during the extrinsic incubation phase (EIP). The virus must survive infection of the midgut, escape from the midgut into the haemocoel and infection of the salivary glands (Figure 1.13). Furthermore, the mosquito immune system is active and shows expression profile changes during infection (Nene *et al*., 2015, Waterhouse *et al*., 2007, Arensburger *et al*., 2007). These barriers impose selection pressures on the quasispecies acting as anatomical bottlenecks.

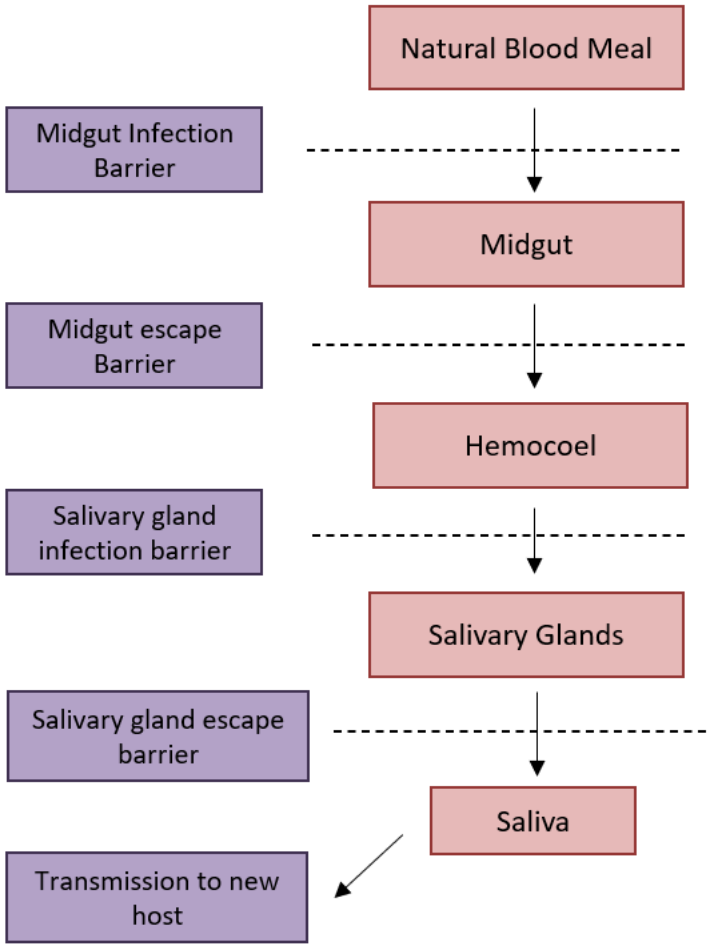


Figure 1.13 Infection bottlenecks between tissues in the mosquito. The mosquito takes an infected blood meal which goes to the mosquito midgut. From here, virus infects midgut cells and disseminates across the midgut infection barrier into the mosquito circulatory system, the hemocoel. Once in the hemocoel, the virus disseminates throughout the mosquito into the legs wings and most importantly, into the salivary glands, where it can then be transmitted from following another blood meal (Red Boxes). Significant barriers to infection have been identified at midgut infection, midgut escape, salivary gland infection, salivary gland escape and transmission to a new host (Purple Boxes). (Adapted from Forrester *et al*., 2014).

1.4.2 The virus faces multiple bottlenecks in the mosquito vector

Experiments using the epidemic IC strain of VEEV have shown that upon reaching the midgut of *A. taeniorhynchus*, an important epidemic vector of VEEV, the majority of epithelial cells are unable to be

infected reducing the number of the virus particles reaching the hemocoel (Kenney *et al*., 2012). However, it is important to note the vectors and strains being used. In the endemic vector Cx taeniopus, midguts there were infected with the endemic IE strain of VEEV showed no discrimination between midgut cells, suggesting long term virus-vector evolution enables the quasispecies to maintain clouds of minority variants adapted to infecting these cells.

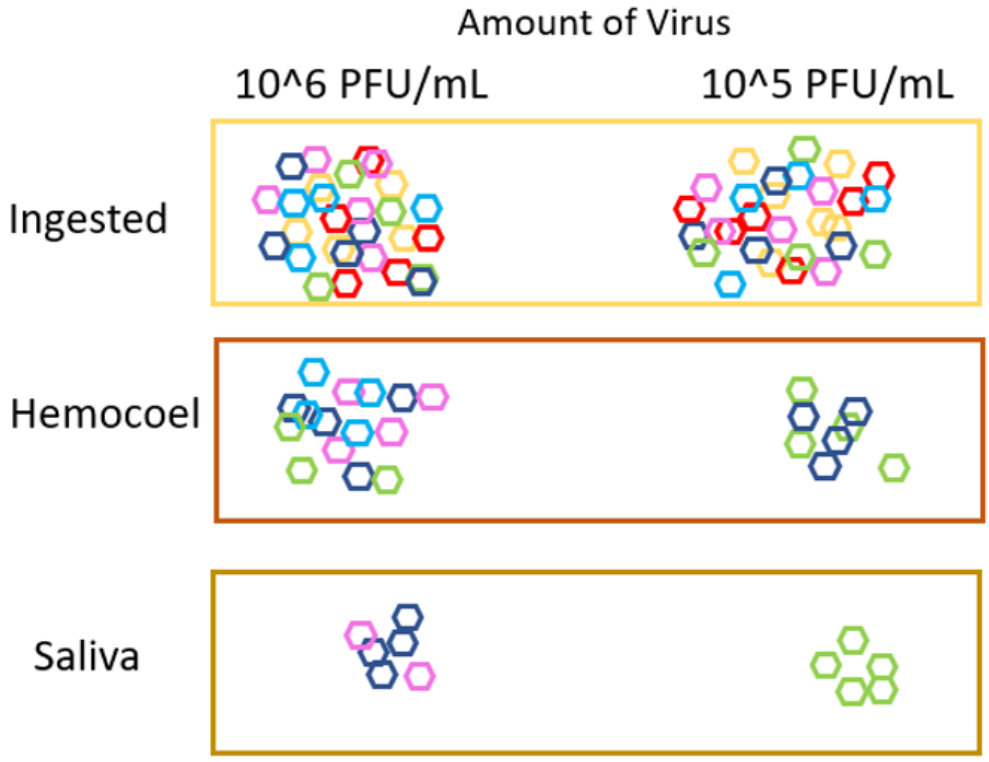


Figure 1.14 The changes in VEEV populations after anatomical bottlenecks in Culex mosquitoes. Eight marked viruses were fed to mosquitoes at high bloodmeal titres of 10^5 or 10^6 pfu/ml. All eight marked viruses were present in infected mosquito midguts, however there was a significant reduction in the number of marked clones in the hemocoel. Following intrathoracic injection to the salivary glands, a third bottleneck was detected. (Adapted from Forrester *et al*., 2014).

Additionally, the number of viruses reaching the midgut is dependent on how many viral particles the mosquito ingests and the viral titre in the infected mammals blood on which the mosquito feeds, with viremia titres often low in rodents and mosquito vectors having limited oral susceptibility to arboviruses (Deardorff *et al*., 2009, Forrester *et al*., 2012). *Culex* mosquitoes were fed a blood meal with 8 marked

VEEV clones in a high dose of 5.7 log10 pfu/ml and a low dose of 4.9 log10 pfu/ml and the number of clones that disseminated throughout the mosquito were measured (Forrester *et al*., 2012) (Figure 1.14). Less than 8 of the clone groups infected the midgut at the lower dose, which more accurately represented the viral titres seen in infection, corroborating that mosquito ingestion of virus may be a significant bottleneck to VEEV transmission in nature.

The midgut represents a bottleneck, with the number of VEEV viral particles escaping the midgut to the hemocoel reduced (Forrester *et al*., 2012). Of the 8 different variants ingested in the experiment, Figure 11 shows the number of variants is reduced upon sampling from the hemocoel, with a previous experiment indicating only 50 viral particles survived the transition (Forrester *et al*., 2012). At 14 days post infection, the number of clones in the mosquito bodies was markedly higher than that isolated from the legs and wings. The above factors generate a strong anatomical bottleneck at both ingestion of a blood meal by a mosquito and escape from the mosquito midgut into the hemocoel.

A third significant bottleneck the viruses face in the mosquito is infection of the salivary glands. In the marked clone experiments, oral infection of the mosquitoes did not show a significant bottleneck upon infection of the salivary glands. In case the bottleneck at the midgut was causing such a severe reduction in viral titre that a drop upon a salivary gland bottleneck was unnoticeable, 1-2ul of 5log10 pfu/ml VEEV was injected intrathoracically into the mosquito to bypass the midgut bottleneck. Following this, they observed a larger number of clones in the legs and wings than in the saliva. Whilst mosquito infection in nature occurs orally, further evidence has arisen to corroborate these observed bottlenecks in nature. To refer to the 2015 study, 267 SNVs in viruses isolated from the human plasma derived DENV populations, only 9.7% of these SNVs were then found in the mosquito tissues (Sim *et al*., 2015). 7.7% of SNVs identified in mosquito abdomen were found in the mosquito salivary glands. Additionally, when mosquitoes were fed WNV >90% of the SNVs detected in mosquito saliva were not found in other tissues (Grubaugh *et al*., 2016) and the mutant spectra of CHIKV transmitted by the mosquito is different from that it originally ingested (Stapleford *et al*., 2014). This suggests that the diversity of the viral

population is significantly altered by the bottlenecks it traverses throughout the mosquito, including into the salivary glands and that the swarm adapts to the selection pressures of the different tissues.

1.4.3 Repeated bottlenecks affect the population fitness of the quasispecies

Despite the continued diversity generated within the quasispecies, repeated bottlenecks are detrimental to the virus. FMDV showed fluctuating fitness for the 20-40 passages, with oscillating fitness around the mean which became decreased following the passages (Domingo *et al*., 2005). Following this, the mean fitness never increased to that observed before passaging, however the oscillations around it increased. This presents a fitness landscape moving away from survival of the flattest as previously discussed. A reduction in diversity could result in attenuation of the viral swarm through lack of replication in the midgut, or short-lived infections in the haemocoel that are neutralised by the host immune system and viral populations with lower diversity are less able to disseminate throughout the mosquito host, which is needed for transmission into the vertebrate host (Patterson *et al*., 2018).

However, this reduction in fitness does not adversely affect diversity in the long term. The greatest diversity of the viral swarm was seen in mosquitoes with successfully disseminated infection, opposed to those with unsuccessful infection (Patterson *et al*., 2018). When *Cx. pipiens* were infected with equal portions of 5 variants of WNV, the diversity increased in all tissues over the 21-day EIP, despite bottlenecks the virus must be faced with (Ciota *et al*., 2012). Furthermore, whilst bottlenecks influenced diversity following dissemination to the legs and wings, the diversity levels were restored 12 days post dissemination (Patterson *et al*., 2018). Given the survival and maintenance of these populations in enzootic cycles, the mutational frequency must be kept at a level that enables the fluid viral quasispecies to survive the endless bottlenecks it faces in those cycles despite the detrimental effect seen *in vitro*. Therefore, generation of diversity is needed to compensate for the reduction in both number and diversity in the quasispecies following a bottleneck (Manrubia *et al*., 2005). This diversity can take up to 4 days to rebuild, however, the remaining viral quasispecies will replicate and produce a diverse swarm before moving into the next tissue. To conclude, each bottleneck reduces the diversity and fitness, which then recovers once infection of new tissue is established.

1.5 Diversity and fidelity mutants

1.5.1 High-fidelity mutants generate fewer mutations than the wild-type

As previously discussed, diversity is an essential strategy for survival. Despite the evolutionary need for diversity and the low fidelity of the viral RdRp, studies have shown that any alteration of the fidelity of the RdRp results in attenuation and extinction of the population *in vivo*. Several papers have shown the detrimental effects that a high-fidelity (HF) mutant has on the survival of the virus. Through treatment with the nucleoside analog, ribavirin, a high fidelity G64S poliovirus mutant was isolated (Vignuzzi *et al*., 2006). The inoculated mice with the G64S mutant via intramuscular injection to allow quick access to the central nervous system, a common tissue infected by poliovirus. In the infected tissue, the HF poliovirus mutant lost its neurotropism, presented an attenuated pathogenic phenotype and despite replicating to the same level as the WT, was unable to compete with it in coinfection (Vignuzzi *et al*., 2006). Using chemical mutagenesis they expanded the quasispecies diversity and showed that the increased diversity restored the neurotropism and pathogenesis to the viral swarm. Furthermore, the 50% lethal dose for the mutant was 300-fold higher than the 50% lethal dose for the WT. The G64S poliovirus HF mutant was unable to create a diverse quasispecies with potentially beneficial mutations and had a greatly reduced fitness. To contradict this, the 3D-G64S HF poliovirus mutant was able to replicate in muscle and spread to the brain, however less often than the WT and with reduced pathogenicity (Pfeiffer and Kirkegaard, 2005). Whilst poliovirus is not an arbovirus, the quasispecies principle remains the same in the viruses, and similar experimental evidence suggests the effects are similar across both.

To corroborate the effect of reduced diversity, when mosquitoes were infected with uneven portions of 5 variants of WNV there was a decrease in variants isolated from the midgut compared to the original meal. This was repeated for the other tissues (Ciota *et al*., 2012). This implies the unequal number of variants may not be providing enough opportunities for beneficial variants to successfully traverse the bottleneck into the midgut, and then between tissues. Interestingly, next generation sequencing of a HF-CHIKV mutant showed increased diversity and replication levels in adult mice than the WT and caused

more acute levels of disease and higher peaks of disease severity. The study proposes this contradiction may be due to experimental differences, with adult mice used instead of neonates (Riemersma *et al*., 2019). Regardless, the results highlight how changes in fidelity can influence infection, and the detrimental impacts of some HF variants.

1.5.2 Low-fidelity mutants generate too many deleterious genomes

Mutations in the viral RdRp that reduce the fidelity of viruses show similar detrimental effects, highlighting the fine balance of fidelity. A study using VEEV showed that four mutations that decreased the fidelity of the TC83 vaccine strain of VEEV, altered the mutation rate in the WT virus 68U201 (Warmbrod *et al*., 2019). They inserted these mutations into the VEEV 68U201 strain backbone to create 3 RdRp mutants. One mutated in G7R, one a 3X mutant containing the three TC83 mutations, and one a 4X mutant additionally containing a mutation found in a low fidelity (LF) CHIKV strain. All mutants showed lower titres than the WT when tested against the nucleoside analog 5' fluorouracil, proving their low fidelity. As stated in other studies (Beaucourt *et al*., 2011, Kautz *et al*., 2018, Coffey *et al*., 2011), they found that all three RdRp mutants produced equivalent amounts of genome copies to the WT virus, indicating that fidelity mutants are capable of replicating to the same levels as the WT. However, whilst the G7R mutant increased diversity in three separate cell types, they found that the other two mutants generated significantly less diversity than the WT in U4.4 cells. As these mutants are attenuated in vertebrate models but show replication to the same levels as the WT, it can be hypothesised that the reduction in survival is owed to the lack of diversity generated by the mutants. In addition, differences in mutation frequency between the WT and CHIKV low-fidelity mutator strains became indistinguishable due to partial or total reversion (Rosen-Gagnon *et al*., 2014). This suggests that interactions within the quasispecies in mosquito cells act to reduce the detrimental effects of certain LF mutants. The generation of diversity is essential to produce minority variants and the quasispecies generated from LF variants may not be able to traverse the bottlenecks seen at ingestion, escape from the midgut, and entry to the salivary glands.

The lethal defection model states a slight increase in the level of defective genomes can lead to viral extinction (Grande-Perez *et al*., 2005). With the VEEV fidelity mutants, they found that despite equivalent copies of genome produced between the mutants and the WT, the mutants showed a reduced specific infectivity compared to the WT, with an SI of 6 x 10-4 for the WT, 9 x 10-6 for the G7R mutant, and 1 x 10-7 and 7 x 10-7 for the 3X and 4x mutants respectively. It could be summarised that the larger amount of non-infectious viral RNA seen with the mutants is leading to defective genomes. Corroborating the lethal defection model, these mutants were attenuated *in vivo*. A decrease in fidelity not only provides too many unfit genomes to traverse bottlenecks or be targeted for degradation, but also impact on competition within the quasispecies.

1.5.3 Defective genomes generated by low fidelity impact quasispecies fitness

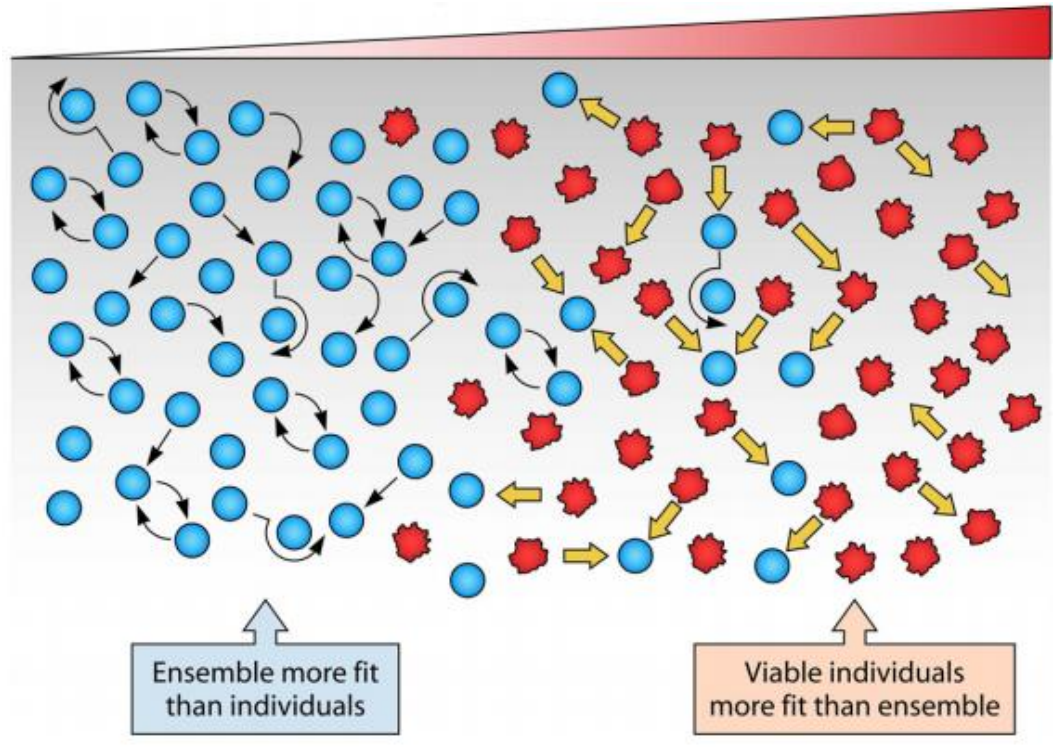


Figure 1.15 A schematic representation of interactions of complementation and interference within mutant spectra (from Domingo *et al*., 2012). The blue spheres on the left represent standard viral genomes where the mutation level is constant, and the red shapes represent DIP's. Thick arrows represent interfering interactions, thin arrows represent complementary.

In a population where the fitness of the ensemble is greater than that of individual viruses, increasing the mutation rate results in interfering interactions dominating. This results in individual variants becoming fitter than the ensemble (Domingo *et al*., 2012). During infection multiple viral genomes are competing for resources within the cellular environment. As the RdRp generates a wealth of variants, DIPs that encode non-functional proteins will arise that are able to use resources that other fully functional viruses need (Arbiza *et al*., 2010), much like the previously discussed cooperation. However, when the numbers are large as in this case, the DIPs use of resources interferes with replication of the viable viruses.

An increase in the mutation frequency not only prevents the viral swarm from overcoming bottlenecks as a result of too many unfit genomes, but it also increases competition with unfit genomes that are unable to be degraded and impair viable virus replication. RNA viruses replicate at the edge of the error threshold, with the fidelity of the viral RdRp crucial to maintaining the mutation level of the quasispecies. Both fidelity variants result in population extinction *in vivo*, showing the fine mutational balance across the diverse quasispecies. These factors enable the virus to replicate and transmit between multiple hosts and vectors, maintaining their enzootic cycle.

1.6 The mosquito immune system plays an important role in infection control

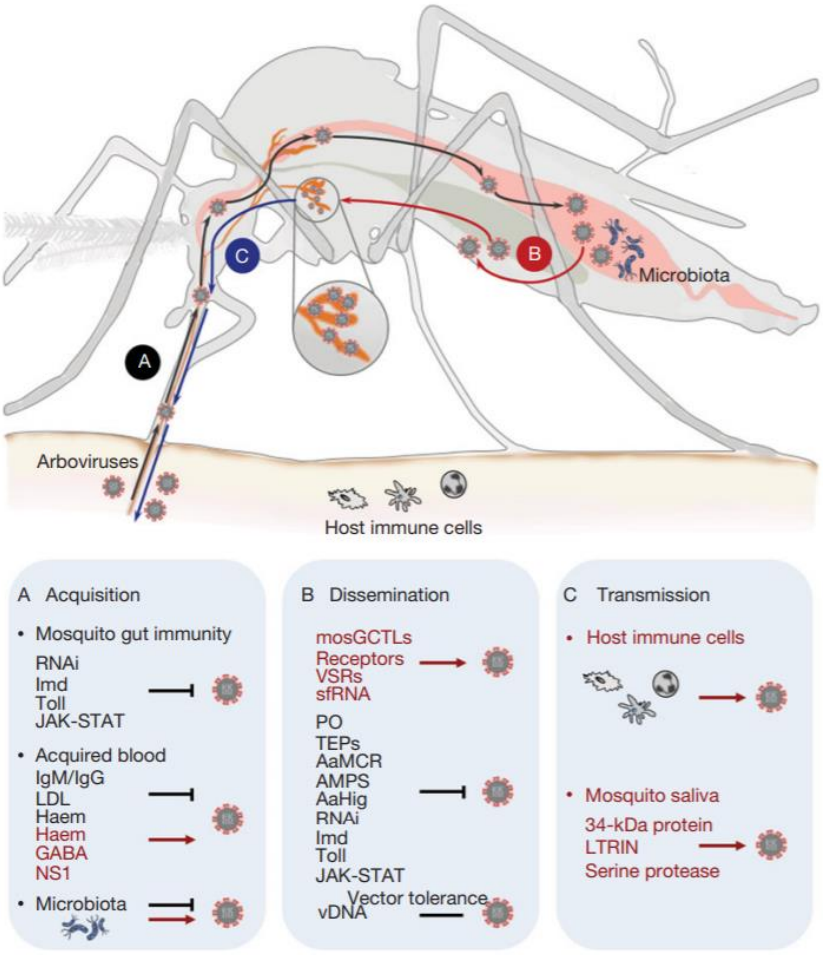


Figure 1.16 Schematic overview of the arbovirus lifecycle in a mosquito (Wu *et al*., 2019). The ability of a virus to infect a mosquito following an infectious blood meal is regulated by mosquito gut immunity, host blood components and the mosquito gut microbiome. A. The Imd, Toll, JAK-STAT and RNAi play a role in inhibiting viral infection. Host blood components such as immunoglobulins can hinder or enhance infection capacity. B. Arboviruses exploit vector/viral factors to facilitate systemic infection, such as viral suppressors of RNAi. Mosquitoes use strategies such as phenoloxidases, to interfere with viral infection. Virus-derived DNA affects tolerance of viral replication, enabling effective transmission. C. arboviruses are transmitted by mosquito bites to human skin. Intradermal immune cells are primary permissive targets in initial infection. Mosquito saliva has been proven to facilitate arbovirus transmission by using salivary proteins to enhance infection of host cells.

It must be remembered that observations can never be limited to one sole factor, and that all these processes are an incredibly complex interplay of different factors. The factors discussed above are finely tuned to deal with rapidly changing host environments, where the hosts and vectors themselves have co-evolved with the virus. Aside from the quasispecies maintaining a specific mutational frequency, the host cells themselves may play a role in the balance for persistence (Blair *et al*., 2015). The mosquito contains a multitude of antiviral defences, including the RNAi response, whilst vertebrates have both an innate and adaptive immune system that employs the likes of interferons, T and B cells and natural killer cells, providing a second challenge for the virus. Both organisms share the JAK-STAT and Toll pathway, all capable of antiviral defences (Cheng *et al*., 2015). Even components of a blood meal from a vertebrate host can alter infection in a mosquito. There are many factors involved in infection, and this multi-host cycle places constraints on the evolution of the virus with multiple bottlenecks throughout infection. Quick adaptation is needed to survive the differing host immune systems and bind to different cell receptors. Whilst generation of the quasispecies is due to the error prone nature of the RdRp, it inadvertently enables mosquito borne viruses to survive multiple bottlenecks in its vectors and hosts.

1.6.1 The JAK-STAT pathway

In the JAK-STAT pathways, a ligand binds to the Domeless (Dome) transmembrane receptor initiating a phosphorylation cascade that ultimately leads to the activation of specific genes in the nucleus. The JAK-STAT pathway has been shown to be a key antiviral defence in *A. aegypti*, with silencing of the negative regulator of the pathway, Protein Inhibitor of Activated STAT (PIAS), reducing DENV-2 infection and depletion of DOME increasing the viral load 3-fold by 7 days post infectious blood meal (Souza-Neto, Sim and Dimopoulos, 2009).

1.6.2 The Toll pathway

The Toll pathway is activated by pattern recognition receptors recognition of pathogen associated molecular patterns. This leads to activation through the MyD88 protein, amongst others to activate transcription in the nucleus of antimicrobial peptides. MyD88, GNBP and Toll5A (all genes of the pathway) were found to be upregulated in the salivary glands of *A. aegypti* following DENV infection

and silencing of MyD88 lead to an increase of DENV in the midgut, implicating the pathway in antiviral immune response (Luplertlop *et al*., 2011).

1.6.3 RNAi is an important antiviral defence in mosquitoes

Unlike mammals, mosquitoes lack immunoglobulin based humoral responses (Wang *et al*., 2006, Arjona *et al*., 2011). Instead, the mosquito has its innate immune system, with an essential antiviral defence among arthropods being interference RNA (RNAi). RNAi, consisting of siRNA, miRNA and piRNA, are stable processes by which small RNA molecules inhibit gene expression, or transcription, by targeting enzyme complexes to specific gene sequences or enzyme complexes methylating complementary sequences respectively.

There are three key aspects of the invertebrate immune response, the siRNA, miRNA and piRNA pathways. For invertebrates the most important anti-viral pathway is the RNAi pathway that is triggered by the presence of double-stranded RNA (dsRNA) during viral infection (Keene *et al*., 2004). RNA viruses naturally generate dsRNA intermediates during their replication, which provide a target for the RNAi pathways of invertebrates (Blair 2011). These RNAi pathways have been extensively studied in Drosophila and have been shown to be critical in maintaining the persistent infection of arboviruses in mosquitoes (Myles, Morazzani and Adelmn 2009, Liu *et al*., 2019). In the siRNA pathway 21nt small RNAs predominantly are recognised and processed in the cytoplasm of mosquito cells. Exonuclease activity is then used to destroy complimentary RNA. Whilst most studies of the siRNA pathway have been conducted in Drosophila, multiple studies have identified 21nt read lengths mapped to arboviruses in mosquitoes and mosquito cell lines (Morazzani *et al*., 2012, Sabin *et al*., 2013, Miesen *et al*., 2016, Ruckert *et al*., 2019).

The miRNA pathway uses Dicer-1, Loquacious and Argonaute-1 to generate 22nt miRNAs in the cytoplasm of cells, used to regulate posttranscriptional gene expression, and the PIWI-interacting pathway (piRNA) pathway processes viral piRNAs of 24-30nt in length that associate with members of the PIWI clade of argonaute proteins including Piwi, Aubergine and AGO3. This pathway is involved in gene and transposon silencing in both the nucleus and cytoplasm and is Dicer independent. Secondary

piRNAs are produced by the ping-pong pathway explained in the reviews below. These pathways are explained excellently in the following: Myles, Morazzani and Adelman, Liu *et al*., 2019, Santos *et al*., 2019, Ruckert *et al*., 2019 and Miesen *et al*., 2016)**.**

A multitude of studies have shown the importance of RNAi during viral infection of arthropods. Virus generated dsRNA is recognised by Dicer2 (DCR2) (Morazzani *et al*., 2012) and acts as a template for cleavage of the RNA through complementary base pairing to the original viral mRNA. (Franz *et al*., 2006, Campbell *et al*.,2008, Cirimotich *et al*., 2009) used a cDNA clone Sindbis Virus chimera, which had 500nt portion of DENV 2 inserted, showed that mosquitoes were less permissive to DENV 2 following the infection. As both Sindbis and DENV replicative intermediates are in the form of dsRNA, this was assumed to relate to the RNAi pathways. Likewise, DCR2 mutants were highly susceptible to infection with DCR2 depleted cells having a shorter EIP and a 10-fold increase in viral load of DENV 2 (Skalsky *et al*., 2010). Recent studies have shown that NS2A of DENV suppresses Dicer dependent siRNA *in vitro* and in context of DENV infection. This is also shown in the NS2As of other *flavivirus*es (Qui *et al*., 2020). Further evidence of the RNAi pathway's role in controlling infection is that depletion of AGO, which forms complementary bp with viral mRNA and cleaves the strand, increased viral titres (Morazzani *et al* 2012, Qiu *et al*., 2020, Costa *et al*., 2009). The fluid nature of the quasispecies results in clouds of beneficial variants able to overcome this defence, resulting in a persistent infection.

1.6.4 Host antiviral genes co-evolve with the viruses

Hosts and vectors within the enzootic cycle have co-evolved with the viruses for a long time, leading to arms race that minority variants within the quasispecies help maintain. Two important genes of the mosquito antiviral RNAi exo-siRNA pathway, *ago2* and *dcr2*, show rapid, positive and diversifying selection in *Aedes* mosquito. Blair (2015) found that *Aedes* populations with lower vector competence for DENV positively correlated with increased diversity indices for *dcr2*. SNVs at the *dcr2* locus for 100 individuals were tracked in an *A. aeqypti* population, identifying 17 genotypes from 10 different *dcr2* alleles and noted that these genotypes, rather than genotypes resulting from changes in flanking loci, correlated with resistance to DENV (Lambrechts *et al*., 2013). They proposed nonsynonymous

polymorphisms in dcr2 lead to differing binding affinities for particular dsRNA on a virus genotype-genotype basis. Furthermore, it's been postulated that selection pressure of siRNA targeting of viral genomes leads to generation of mutations within highly targeted sequences of the virus (Carine *et al*., 2012). This increases diversification and therefore allows the beneficial variants to escape the immune response. WNV also shows increased diversity in mosquitoes compared to its avian host which may be due to its need to evade the RNAi response in mosquitoes (Forrester *et al*., 2014). Given RNAi is one of the major antiviral responses the viruses face in the mosquitoes, this shows how the quasispecies uses selection pressures and beneficial variants to escape elements of the host and vector immune response.

Whilst this study is done in *Drosophila* rather than mosquitoes, *Drosphila* has proven to contain mosquito analogues and follow similar processes. DNA forms of non-retro virus Flock House Virus (FHV) have been detected and in cells treated with azidothymidine, a reverse transcriptase inhibitor which inhibits FHV DNA production, viral load increased 1,000-fold (Goic *et al*., 2016). FHV in cells lacking FHV DNA form or cells depleted in DCR2 failed to establish persistent infection or reached an acute stage of infection. Not only does this suggest that some cells produce DNA copies of RNA viruses to increase the success of the RNAi siRNA pathway. When ONNV virus infects *Anopheles gambiae* it takes 9 days for the virus to reach the salivary glands. When co-infected with dsRNA of the same virus that stimulates RNAi response, the infection slows and it takes longer for the virus to reproduce. When co-infected with RNA that suppresses the RNAi response the virus replicates faster and to higher titres, and leads to increased mosquito mortality (Keene *et al*., 2004). The above highlights the balance that is kept between the mosquito and virus with the RNAi response.

Therefore, this infers the importance of the fine balance between resistance and susceptibility needed with the RNAi antiviral pathway in arthropods. Whilst it could be presumed any increase would lead to attenuation of the virus, increased resistance to RNAi can lead to failed persistence and a highly pathogenic infection, which leads to attenuation and extinction of the viral swarm. It highlights the balance of mutations incorporated by the RdRp in its generation of minority variants and suggests that the addition of some mutations is not a stochastic process, rather a controlled process. The mosquito immune system is constantly evolving to keep up with the viral swarm, presenting an arms race between

the mosquito and viral swarm. Complete suppression of RNAi would lead to damaging pathogenesis and therefore be detrimental to the viral swarm, potentially leading to extinction. However, as discussed, the viral quasispecies is selected for as a whole, so an advantage of a multitude of different variants across the fitness landscape allows some sequences to escape the antiviral RNAi, whilst still providing enough RNA transcripts, unable to evade the RNAi response, to be targeted so as to remain in permissive opposed to virulent infection.

1.7 Beneficial Minority Variants aid viral infection

1.7.1 Minority variants enable quasispecies to infect multiple different tissues and hosts

Beneficial minority variants are key for quasispecies survival in vector and host infection. Given the multitude of differing tissues the virus must infect, the virus must be able to bind to multiple receptors to gain entry to multiple different cells. Whilst the consensus sequence may not enable this differential binding, minority variants provide the diversity that enables this.

Different organs and tissues have a different mutational spectrum (Patterson *et al*., 2018), and mutational adaptation occurs as viruses move into new tissues and hosts. One of the ways beneficial variants can benefit the quasispecies is by enabling modification of host cell tropism via receptor shifting. Single amino acid substitutions can occur in surface proteins that enable recognition by alternative receptors (Domingo *et al*., 2012). Alternatively, this can occur via recombination events that exchange molecules. In FMDV infection, the integrin recognition RGD amino acid triplet on the viral capsid is considered essential, however was not required when FMDV was passaged in BHK-21 cells. Instead, FMDV used heparan sulfate or another unidentified receptor to gain entry to cells during the passages (Acharya and Baranowski *et al*., 2003 and 2000). Amongst others, changes to the antigenic loop of the viral capsid protein were identified when a swine strain of FMDV was replicated in guinea pigs (Nunez *et al*., 2001). Interestingly, these variants remained when the virus was then alternatively passaged in swine, alluding to quasispecies memory, where low frequencies of specific clouds of variants are maintained. These examples indicate groups of variants within the quasispecies can alter receptor recognition, providing a selective advantage in new cell types.

Structural studies of paramyxovirus surface glycoproteins and receptors found at least 3 different mechanisms for functional interactions, showing how variants aid the infection capacity of the quasispecies (Bowden *et al*., 2010). However, these changes may inadvertently bring fitness losses (Reiter *et al*., 2011). The high number of amino acid substitutions, mutations and range of the type of mutations involved in creating a new receptor binding site may negatively impact other aspects of the virus mutant cloud, reducing the overall fitness of that subset of viruses. This may be where cooperation or complementation come into play, allowing these clouds of minority variants to progress infection to the new cell.

The combination of complementation and deletions can lead to the formation of segmented forms of the full-length genome, that can show increased fitness in each environment (Domingo *et al*., 2012). When FMDV was passaged for over 400 passages in BHK-21 cells, after the initial increase in fitness, the viral population changed drastically, with the full-length consensus sequence no longer at detectable frequencies after passage 240 (Garcia-Arriaza *et al*., 2004). Instead, genomic forms of the virus with internal deletions had replaced the full-length genome. These deletions were found in the L- coding region and the capsid protein. They determined that it was the new stability of the capsid that gave the selective advantage over the standard genome size, and others further hypothesised that the shorter RNAs showed increased stability due to relaxation of packaging constraints that hindered the full length genome in that specific environment (Mateo *et al*., 2008, Ojosnegros *et al*., 2011). This shows that within the quasispecies mutant clouds assume dominance based on fitness selected for by the environment. When these genomes were passaged at low MOI, the full-length consensus sequence was rescued by recombination between the shorter genomic forms. This shows that recombination actively acts between variant clouds within the quasispecies to alter sequences when the environment allows.

Diversity allows the quasispecies to evade immune responses of the hosts and vectors. Not only can beneficial variants reduce the impact of the siRNA response to gain persistent infection, but previous sections alluded to how beneficial variants can evade host antibody responses; The human NS/S SNV ratios were significantly higher for prM, E and NS1 genes, genes involved in the human antibody

response, than in mosquitoes. Furthermore, the ratio for C in humans was nearly double that of C in mosquitoes, with hotspots identified in the envelope protein coding region in several isolates. Variants with diversity in these genes have an advantage in evading the antibody response.

1.7.2 Defective interfering particles provide variation within the quasispecies

Another mechanism of minority variant generation within the quasispecies is through the production of DIPs. These are truncated forms of the virus that are unable to replicate alone, however upon coinfection with the WT virus they use the functioning replication machinery to produce defective genomes and particles. Large sections of the parental genome are deleted, leaving the remaining sub-genomic fragments to be replicated and maintained by complementation and coinfection (Li *et al*., 2011). When VSV was deep sequenced following infection of BHK-21 cells, there was simultaneous emergence and enrichment of at least two distinct truncated-genome variants of the virus that co-existed with the full-sized particles in infection (Timm *et al*., 2014). As mentioned previously, cooperation, and in some cases interference, enables the DIPs to replicate via replication machinery or other useful products of the fully functioning viral particles. A deletion in the VEEV 6k protein resulted in higher diversity (Forrester *et al*., 2011), and a deletion in the VEEV capsid protein that resulted in truncation was repeatedly isolated throughout mosquito infection (Patterson *et al*., 2018).

Negative stranded viruses generate DIPs more readily than positive stranded viruses such as *alphavirus*es and *flavivirus*es. In DENV infection of humans, sub genomic DENV RNAs were isolated that represented the four subtypes of DENV and fulfilled the DIP criteria are arose in *de novo*, rather than being maintained in the transmission cycle (Li *et al*., 2011). They hypothesised that DENV DIPs and full-length virions with defective genomes are unable to generate infectious progeny whilst interfering and competing with the WT. This imposes a fitness burden on the population and subsequently reduces the severity of the disease (Aaskov *et al*., 2006, Stoddard *et al*., 2009). Human mobility has been shown to be critical to maintenance of arbovirus transmission cycles (Stoddard *et al*., 2009), and the reduced infection led by DIPs has the potential to further human to human transmission. The DIPs may also enable the quasispecies to evade the RNAi, leading to a persistent infection. Whilst

the siRNA response in mosquito cells is occupied by DIPs, infectious progeny infect further cells, corroborating the potential benefits of DIPs to viral infection.

It must be remembered that a viral particle may not be capable of initiating infection and producing progeny at a given time, but the same particle may immediately be exposed to a different environment that triggers its replication. When the appropriate environment arises, groups of these minority variants will then be selected to gain entry to new cells and tissues, allowing the quasispecies diversity to be rebuilt in the next tissue following the bottleneck.

1.8 Conclusion

To conclude, arboviruses including both alpha and *flaviviruses* are important viral pathogens with expanding geographical range that cause significant mortality. These viruses have a multi-host cycle that requires them to infect multiple different tissues and cells. Due to the error prone nature of the RdRp, the viruses are replicated into a vast mutant swarm known as the quasispecies. The viral progeny are all genetically related variants containing one or a few mutations each, leading to a huge amount of diversity within the swarm. This diversity of the quasispecies is essential for viral survival. In both intra-host and inter-host infection the viruses are faced with multiple different tissues, each with their own selection pressures and barriers to infection. In the mosquito vector of *alphaviruses*, infection of the midgut, escape from the midgut, and infection of salivary glands have been shown to act as anatomical and genetic bottlenecks, reducing the size and diversity of the viral population. Variation within the quasispecies enables the swarm to traverse these bottlenecks by binding to alternative receptors or evading the host immune system. after the bottleneck has been traversed, the RdRp rebuilds diversity through its error prone replication, allowing the viral swarm to infect new tissues and traverse future bottlenecks.

Whilst the RdRp is key to maintenance of the vast diversity seen across the quasispecies, interactions and purifying selection lead to a fine balance of the resulting effects. To maintain a balanced fitness, the

effects of the fidelity of the polymerase are tightly regulated from a population level, with any increase or decrease leading to extinction of the viral population. Complementation, cooperation, and interference between the variants, coupled with mutational load ensures that beneficial variants or unfit viral genomes do not have drastically higher or lower fitness than the population. This maintains a survival of the flattest, a fitness landscape that ensures the variants from the consensus sequence have vastly altered fitness from the consensus sequence which would ultimately result in their extinction. Finally, genomes containing too many mutations are purified out, preventing their accumulation. This balance of fitness ensures the diverse population is continually able to produce infectious progeny in their given environments.

The beneficial variants generated within the quasispecies are key to maintaining fitness and traversing bottlenecks during transmission. The variant enables binding to alternative receptors to gain entry to new cells, altered recognition by immune cells and evasion of responses such as the RNAi, and modulation of the immune response to prevent severe infection allowing further spread. These beneficial minority variants are generated throughout infection as a by-product of the quasispecies, and certain variants of VEEV are found to be continually selected throughout mosquito tissues. Given their importance to infection, it is imperative that we understand how these variants contribute to infection.

RNA viruses pose a continual threat to human populations, increasing as populations expand and the vector range widens. Lack of treatments mean these viruses are hard to control and outbreaks result in a huge number of fatalities. These viruses are continually circulating in the environment leading to an increasing chance of an epidemic outbreak that will affect humans and livestock. Variants within the quasispecies are essential to transmission of the viruses and understanding how the variants contribute to the above factors is crucial to controlling and understanding how these outbreaks occur.

Chapter 2 Methods

2.1 Cell Culture

African Green Monkey Kidney Cells (Vero) were obtained from ATCC through MERCK. Mouse microglial cells (BV-2) were obtained from Dr Ruoli Chen and Mr John Bennett of the Keele chemistry department. C636 and U4.4 cells were obtained from Pirbright Virology Institute. Vero and BV-2 cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 2.5ug/mL gentamicin at 37 degrees with 5% CO2. U4.4 and C636 cells were maintained in EMEM supplemented with 10% FBS, 2.5ug/mL gentamicin and 2.5% tryptose phosphate at 30 degrees with 5% CO2. Cells were rescued by thawing quickly at 37 degrees followed by addition to 5mL of media. This was centrifuged at 500rpm for 5 minutes and supernatant removed. Cell pellet was resuspended in 5mL of fresh media (appropriate to the cells) and added to a T25 vented tissue culture flask. Cells were transferred to a T75 or T180 and passaged as required, 2-3 days for vero and BV-2 and 5-7 days for insect.

2.1.1 Cell Passaging

Vero and BV-2 cells were passaged every 2-3 days when fully confluent or as required. All media was removed from the flask and cells were washed once with 5mL of PBS (10mL for a T180 flask). 1 or 2ml trypsin was added (depending on flask size) and incubated for 5 minutes or until the cell monolayer lifted. 4 parts media were added to inactivate the trypsin. All media was removed and cells were split as needed. 8-10mL media was added to T75s and 20mL media to T180s. C636 and U4.4 cells were scraped when fully confluent and split as appropriate. For treatment experiments, C636 and U4.4 were passaged three times in GLY or TRI at 0.5mg/L.

2.1.2 Cell Culture Plates

Cells were harvested and 20uL mixed with 20uL trypan blue for counting using a hemocytometer. Unless specified otherwise, cells were seeded in well plates at 2.5 x 10^5 cell density. Mammalian cells were

seeded 24 hours before needed, insect cells were prepared on the day required. Viral infections were carried out within the plates for mammalian cells or in suspension for the insect cells.

2.1.3 Cell culture flask infection for viral stock generation

Cells were prepared as described above. Media was removed from mammalian cells and virus was added. For insect cells, virus was added to the cell suspension. These were incubated for 1 hour with gentle rocking every 15 minutes. Media was added to cells to fill the flask and incubated per specific virus. Once the incubation period was finished media was harvested from the flask and centrifuged for 5 minutes at 2.5 x G. supernatant was aliquoted into tubes and stored at -80.

2.1.4 Electrocompetent cells

Chemically competent cell stocks were generated from Thermofisher TOP10 chemically competent cells. 5mL of LB broth was inoculated with one colony of *E. coli* and grown at 37 degrees in a shaking incubator at 225rpm. The following day, 100ml of LB broth was inoculated with 1ml of the culture and grown at 37 degrees with shaking until an OD600 of 0.4 was reached (2-3hr). The culture was incubated on ice for 15 minutes. Cells were kept chilled for the rest of the protocol. The culture was split between two pre-chilled 50mL tubes and centrifuged at 2700 x g for 10 minutes at 4 degrees. The supernatant was removed and the cell pellet gently resuspended in 10ml of sterile 100mM MgCl2. This was incubated on ice for 10 minutes. The previous centrifugation step was repeated. The supernatant was removed and pellets resuspended in 1ml of sterile CaCl2 and combined. 500uL of sterile 60% glycerol was added and swirled to mix. 100uL aliquots were prepared in pre-chilled microfuge tubes with chilled tips and snap frozen using liquid nitrogen. Cells were stored in the -80 freezer.

2.2 Viruses

Plasmids for Venezuelan Equine Encephalitis Virus (VEEV), Chikungunya Virus (CHIKV) and Negev virus (NEGV) were obtained from the University of Texas Medical Branch on filter paper. Zika Virus (ZIKV) was obtained from the University of Glasgow. Plasmids were eluted from filter paper into 50uL of RNase/DNase-free water and transformed into chemically competent *E. coli* as described below.

2.2.1 Transformation

Transformations of viral plasmids were done using the chemically competent E. coli cells generated in the previous step. This was done according to the thermofisher one shot chemically competent TOP10 cells protocol: The vial containing the plasmid was centrifuged and briefly placed on ice. A 50-100uL aliquot of competent cells were thawed on ice. 1-5uL of the ligation reaction was added to the competent cells and mixed by gentle tapping. The vial was incubated on ice for 30 minutes. This was then incubated at 42 degrees in a water bath for 30 seconds with no mixing or shaking and placed on ice. 250uL of pre-warmed S.O.C media was added and the vial was placed on its side in a shaking incubator (225rpm) at 37 degrees for 1 hour. 20-200uL of the culture was added to a neat LB agar plate and an LB agar plate containing 100ug/mL or 50ug/mL of the selective antibiotics, ampicillin or kanamycin respectively. The plates were incubated at 37 degrees overnight. Colonies were tested by PCR to confirm presence of plasmid.

2.2.2 Transfection of plasmids into vero cells

Preceding transfection, each viral plasmid was digested using NEB EcoR1-HF as described in their protocol. 5uL of plasmid DNA was added to 5uL cutsmart buffer, 1uL EcoR1-HF, 5uL Antarctic phosphatase buffer, 2.5uL Antarctic phosphatase and 31.5uL H2O. The DNA was ethanol precipitated overnight with $1/10^{th}$ volume of Sodium Acetate, 150uL ethanol and 1uL of glycol blue to visualise the pellet. The DNA was pelleted for 15 minutes in a microcentrifuge at top speed. The supernatant was removed and any residual fluid was removed with a fine-tipped pipette. Pellet was allowed to air dry for 5 minutes. Pellet was resuspended in 10uL dH2O.

The protocol for the mMessage mMachine was followed as per manufacturers instructions. 4uL of plasmid from the previous step was added to 10uL 2Xntp/cap, 2Ul 10X Reaction Buffer (thawed to room temperature before use), 2uL GTP and 2uL Enzyme mix. This was incubated at 37 degrees for 1-2 hours.

For the transfection, 3.75uL lipofectamine was added to 125uL of serum free DMEM and incubated for 10 minutes at room temperature. 10uL of mRNA from the mMessage step was diluted into 250uL of

DMEM. 125uL of this was then added to the lipofectamine mix and incubated for 5 minutes at room temperature. 250uL of this final solution was added in a drop wise fashion to an individual well in a 6 well plate. Plates were left as per the time required for each virus before the full 2mL was harvested from each well and plaque assayed.

2.2.3 Venezuelan Equine Encephalitis Virus

Clones were rescued as described above. Wild-type enzootic subtype strain IE 68U201 was cloned from the 9980T plasmid using the NEB Q5 Mutagenesis kit to clone outwards from an additional T inserted at position 9980T in 68U201 (put in cloning kit protocol here- qPCR protocol). The primers used for this are Q5_SDM_5/5/2022_F 5'-TAGTCGTGGCCGGCGCCG-3' and Q5_SDM_5/5/2022_R 5'-AAAAAGGCACAACGCAACACACACATTTCAGC-3. Resulting plasmids were transformed into *E. coli* and screening with Illumina sequencing to confirm the deletion and generation of the 68U201 wild-type. qPCR primers were as follows Fwd: 5'-TGTCCATGAGCGGTAGTTCT'3 and Rvs: 5'-GGTATGCCCTGCACTGAGAT-3'.

2.2.4 Chikungunya Virus

Chikungunya virus plasmid OPY2006 La Reunion ECSA strain was used in these experiments. Virus was rescued as described above. Infections were carried out for three days. If needed, qPCR primers sere as follows: FwD 5'-GAACACTGACCTGCTGCTGTCTATG-3' and RvS: 5'-CTGCTGTCATGACCTCGTATCCAC-3'.

2.2.5 Zika Virus

Zika virus strain PE243 was obtained from the University of Glasgow. Stocks were generated following incubation in vero cells for 5 days. Plaque assays were incubated for 4 days. qPCR primers used were as follows Fwd: 5'-TCAACGAGCCAAAAAGTCAT-3' and Rvs: 5'-CAGGTCCCACCTGACATGC-3'.

2.2.6 Negev Virus

M30957 isolated from *Culex coronator* in Harris County, TX, USA. qPCR primers used were as follows Fwd: 5'-CCACAACTCACCGAAGACG-3' and Rvs: 5'-GCGGCGAATGATTTAACCCC-3'.

2.3 Infections

2.3.1 Cell culture Passage Infection

Cells were prepared as described in section 2.1.1. Media was removed from mammalian cells and virus was added as per experimental requirements. For insect cells, virus was added to the cell suspension. These were incubated for 1 hour with gentle shaking every 15 minutes. For mammalian cells, the cell monolayer was washed with PBS twice before media added to each well. For insect cells, these were centrifuged at 5000rpm, and the supernatant removed before washing once with PBS. The pellet resuspended in the appropriate amount of media.

2.3.2 Plaque Assays

Vero cells were seeded in a 12 well plate at 2.5 x 10^5 as described in section 2.1.2. Viral sample underwent a 10-fold dilution series to 10^-6. Media was removed from wells and 120uL of each sample was added to the appropriate well. This was incubated at 37 degrees for 1 hour with gently shaking every 20 minutes. Following the end of the incubation, wells were overlaid with plaquing agar consisting of 0.4% gel agarose and DMEM. Plates were incubated for the viral incubation period as mentioned previously. At the end of the incubation period formalin was added to each well to fix the plates and left for at least 1 hour. After fixation, the agar was removed and 1% crystal violet was used to stain the cells and read the assay.

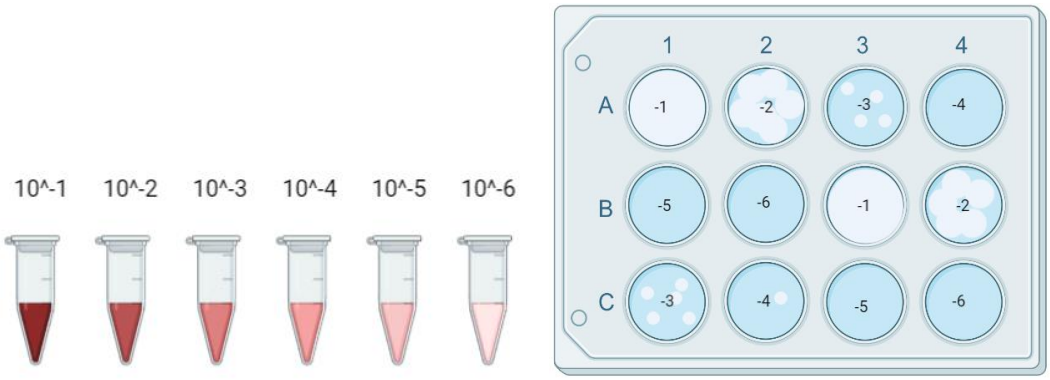


Figure 2.1 The dilutions and layout of a plaque assay. A. The dilution series is generated (1:10) from the original stock of virus in DMEM. 120uL of each solution is then added to the appropriately labelled well in the cell culture plate; B. The 12 well plate layout used for plaque assays. Following incubation, inactivation and staining, plaques are visible in the wells and counted to determine pfu/ml. Images made on Biorender.

2.3.3 Growth Curves

For the passage experiments at an MOI 0.01, viral samples were diluted 10-fold dilutions to ensure more than 30uL of virus was taken for each infection to cover as much of the quasispecies as possible. Mammalian cells were seeded at 2.5 x 10^5 in each well and insect cells were counted to 3.25 x 10^5 (confluency of mammalian cells on day of infection). For each sample, the amount of virus for an MOI of 0.01 was calculated. The viral stock then underwent several serial dilutions in a 96 well plate to ensure that a wide enough variety of viral particles was taken for infection. This is to prevent only defective interfering particles from being take up in such a small volume. Cells were then inoculated for 1 hour with gently shaking every 15 minutes. At the end of the hour virus was washed off of the cells with PBS twice before 700uL of media added to each well. The cells were incubated for 48 hours and 72 hours at 37 and 27 degrees for mammalian and insect cells respectively. Samples were taken at 2, 6, 12 and 48 for mammalian and 6, 12, 24 and 72 for insect. For harvest, 700ul of sample was taken from each well and centrifuged at 2500rpm for 5 minutes and left to sit for five minutes. 30uL was removed for plaque assays and all samples were stored at -80 short term.

2.3.4 Cellular growth curves with treatments

To understand how the treatments may affect viral infection of mosquitoes at the cellular level, U4.4 cells were passaged three times in each treatment (0.5mg/mL) or control as previously mentioned. On the day of infection, cells were counted and infected with CHIK passage 1 at an MOI of 0.01 and 1. The cells were then plated in a 24 well plate at 2.5 x 10^5 with each well representing a time point and three repeats for each time point. A row of uninfected cells was maintained in each plate for a negative control. Supernatant was harvested at five time points, 0, 6, 24, 36 and 72hpi and 0, 24, 48, 72, 96 and 120hpi for CHIKV and ZIKV respectively with three repeats for each. Supernatant was spun at 5000rpm for 5 minutes to collect cell debris at the bottom and 50uL of each sample added to 150uL TRIzol for RNA extraction. 30uL of each sample was collected for plaque assays. This experiment was also carried out with ZIKA virus PE243 with samples taken every 24 hours over a 5-day period.

CHIKV ZIKV

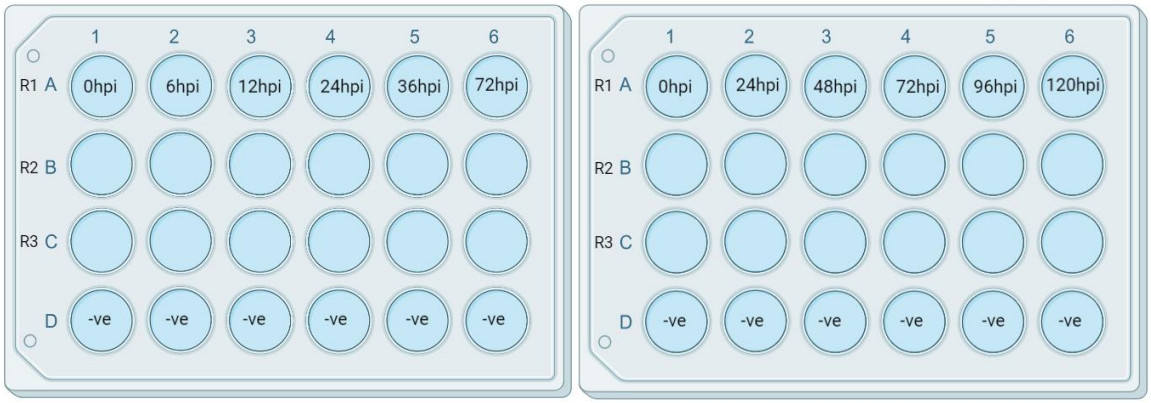


Figure 2.2 The 24 well plate layouts used for the agricultural treatment cellular growth curves of CHIKV and ZIKV at a total of 72 and 120hpi respectively. Experimental repeats were done in rows A, B and C whilst negative treated cell controls were done in row D.

2.3.5 Standard Curves

Passage 0 was grown in vero cells as described above to generate a stock. 1-part viral sample was added to 3 parts trizol and extracted as described below. This was then diluted 10-fold to qPCR for the growth curves. To determine the genome copy number from the qPCR results, a standard curve was created for each of the plasmids. To do this, the viral plasmid stocks were quantified using the qubit 3.0 and the Genome molecular weight was worked out using the RNA molecular weight calculator

https://www.aatbio.com/tools/calculate-RNA-molecular-weight-mw. From this, the number of genomes correlating to each neat plasmid stock was calculated by converting the weight to ng/ul. The number of genomes was then calculated for the neat stocks of each plasmid. This was then matched to the dilution series, giving a genome copy number for each qPCR Ct value.

2.3.6 Specific Infectivity

The specific infectivity for a virus was determined by dividing the genome copy number of the virus by the pfu/ml of the virus using the standard curves explained in the previous section. Genome copy number was determine as described in section 2.3.5 whilst pfu/ml was determined as described in section 2.3.2.

2.3.7 Cytopathic Effect Assays

Vero cells were seeded in a plate at 2.5 x 10^5 as described above. The following day, 50uL of sample was added to each well and allowed to incubate for 1 day longer than the normal incubation period. After this the wells were checked for CPE and compared to control wells. CPE was decided if the cell monolayer had lifted. If, after the full time, the cells looked bad compared to the control well but did not show any lifting, this was counted as negative for CPE.

2.4 Molecular Techniques

2.4.1 RNA Extraction

RNA was extracted from samples following the ZYMO RNA Microprep Kit Protocol, with an additional spin step to remove residual wash buffer. All centrifugation steps were performed for 30 seconds at 13,000 x g unless stated otherwise. 3-parts TRIzol were added to 1-part sample. Equal parts ethanol were added and the sample was transferred to a spin column. This was centrifuged and the spin columns transferred to new collection tubes. 400uL of RNA wash buffer was added and centrifuged. 5uL DNase 1 and 35uL of DNA digestion buffer was added to the centre of each column and incubated at room temperature for 15 minutes. 400uL of RNA Pre-wash was added and centrifuged. Flow through was

discarded. This last step was repeated once more. 700uL of RNA wash buffer was then added to each column and centrifuged. Flow through was discarded and the column was spun again to remove any residual wash buffer. Samples were eluted into 50uL of DNase/RNase Free Water and stored in the -20 or -80 for long term storage.

2.4.2 qPCR

SensiFAST SYBR Lo-ROX One-Step Kit from Meridian Bioscience. For standard curves from plasmids, no RT was added, and instead 1.9uL H20 was added. Cycles were as follows: 1 cycle of 45˚C for 10 minutes, 1 cycle of 95˚C for 2 minutes, 40 cycles of 95˚C for 5 seconds, 60˚C for 10 seconds and 72˚C for 5 seconds.

Component	**To add to one reaction (uL)**
Master mix	5
Forward Primer	0.5
Reverse Primer	0.5
Reverse Transcriptase	0.1
Rnase Inhibitor	0.1
RNase/DNase Free Water	1.8
RNA	2

Table 2.1 Reaction components for a qPCR.

2.4.3 Immune gene testing

Three samples from each experimental group were tested for immune gene expression to look at changes in expression following treatments. We looked at the JAK-STAT, Toll and RNAi pathways with two genes: DOME and PIAS, MyD88 and CACTUS and DCR2 and AGO for each pathway respectively. RPS17 was used as the house keeping (HK) genes. RNA was extracted and underwent qPCR for each

of the genes as described. Expression data was normalised to the housekeeping gene using the Double Delta Ct method. Primer information is listed below.

Gene	Primer Sequence (5'-3')	Pathway	Primer Type
RPS17	F- AAGAAGTGGCCATCATTCCA	HK	qPCR
RPS17	R- GGTCTCCGGGTCGACTTC	HK	qPCR
Dome	F- AAACGGTGGCAAAATGAACT	JAK-STAT	qPCR
Dome	R- CTCCAGACCGGTGAGATTGT	JAK-STAT	qPCR
PIAS	F-GCTGCAACGCATGAAAACTA	JAK-STAT	qPCR
PIAS	R-CAGACGGGACAGTTCCAAGT	JAK-STAT	qPCR
MyD88	F-CGATGCGTTCATTTTGTTTG	Toll	qPCR
MyD88	R-CACCGCTCAGAAATCAGCTT	Toll	qPCR
CACTUS	F-TCTTGCGTTGAAGTGAGTGG	Toll	qPCR
CACTUS	R-GACCCTCTGAAAGGGAAAGG	Toll	qPCR
DCR2	F-GAATTCCTCGGCGATGCGGTATTA	RNAi	qPCR
DCR2	R-GATGCCGACTCTGCCAGGATG	RNAi	qPCR
AGO	F-CTGGCCTTTAGCGAGTTCAC	RNAi	qPCR
AGO	R-CCTTGACCTCCTGCTCGTAG	RNAi	qPCR

Table 2.2 A list of the immune genes and their primers. RSP17 is the housekeeping gene for mosquito samples. Dome and PIAS are of the JAK-STAT pathway, MyD88 and CACTUS are of the Toll pathway and DCR2 and AGO ae of the RNAi pathway.

2.5 Mosquito Rearing

Aedes aegypti were obtained from Liverpool school of tropical medicine and hygiene. Mosquitoes were grown at 27 degrees with 77% humidity and a 12-hour light and dark cycle. Eggs were placed into 1.5L

distilled water and one pellet of chinchilla feed was added to the tray. Larvae emerged over a 5-day period and pupae were collected into pots and placed into large cages for adult emergence.

Upon adult emergence, 10% sugar water was added to the cages in tubes with soaked paper towels for the mosquitoes to feed upon. Once the cage was full, mosquitoes were fed a blood meal of defibrinated horse blood warmed to 37 degrees for 1 hour. Following the feed, an egg collection pot consisting of ½ dH2O and a paper towel was added to the cage for the mosquitoes to lay eggs on.

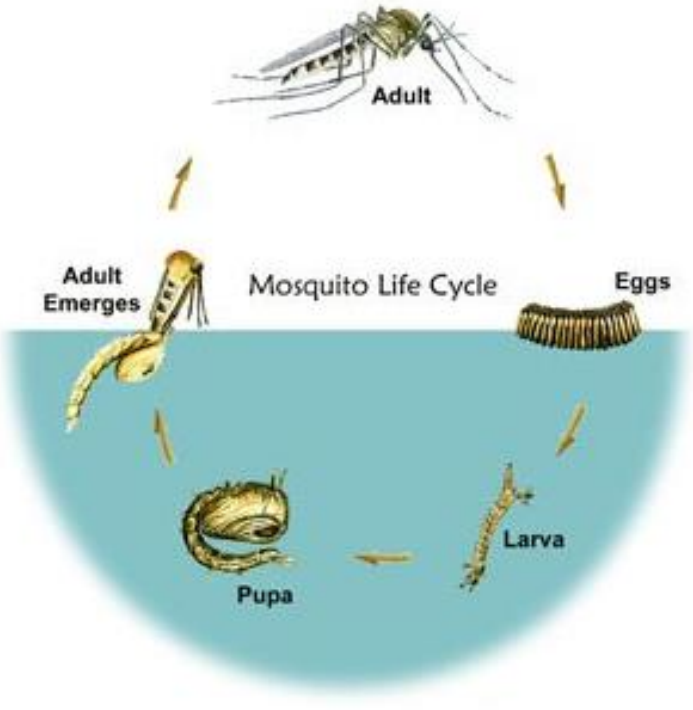


Figure 2.3 The mosquito life cycle. EPA US Environmental Protection Agency https://www.epa.gov/mosquitocontrol/mosquito-life-cycle. Mosquito eggs hatch when exposed to water. Mosquito larvae (*Aedes*) go through four stages of moulting before becoming Pupae. After a short while the pupae emerge into adult mosquitoes, which can fly from the water once their bodies have hardened. After an infectious blood meal, adult female mosquitoes lay their eggs on the water surface or materials in the water.

2.5.2 Mosquito Treatments

For treatments, mosquito eggs were placed into 250ml of either control dH20 or dH20 treated with 0.5mg/L of glyphosate or triticonazole. As larvae emerged they were separated to 20 larvae per tub. Six tubs for each treatment/control were maintained for each repeat. The water, treatment and feed were replaced every 3 days. Upon the day of emergence, pupae were sexed and placed into polystyrene cups. Pupae were then blot dried on paper towels and weighed to the neared .1mg using an electronic scale. Pupae were transferred back into pots containing a small volume of liquid and placed in appropriate cages for emergence. Mosquitoes were reared in the same manner for the coinfections.

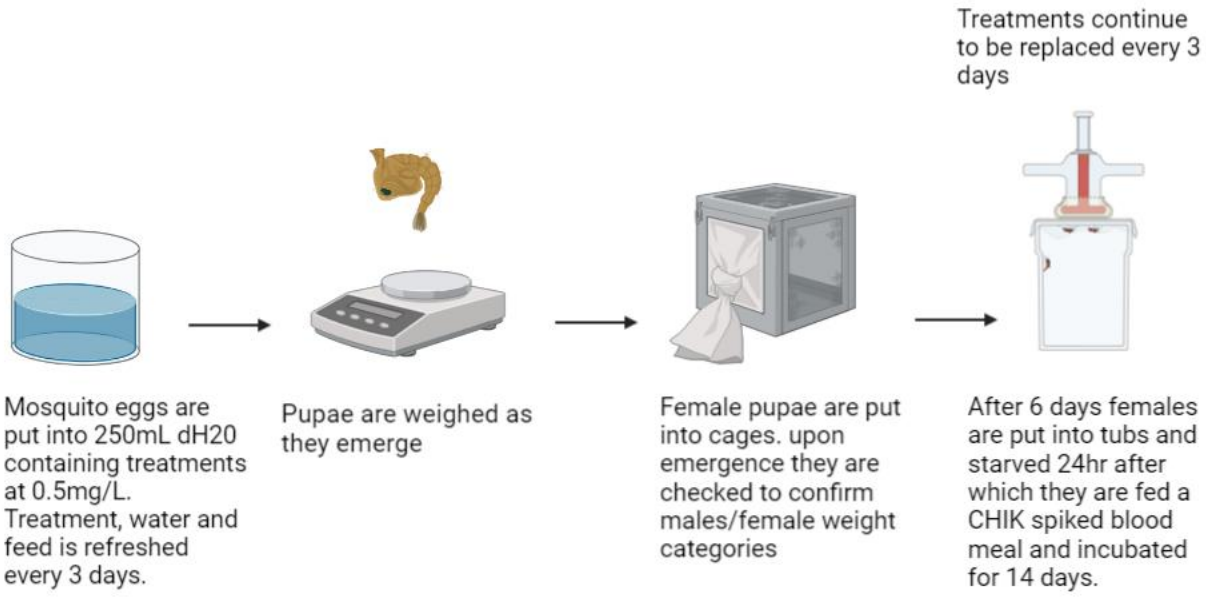


Figure 2.4 The process of rearing mosquitoes in their agricultural treatments, from eggs to an infectious blood meal. Mosquito eggs are hatched into treatments. Upon emergence, pupae are weighed and placed into separate cages based on sex. Adult female mosquitoes are provided an infectious blood meal and incubated for 14 days with treated sugar feeds replaced every 3 days.

2.5.3 Mosquito Infection

Three repeats were done for the mosquito infection experiments. For infection, mosquitoes were anesthetised and put into ice cream tubs and starved for 24 hours. Post infection mosquitoes were stored in a 27-degree incubator with 77% humidity and 5% CO2 and were fed sugar water with treatments every 2-3 days.

Virus was mixed with defibrinated horse blood to achieve a titre of 1-11 x 10^5 pfu/ml. This was heated to 37 degrees using the hemotek blood feeder and mosquitoes were allowed to feed for 1 hour. After feeding, mosquitoes were briefly anesthetised and blood fed females were collected and put back into their pots. Mosquitoes were incubated for 14 days. For coinfection, mosquitoes were fed 1:5 dilution of NEGV mixed with 10% sugar water on a paper towel for 1hour and engorged females were separated into tubs 24hour prior to the second infectious blood feed.

2.5.4 Mosquito Harvest

To harvest, mosquitoes were placed in the -20 for 1 hour. Heads and legs and bodies of each mosquito were put into individual tubes containing 350uL of mosquito storage media (DMEM, 10% FBS, 2.5ug/mL gentamicin, 2.5ug/mL amphotericin B) and stored short term at -20. 5mm steal beads were added to each tube and mosquito samples were triturated at 225rpm for 5 minutes. Samples were centrifuged at 16,000 x g for 5 minutes and 50uL of each sample was added to 150uL TRIzol. RNA was extracted using the ZYMO RNA microprep kit according to manufacturers instructions (as described above). Presence of virus was tested using qPCR and the SYBR LOW-ROX One step qPCR kit (list primers and qPCR setup). Mosquito samples with positive bodies were then tested for dissemination by the same method in the heads and legs. We monitored the survival rates of the mosquitoes in each treatment over the 14-day period.

Small RNA Sequencing

Following 24 hours of infection in cell culture, total cellular RNA was extracted from U4.4 or C7/10 cell pellet and sent for in house Illumina miRNA sequencing at UTMB. Bam files were then analysed using the viRome program on R.

2.6 R Code for RNAi analysis along TC83 and TC83_3X genomes

R-3.6.0, RStudio. To be performed on BAM files.

```{For viRome}
```

```
install.packages(c("BiocManager","Rcpp","seqinr","plyr","gsubfn","Rsamtools","reshape2","seqLogo
", "motifStack", "S4Vectors"))
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
library("seqinr")
library("plyr")
library("gsubfn")
library("Rsamtools")
library("reshape2")
library("seqLogo")
library("motifStack")
library("S4Vectors")
library("Rcpp")
source("https://raw.githubusercontent.com/mw55309/viRome_legacy/main/R/viRome_functions.R")
setwd("")
bam <- read.bam(bamfile = ".bam")
bamc <- clip.bam(bam)
read.distribution <- position.barplot(vdf=bamc,minlen=, maxlen=, reflen=12000)
pwm <- make.pwm(bamc, minlen=, maxlen=, strand="")
seqLogo(pwm)
```

Legacy R code for the R package viRome DOI:10.1093/bioinformatics/btt297

2.7 Equipment list

Item	**Manufacturer**
12 Well Plates	ThermoFisher Scientific
24 Well Plates	ThermoFisher Scientific
T25 Tissue Culture Flask	BioLite
T75 Tissue Culture Flask	BioLite
T180 Tissue Culture Flask	BioLite

10ml Serological Pipette	VWR
50ml Falcon Tubes	Fisher Scientific
15ml Falcon Tubes	Fisher Scientific
2ml Safe Lock Tubes	Eppendorf
1.5ml Screw Cap Tubes	Ependorf
96 Well Plate	Thermo Scientific
1.5ml Eppendorf Tube	Eppendorf
10/20uL Filter Pipette Tips	Starlabs
200uL Filter Pipette Tips	Starlabs
1000uL Filter Pipette Tips	Starlabs

Table 2.3 Equipment list used throughout the thesis.

2.8 Materials

Material	**Manufacturer**
Dulbecco's Modified Eagle Medium	ThermoFisher
EMEM	ThermoFisher
Gentamicin	
FBS	ThermoFisher
Trypsin	ThermoFisher
Tryptose Phosphate	ThermoFisher
DMSO	Sigma-Aldrich
Plaque Assay Agar	ThermoFisher
Crystal Violet	Sigma-Aldrich
Defibrinated Horse Blood	Darwin Biological
Sugar	Tate & Lyle

Table 2.4 List of manufacturers for the equipment and materials used throughout the thesis.

Chapter 3 The importance of viral diversity in the establishment of infection of medically important arboviruses

3.1 Introduction

VEEV generates a cloud of genetically diverse variants known as the quasispecies. This diversity arises largely due to the error prone replication of the RNA-dependent RNA polymerase which produces an error frequency of 10^-3 to 10^-5 misincorporations per nucleotide copied (Forrester *et al*., 2012). This genetic diversity within the population can confer advantages such as increased fitness and adaptability to changing environments which are common in the arboviral life cycle. Genetic diversity is particularly important in viral evolution to facilitate positive selection (to increase viral fitness) or genetic drift (stochastic mutations in a small population) needed to survive the population bottlenecks that arboviruses face during transmission and infection (Forrester *et al*., 2012). Mosquitoes are the primary vector of the VEEV, and the viral quasispecies and its diversity are key to successful infection of the vectors. Understanding how altering viral diversity impacts infection is key for predicting transmission and developing effective control strategies to minimise the threat.

In 2018, Kautz passaged the VEEV vaccine strain TC83 with 5-flourouracil to generate fidelity mutants that produce an altered mutation rate compared to the parent virus. They then created three low-fidelity variants; two clones with one or three of these mutations, and a fourth that contained an additional CHIKV high fidelity mutation. The low fidelity of the 4X only becomes apparent after multiple passages and this was hypothesised a result of the CHIKV high-fidelity mutation compensating for the other mutations. The low-fidelity viruses were unable to surpass the WT virulence after multiple passages and all showed a reduced risk of reversion to the WT compared to TC83.

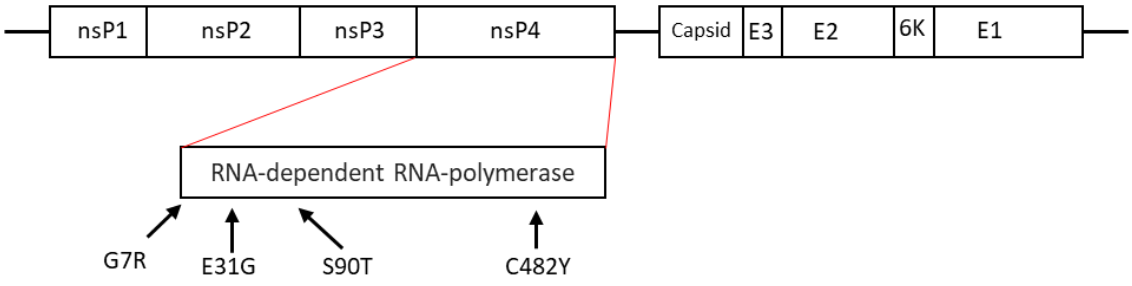


Figure 3.1 The genome of VEEV strain 68U201 showing the four mutations inserted into the viral RdRp during the study. The G7R mutant contains the G7R mutation, the 3X mutant contains the G7R, E31G and S90T mutations and the 4X virus contains the G7R, E31G, S90T and C482Y mutations, all within the RdRp. The number denotes the position along the RdRp sequence. Adapted from Warmbrod *et al*., 2019.

	WT	**G7R**	**3X**	**4X**
G7R		X	X	X
E31G			X	X
S90T			X	X
C482Y				X

Table 3.1 A table denoting the mutations found in each of the viruses. The WT contains no mutations, the G7R contains the G7R mutation, the 3X virus contains the G7R, E31G and S90T mutations and the 4X virus contains the G7R, E31G, S90T and C482Y mutations.

To study the effect these polymerase mutants had on viral diversity and infection, Warmbrod *et al* created three mutants of VEEV strain 68U201 by adding these same mutations as indicated in figure one. These were referred to as the G7R, 3X and 4X based on the position and number of mutations as can be seen in figure 3.1 and table 3.1 Mosquitoes and mice were used to mimic the enzootic infection cycle of VEEV. Firstly, they looked at whether the altered diversity affected viral dissemination in the mosquito vector. When infectious clones were orally fed to mosquitoes, there was no significant difference between the number of bodies infected, however there was a significant decrease in the virus in the legs and saliva suggesting these viruses could not disseminate throughout the mosquito. Figure two represents this.

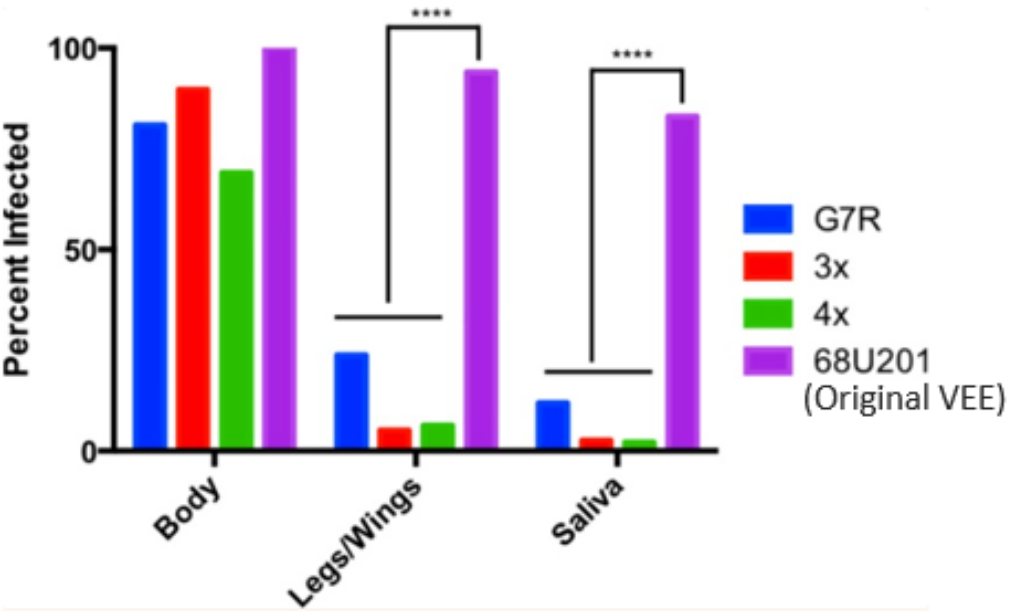


Figure 3.2 The percentage of bodies, legs/wings and saliva infected in mosquitoes following feed with each of the mutants and the WT. Mosquitoes were harvested at 4, 8 and 12 when 95% of mosquitoes are infected with the WT (Warmbrod *et al*., 2019).

To study how the altered diversity impacts the mutant viruses compared to the WT in mammalian infection, the mutant clones and wild-type were individually injected subcutaneously into mice and different tissues were tested for viral infection. The mice injected with the low-fidelity mutants showed increase survival and increased time to death. Additionally, there were significantly fewer clones in the lymph nodes and brain showing a decreased ability to disseminate throughout the mice or cross the blood-brain barrier compared to the WT 68U201. Together with the lack of observed dissemination in the mosquitoes, the results suggest that due to altered diversity, these mutants are unable to traverse the bottlenecks associated with arboviral infection, as indicated in figure two.

This works shows that altering the diversity of a viral population heavily impacts its ability to successfully infect its mammalian host and invertebrate vector. It is imperative to further understand why altering the diversity leads to attenuation of the viral population. With live attenuated vaccines such as TC-83 based on low-fidelity variants, it is important to understand how they may respond should they end up circulating in wild mosquito populations. This research project aimed to test the hypothesis that

the attenuation seen with these polymerase mutants was a direct result of altered diversity preventing the viruses from surpassing bottlenecks. To test our hypothesis, we performed serial passages in neat (one cell type only) or alternating mammalian and insect cells. These experiments would also show us if the G7R, 3X and 4X were able to adapt to cell types or experience fitness losses over five passages. We used five passages as this would be long enough to identify extinction events. We used a low MOI to replicate the bottlenecks faced in nature and identified extinction events in mosquito cell lines, both in neat and alternating passages. Our results corroborate that generation of viral diversity in mosquitoes is time-dependent and that mammals are key for providing titre and diversity to initiate arboviral infection in mosquitoes.

To study this, we passaged each of the viruses in vero (African Green Monkey Kidney) and BV-2 (mouse microglial) cells for the mammalian cells and C636 and U4.4 (both *A. albopitus*) cells for the insect cells. We chose vero and C636 cells for their largely-deficient interferon and siRNA responses respectfully. These provided controls for the experiment to compare the impacts of the immune response on the altered diversity. We chose BV-2 and U4.4 cells for their competent interferon and siRNA response respectively, to understand how these responses impacted the low-fidelity mutants ability to traverse bottlenecks compared to the WT virus. BV-2 cells have previously been shown capable of infection by alphaviruses (Weger-Lucarelli, *et al*., 2016).

3.2 Results

3.2.1 The low-fidelity mutants exhibit several extinction events across five passages

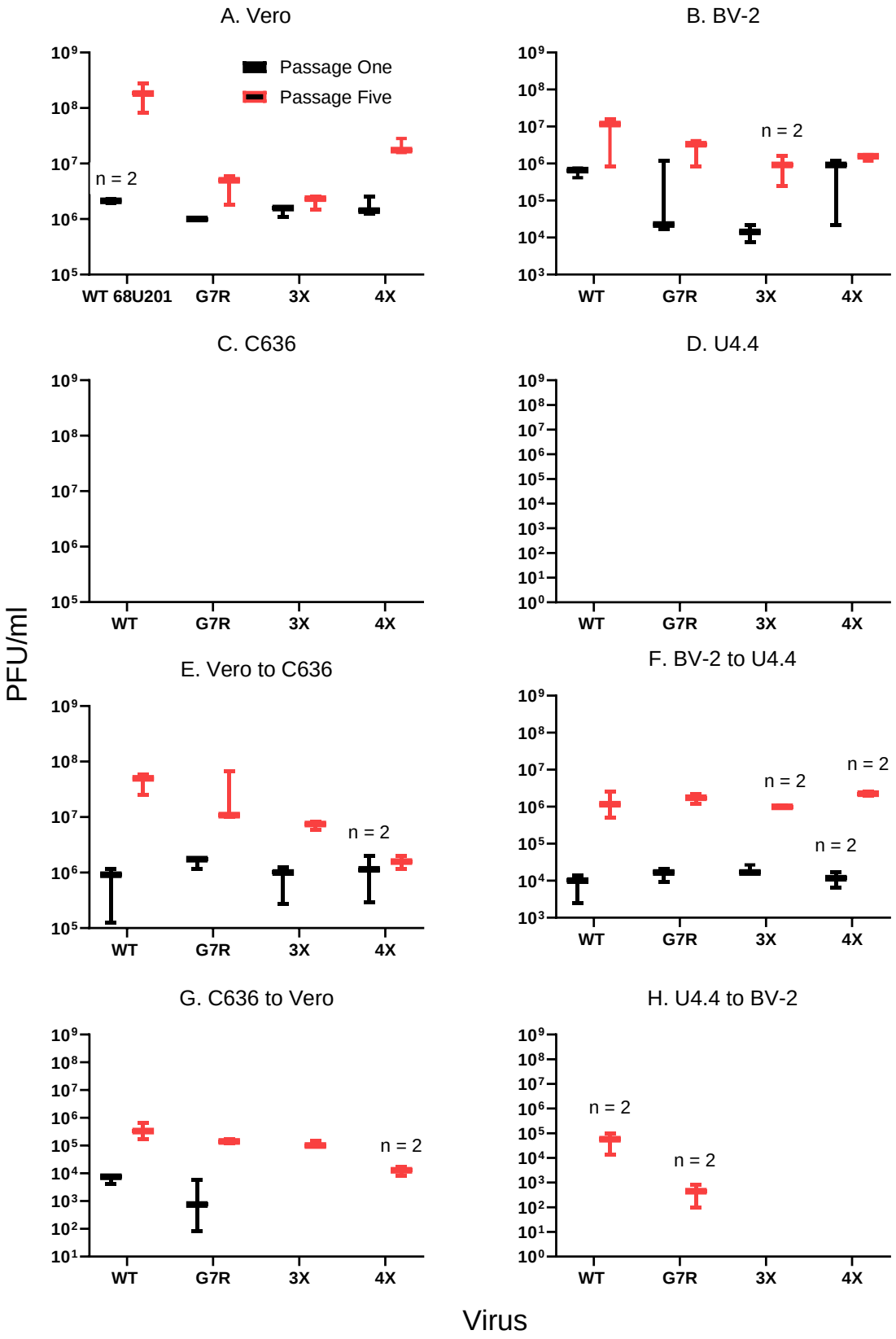

A. Vero
Passage One
Passage Five
n = 2
WT 68U201
G7R
3X
4X
B. BV-2
n = 2
WT
G7R
3X
4X
C. C636
WT
G7R
3X
4X
D. U4.4
WT
G7R
3X
4X
E. Vero to C636
n = 2
WT
G7R
3X
4X
F. BV-2 to U4.4
n = 2
n = 2
n = 2
WT
G7R
3X
4X
G. C636 to Vero
n = 2
WT
G7R
3X
4X
H. U4.4 to BV-2
n = 2
n = 2
WT
G7R
3X
4X
PFU/ml
Virus

Figure 3.3 Passage one (black) and passage five (red) titre for each virus determined by viral plaque assay for each sample (n = 3). (A) Vero cells, (B) BV-2 cells, (C) C636 cells, (D) U4.4 cells, and the alternating passages (E) Vero to C636 cells, (F) BV-2 to U4.4 cells, (G) C636 to Vero cells and (H) U4.4 to BV-2 cells. The series of passages included a total of 5 passages, with alternating passages having 3 passages in one cell type and 2 in the other (C636-Vero-C636-Vero-C636).

To identify whether population bottlenecks were the reason the low-fidelity viruses were attenuated in mosquito and mouse infection, we conducted a five-passage experiment in neat or alternating mammalian and insect cells. In the initial experiment, each passage was performed for 48 hours and titres were measured using plaque assays at passage one and passage five. When repeated at 72hpi, CPE assays showed the same survival rates (data not shown). Plaque assay data was obtained for passage one and five only, to see titre changes across the five passages, whilst CPE assay data was obtained for every passage to confirm presence of infectious virus (data not shown). In later experiments, repeats of the WT survived to passage five of C636 and U4.4 neat passages.

The WT, G7R, 3X and 4X viruses survived the initial passage in neat vero and BV-2 cells and increased in titre over five passages. The WT and the 4X virus increase by 100 and 10-fold by the end of the neat vero cell passages, reaching titres of 10^8 pfu/ml and 10^7 pfu/ml respectively. Both the G7R and the 3X virus increased by >10-fold, remaining at 10^6 pfu/ml. Similarly in neat BV-2, the WT, G7R and 3X all increased roughly 100-fold in titre to 10^7 pfu/ml and 10^6 pfu/ml, whilst the 4X virus showed limited change in titre, remaining around 3 x 10^6 pfu/ml. None of the fidelity mutants surpass the WT in titre for either of the neat mammalian cell passages.

When passaged in neat insect cell passages for either C636 or U4.4 at an MOI of 0.01, no infectious virus was detectable at passage one or five for any of the viruses, including the WT. Interestingly, in alternating passage where the mammalian vero cells were the predominant cell line, all viruses except for the 4X virus were able to increase in titre almost 10-fold, with the WT almost 100-fold, throughout the five passages. In comparison, the 4x virus was only able to increase 2-fold, reaching 1 x 10^6-2 x

10^6 pfu/ml. The titre of the 4X virus went from roughly 1 x 10^6 to 2 x 10^6 PFU/ml. BV-2 to U4.4 alternating passage is the only passage series where the titre of the WT virus is surpassed by the G7R and 4X viruses by the end of the five passages, though the difference is minimal. The 3X virus shows the smallest increase in titre whilst the 4X shows the largest, increasing from roughly 8 x 10^8 pfu/ml to 4 x 10^6 pfu/ml by the end of the five passages. This surpasses the WT titre of 1 x 10^6 pfu/ml, as does the G7R virus which has reached 3 x 10^6 pfu/ml. Both the 3X and 4X viruses experience extinction events.

However, although some of the viruses show an increase in titre in passages where the mammalian cell is predominant, we also see that they have extinction events, as can be seen with the 3X and 4X viruses in the BV-2 to U4.4 cell alternating passage (F). The WT and G7R viruses were rescued by passage five of the U4.4 to BV-2 alternating passage, reaching titres of 7 x 10^4 pfu/ml and 6 x 10^2 pfu/ml, with no infectious particles detected at passage one. Unlike the WT and G7R viruses, the 3X and 4X viruses were not rescued by the end of the 5 passages in U4.4 to BV-2 cell alternating passage.

In alternating C636 to vero cell passages, both the WT and G7R increased 10-fold to 5 x 10^5 pfu/ml and 1 x 10^5 pfu/ml whilst the 3X and 4X viruses were both rescued to 10^5 pfu/ml and 10^4 pfu/ml despite having no infectious particles at passage one. These titres are at least 10-fold lower for all viruses except the 4X compared to the vero to C636 alternating passage. This suggests C636 cells are limiting viral replication.

The consistent increase to higher titres of the WT compared to the low-fidelity mutants suggests that the WT is better at adapting to neat and alternating cell passages. The exception here is the BV-2 to U4.4 cell alternating passage, which suggests that when both the IFN and RNAi responses are active the fidelity mutants have an advantage over the WT. This may be a result of the low-fidelity viruses producing a larger amount of diversity that prevents the RNAi pathway from targeting the viruses with the same intensity as the WT virus, given RNAi targets common sequences. Titres reached in C636 to vero cell alternating passage were lower than vero to C636 cells, suggesting the insect cells are limiting replication, with the lack of purifying response (RNAi) in C636 cells resulting in the viral population producing too many deleterious genomes and replicating to extinction. The same is seen in U4.4 to BV-

2 cell passage, with the fidelity mutants struggling with the strong purifying selection in comparison to the WT virus. Statistical analysis was not performed due to extinction events across the passages.

3.2.2 Specific Infectivity of the viruses

Average Specific Infectivity Passage One								
Cell Type	**Virus**							
	WT	**SE**	**G7R**	**SE**	**3X**	**SE**	**4X**	**SE**
Vero	2 x 10^6	2.5 x 10^6 (n=2)	2.7 x 10^7	4.5 x 10^7 (n=3)	1.2 x 10^5	5.1 x 10^4 (n=3)	2.3 x 10^5	1.4 x 10^5 (n=3)
BV-2	9.4 x 10^5	1 x 10^6 (n=2)	1.2 x 10^12	2E+12 (n=3)	1.7 x 10^7	1.7 x 10^7	6.9 x 10^6	(n=1)
Vero to C636	6.5 x 10^6	6.6 x 10^6 (n=3)	1.7 x 10^7	8.3 x 10^6 (n=3)	3.6 x 10^6	4.8 x 10^6 (n=3)	2.1 x 10^6	1.1 x 10^6 (n=3)
BV-2 to U4.4	6.6 x 10^9	6.00E+09	4.8 x 10^8	2.8E+08 (n=3)	2 x 10^8	8.6E+07 (n=3)	8.3 x 10^8	(n=1)
C636 to Vero	1.4 x 10^2	1.2 x 10^2 (n=3)	7.2 x 10^4	1 x 10^4 (n=3)	(n=0)	(n=0)	(n=0)	(n=0)
U4.4 to BV-2	(n=0)	(n=0)	(n=0)	(n=0)	(n=0)	(n=0)	(n=0)	(n=0)

Average Specific Infectivity Passage Five								
Cell Type	**Virus**							
	WT	**SE**	**G7R**	**SE**	**3X**	**SE**	**4X**	**SE**
Vero	5.6 x 10^1	1.9 X 10^1 (*n*=3)	5.3 x 10^3	3.8 X 10^3 (n=3)	1 x 10^5	1.5 X 10^5 (n=3)	5.6 x 10^1	2.4 X 10^1 (n=3)
BV-2	3.9 x 10^5	5.3 X 10^5 (n=3)	1.5 x 10^5	2.4 X 10^5 (n=3)	4.9 x 10^5	2.9 X 10^5 (n=3)	1 x 10^6	8.7 X 10^5 (n=3)
Vero to C636	4.4 x 10^4	2.1 X 10^4 (n=3)	2.5 x 10^5	1.8 X 10^5 (n=3)	1.3 x 10^5	5.5 X 10^4 (n=3)	1.5 x 10^6	1.4 X 10^6 (n=3)
BV-2 to U4.4	1.5 x 10^6	1.7 X 10^6 (n=3)	3.1 x 10^5	2.1 X 10^5 (n=3)	3.1 x 10^4	3.3 X 10^4 (n=2)	7.7 x 10^4	7.6 X 10^4 (n = 2)
C636 to Vero	1.1 x 10^2	1.8 x 10^2 (n=3)	6.7 x 10^2	2.4 x 10^2 (n=3)	6.5 x 10^1	1.4 x 10^1 (n=3)	3 x 10^1	2.9 x 10^1 (n = 2)
U4.4 to BV-2	1.8 x 10^2	1.8 x 10^2 (n=3)	4.4 x 10^3	4.4 x 10^3 (n=3)	(n=0)	(n=0)	(n=0)	(n=0)

Table 3.2 The specific infectivity of each of the viruses at passage one and passage five of each of the cell passages. Genome copy number was determined by qPCR for two repeats of each viral sample. Pfu/ml was calculated for each sample as previously described. Standard error (SE) is shown to the right of each SI value. *n* = 3 experimental and 6 technical, unless stated otherwise.

To determine the specific infectivity (SI) of each of the viruses in the different cell types, we identified the ratio of genome copies to infectious particles. This would allow us to see how many defective particles the viruses were producing during infection.

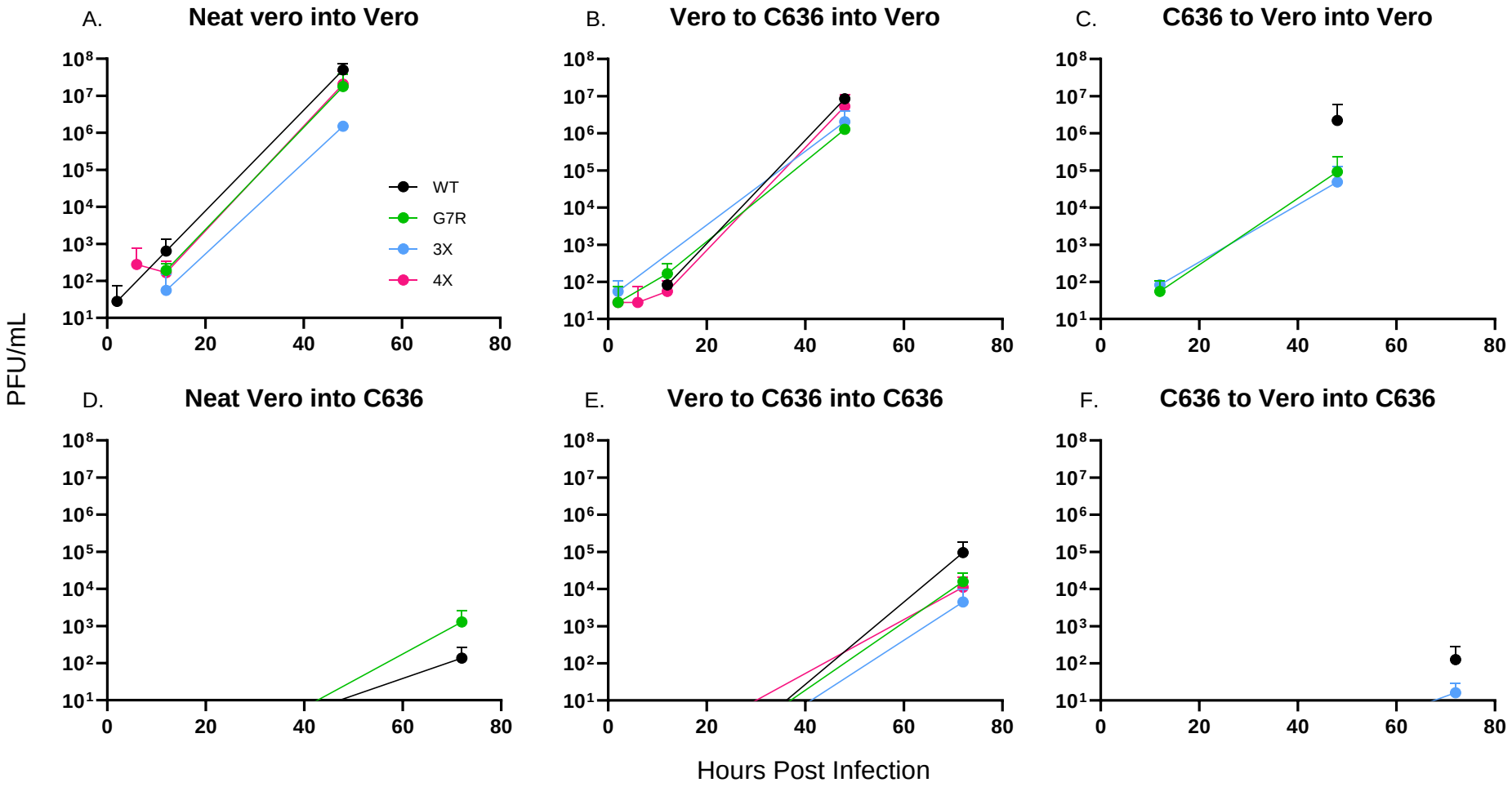
In passage one in vero cells the WT produced the second highest specific infectivity (SI) of 2 x 10^6, meaning a high number of defective genomes were produced, compared to 1.2 and 2.3 x 10^5 of the 3X and 4X viruses respectively. G7R produced the highest SI of 2.7 x 10^7. In neat BV-2 cells, the WT had the lowest SI of 9.4 x 10^5, compared to 1.2 x 10^12 for the G7R virus. The 3X and 4X viruses produced a similar SI of 1.7 x 10^7 and 6.9 x 10^6 in neat BV-2 cell at passage one. There is less than a 10-fold difference in SI between the viruses in vero to C636 cell alternating passage, ranging from .1 x 10^6 for the 4X virus and 1.7 x 10^7 for the G7R. The WT produces the highest SI of 6.6 x 10^9 in the first BV-2 cell passage for the BV-2 to U4.4 cell alternating passage, whilst the other three viruses produce an SI at 10^8. Finally, in C636 to vero cell passage one the WT produces an SI of 1.4 x 10^2 whilst the G7R is higher at 7.2 x 10^4. The G7R is consistently amongst the highest SI produced throughout the passage one infections whilst the 3X and 4X viruses are similar in the genome copy number to infectious particle ratio.

In vero cells, vero to C636 cells and U4.4 to BV-2 cells, and almost neat BV-2 cells, the WT had the lowest ratio of genome copy numbers to infectious particles, suggesting it produced the lowest amount of infectious particles compared to the other viruses (SIs of 5.6 x 10^1, 4.4 x 10^1, 1.8 x 10^2 and 3.9 x 10^5 respectively). In neat vero and BV-2 cells, the SIs of the WT are similar to the SI of the WT at passage one, suggesting it is maintaining a steady infectious particle to genome copy ratio throughout infection in both neat immune competent and incompetent cell lines. In vero to C636 cell alternating passage the WT has decreased its SI over the five passages (from 6.5 x 10^6 to 4.4 x 10^4), suggesting that to adapt to the alternating passage the WT's replication is becoming more efficient with a higher number of genomes able to initiate infection. This effect is more drastic with the WT in BV-2 to U4.4 cell alternating passage, with a 3-fold drop in SI, suggesting purifying selection may play a role in the reduction of defective particles (from 6.6 x 10^9 at passage one to 1.5 x 10^6 at passage five).

The G7R shows a strong decrease in SI in all cell passages tested at passage five, suggesting it is reducing the number of defective particles it is producing for more efficient replication, with its SIs ranging from 10^2 to 10^5 at passage five compared to a range of 10^4 to 10^12 at passage one). Again, this drop is more drastic in immune competent cells suggesting purifying selection is playing a role (for example 1.2 x 10^12 in neat BV-2 passage one to 1.5 x 10^5 at passage five). Both the 3X and 4X follow the same trend with decreasing SI, though for the 4X there is only limited decrease in BV-2 and vero to C636 passages, from 6.9 x 10^6 and 2.1 x 10^6 to 1 x 10^6 and 1.5 x 10^6 respectively, whilst the 3X virus shows a reduction from 1.7 x 10^7 and 3.6 x 10^6 at passage one to 4.9 x 10^5 and 1.3 x 10^5 at passage five in those cell types respectively.

To conclude, all viruses show a decrease in specific infectivity over the passages, producing more infectious particles to genome copies. This effect is particularly strong in immune competent cells suggesting that purifying selection is aiding the removal of defective particles. As can be expected, this suggests that repeated bottlenecks remove defective particles from the population.

3.2.3 Replication efficiency



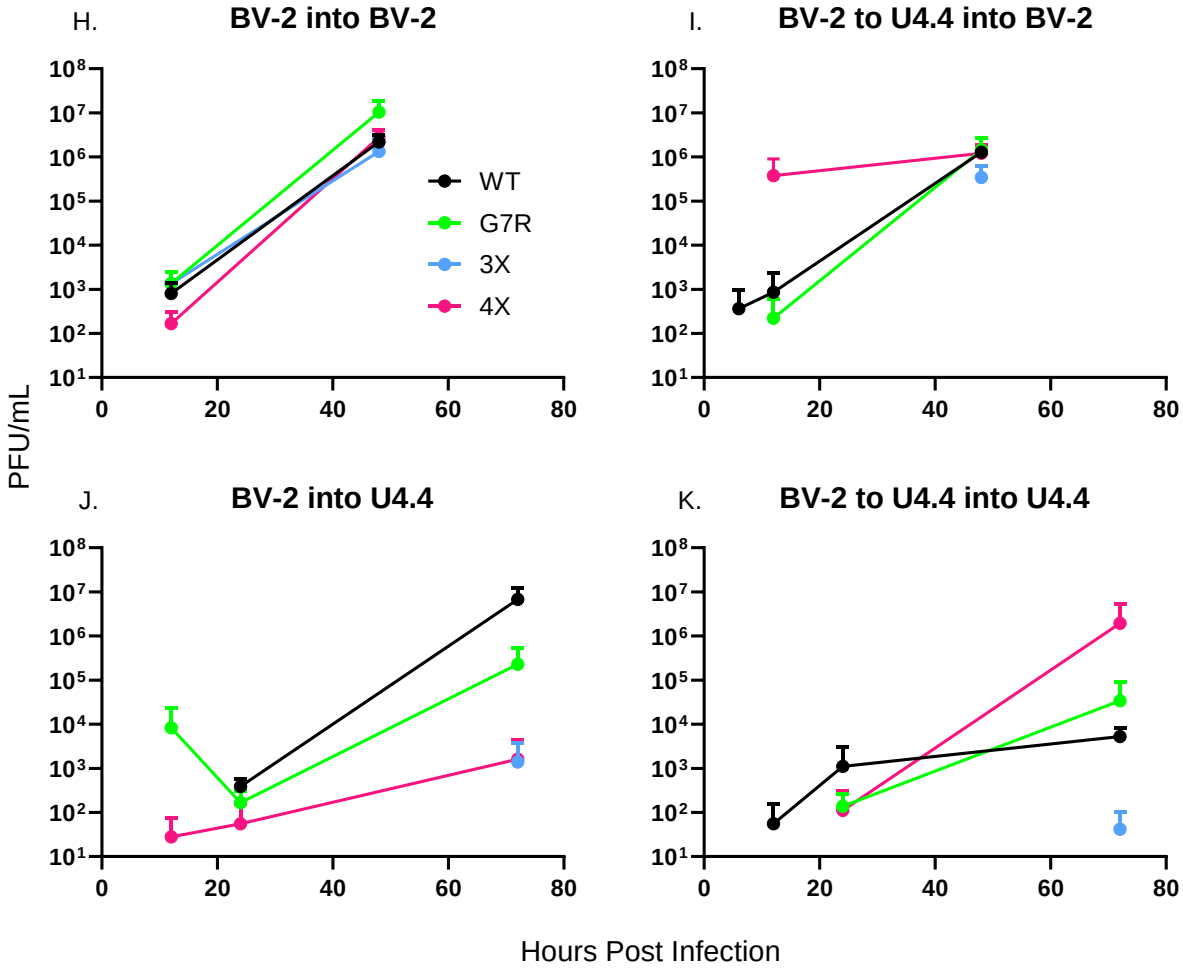


Figure 3.4 Replication kinetics of each of the viruses in their original cell or in the opposing cell line. (A) Vero cells into C636 cells, (B) Vero to C636 cells into C636 cells, (C) C636 to vero cells into vero cells, (D) Vero cells into C636 cells, (E) Vero to C636 cells into C636 cells, (F) C636 to vero cells into C636 cells. Results have been converted to log10 for ease of viewing and were analysed using a one-way ANOVA. Statistical significance was not determined due to extinction events within the repeats. Samples appear on the graphs as and when they were able to be plaque assayed. Missing points indicate no quantifiable virus by plaque assay.

To determine the replication efficiency at early time points of infection for each of the viruses in their passaged and novel cell lines, we took passage five and infected either the neat/final cell line of the passage or the novel cell line. We then monitored the replication efficiency at multiple time points for each of the viruses using plaque assays (Fig 3.4).

Replication of all four viruses in vero cells (fig 3.4 graph A) produces higher titres than in C636 cells with replication starting earlier in infection with the mammalian cells as expected. When viruses adapted to neat vero cells infect C636 cells (graph D), there is only a small increase in titre by the end of the passage, with only the G7R and WT reaching 10^3 pfu/ml and 10^2 pfu/ml respectively. We could not determine accurate statistical significance between the viruses due to extinction events within the repeats.

Viruses that have alternated between vero and C636 reach a higher titre in the C636 cells by 72hpi with a range of 10^3 pfu/ml to 10^5 pfu/ml suggesting that alternating passage is adapting the virus to both cell types (graph E), with the WT achieving the highest titre. In C636 to vero cell alternating passage, where C636 cells are the predominant passage, the viruses reach lower titres in both vero (graph C) and C636 cells (graph F), though still titres of around 10^5/6 pfu/ml in vero cells, compared to the 5 X 10^7 pfu/ml of the WT adapted to vero cells alone (graph A). This suggests that multiple passages in insect cells come at a fitness cost in mammalian cells, though only slight. Viral titres in the C636 cells following this alternating passage are very low, with the WT reaching 10^2 pfu/ml and the 3 X 10^1pfu/ml, despite C636 cells being the dominant cell line (graph F). The WT titre here is similar to when the WT had been put into C636 cells following adaptation to veros. This suggests that without the higher titre generated from the mammalian passage, adaptation to C636 cells alone is not enough to sustain efficient passage. This further implies that the increase in titre in the C636 cells following vero to C636 cell alternate passage is largely due to the higher titre produced by the dominant vero cell line in combination with adaptation.

When viruses adapted to BV-2 cells over five passages are passaged into BV-2 cells (graph H), all viruses reach a titre around 10^6 pfu/ml to 10^7 pfu/ml. When these same viruses are passaged into U4.4 cells (Graph J), the WT reaches a similar titre of around 5 x 10^6, whilst the G7R virus is 100-fold lower, and the 3X and 4X viruses are 1000-fold lower compared to when passaged in BV-2 cells. This is similar to when vero adapted viruses were used to infect C636 cells, though not as drastic and could be a result of the purifying forces of the RNAi response in the U4.4 cells.

When viruses that have been passaged alternately in BV-2 to U4.4 cells were passaged back into BV-2 cells (graph I), all titres were roughly 10-fold lower than from the passages in neat BV-2 cells, matching the results we see with the predominant vero passage. This corroborates the idea that the insect cells reduce the titre slightly overall on these time scales of infection. When the WT, G7R and 3X viruses were passaged into U4.4 cells (graph K), as we saw with the C636 cells there is a decrease in titre. This is except for the 4X virus which maintains the same titre, suggesting that alternating passage in immune competent cells may adapt the virus to both cell lines when the mammalian cell line is predominant. Interestingly for the other three viruses, including the WT, titres in U4.4 cells (graph K) following alternating passage in BV-2 to U4.4 cells, are lower than when neat BV-2 were passaged into U4.4. This suggests that adaptation to both cell line leads to overall fitness decreases in the insect cells. There were no differences between viruses as determined by a one-way ANOVA.

It is worth noting, neither the 3X or 4X viruses survived to passage five of the U4.4 to BV-2 cell alternating passage, though the WT and G7R viruses were rescued by the final passage. This suggests that, as seen to a reduced extent with the C636 to vero cell passage, when the insect cell line is predominant, or the cell line the passage series finishes on, the viruses struggle to maintain efficient replication, which also suggests adaptation is not able to occur when the insect cell line is predominant. As we did not have all of the viruses survive to passage five of the neat insect or alternating U4.4 to BV-2 cells, there is no replication efficiency data for these.

To summarise, the viruses replicate to higher titres in mammalian cells than in insect cells. In the immune incompetent cells, the viruses adapt better/replicate to higher titres in both cell lines when the mammalian cell line is dominant, though there are more fitness losses than when viruses adapted to the single mammalian cell line are passaged into the insect cell line. This may be a result of increased purifying selection in the insect cell line following repeated passage.

3.2.4 Percentage survival at different MOIs

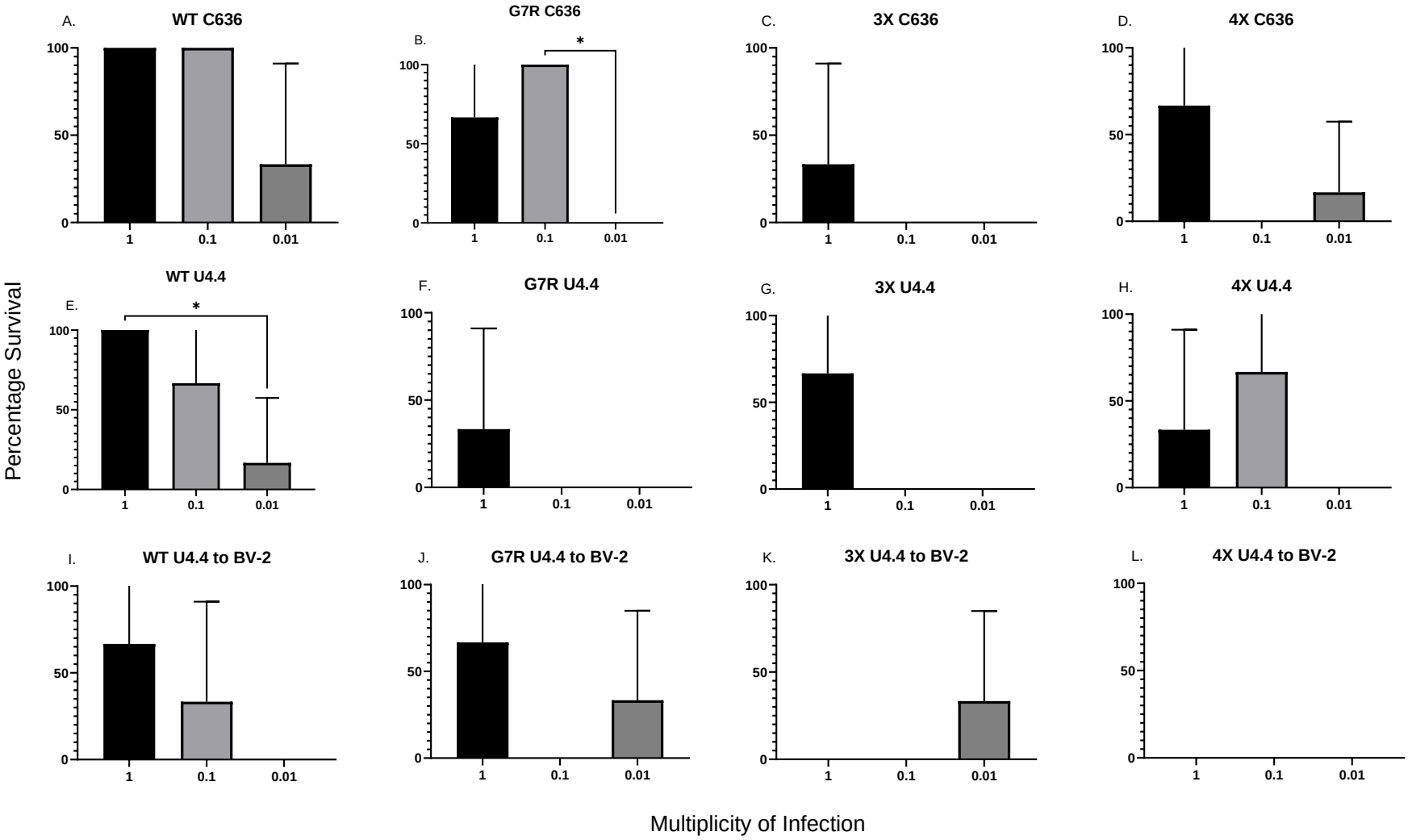


Figure 3.5 The percentage survival of repeats in neat C636 and U4.4 or alternating U4.4 to BV-2 at an MOI of 1, 0.1 and 0.01 for a 3-day infection. Survival was concluded in samples that produced significant CPE on vero cells following a 2-4 day incubation. A-D. WT, G7R, 3X and 4X viruses in C636 cell passage, E-H. WT, G7R, 3X and 4X in U4.4 cell passage, I-L. WT, G7R, 3X and 4X viruses in U4.4 to BV-2 passage. Percentage determined from n = 3. Statistical significance was determined using a one-way ANOVA.

I hypothesised that the higher titre of virus produced in mammalian cells was the reason for the increased survival and that this would increase the chances of surviving the bottlenecks in insect cells. To understand the impact titre would have on surviving multiple bottlenecks, we performed a series of 3-day passages whereby we infected each virus at an MOI of 1, 0.1 or 0.01 to represent the bottlenecks. We recorded the survival at each passage via CPE assays and looked at the percentage survival to passage five for the three repeat infections (Fig 3.5). We chose to focus on neat C636 and U4.4 cells and alternating U4.4 to BV-2 cells as these are the cell types and passages in which we saw consistent extinction events in the previous experiments.

The WT shows a positive trend of decreasing percentage survival with decreasing MOI. With 100% survival at an MOI of 1 for both C636 (graph A) and U4.4 (graph E) cell neat passages and 66.66% in U4.4 to BV-2 cell alternating (graph I), which drops down to 33.33, 16.665 and 0% for an MOI of 0.01 in those cell passage respectively. In U4.4 cells, the percentage of repeats surviving at an MOI of 1 is significantly higher than those at an MOI of 0.01 ($P = 0.0431$). The G7R virus also shows a trend of increased survival at an MOI of 1 for all three cell types, except for 100% survival at an MOI of 0.1 in C636 cells (graph B) and 33.33% in U4.4 to BV-2 cell alternating passage (graph J). The 100% survival at an MOI of 0.1 is significantly higher than the percentage survival at an MOI of 0.01 ($P = 0.0242$). For both C636 (Graph C) and U4.4 (graph G) cells the 3X virus only has repeats surviving to passage five at an MOI of 1. The U4.4 to BV-2 (Graph K) cell alternating passage has 33.33% survival of the 3X virus at an MOI of 0.01, though it is possible this is experimental error due to the lack of survival at the MOIs of 1 and 0.1. Finally, the 4X virus shows increased survival at an MOI of 1 in C636 cells (graph D), though 16.665% survival for an MOI of 0.01. In U4.4 cells (Graph H) there is no survival at an MOI of 0.01, however an MOI of 0.1 shows the highest survival. The 4X virus does not survive the U4.4 to BV-2 alternating passage (Graph L) for any of the MOIs tested suggesting that the 4X virus cannot survive repeated bottlenecks in immune-competent cells regardless of the titre. This suggests another reason for its attenuation in infection.

As we expected, the results suggest in general there is a trend of increasing survival with increasing MOI. We believe this shows that the higher titre produced in mammalian cells may aid survival in insect cells.

3.2.5 Percentage survival at different times of infection

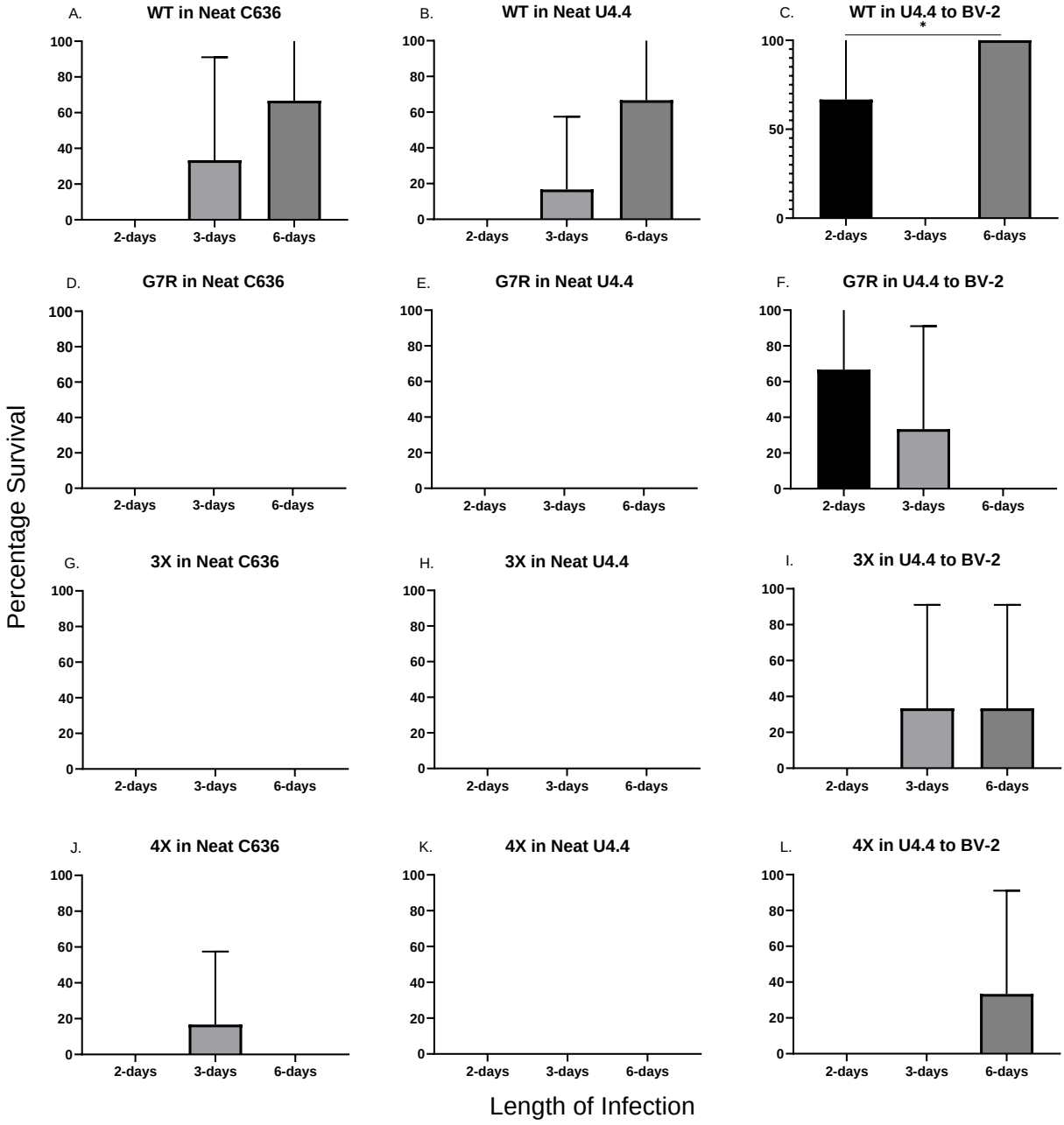


Figure 3.6 The percentage of viral repeats that survived through to passage five in neat C636, U4.4 and alternating U4.4 to BV-2 cell culture passages (Percentage determined from n = 3/6). Graphs show viral

survival in neat C636, U4.4 and U4.4 to BV-2 passage respectively. A-C. WT virus, D-F. G7R virus, G-I. 3X virus, J-L. 4X virus. This includes data from 2 (48hr), 3 (72hr) and 6 (144hr)-day infections. For 2 and 6- day n = 3, for 3-day n = 6. These results are from an MOI of 0.01 and were determined using CPE assays. Significance was determined using a one-way ANOVA.

Following on from the previous results, we hypothesised that the higher titre in mammalian cells may produce more diversity, providing benefit for the viruses when traversing the initial midgut infection bottleneck. To test this, we passaged each of the viruses for five passages in C636, U4.4 or alternating U4.4 to BV-2 cells. We varied the time for the insect cell passage by 3 or 6 days. We also compared this to the 2-days of the initial experiment. We again chose to continue focusing on the cell types and passages in which we were experiencing consistent extinction events (neat U4.4 and neat C636 cells and U4.4 to BV-2 cell alternating passage) and this was done at an MOI of 0.01 and was measured using CPE assays for each repeat. Survival was considered at passage five if samples produced significant CPE.

For the WT, there is a positive correlation between increasing percentage survival to passage five and increasing time of infection. At an MOI of 0.01, the WT does not survive to passage five in either C636 (graph A) or U4.4 cells (graph B), though there is over 50% survival of the three repeats in U4.4 to BV-2 (graph C) alternating passage at an infection time of 2-days, where the virus was rescued by the end of the passage series. Interestingly, there is no survival at 3-days but 100% survival at 6-days ($P = 0.0242$), showing that the longer period of infection is still beneficial in alternating passage.

Unexpectedly, neither the G7R or the 3X virus replicated in C636 (graphs D and G) or U4.4 (graphs E and H) cells at any of the time frames used in the infection, suggesting the time the infection runs for does not impact the survival of these two fidelity mutants. The G7R virus shows a negative correlation with increasing percentage survival and increasing time, with >60% surviving to passage five at 2-days, >20% at 3-days and 0% at 144 hours, whilst the 3X virus shows equal percentage survivals of >20% for each of the three-time frames. The 4x shows >20% survival to passage five at 3-day infection times in C636 cells (graph J), with no survival at any other time frames alongside 0% survival to passage five in

U4.4 cells (Graph K). The 4X virus does show >20% survival at 6-day infection times in U4.4 to BV-2 (graph L) alternating passage compared to 0% survival at either of the other two-time frames.

In general, the WT shows increased chances of survival with increasing length of infection. This is the trend for neat C636, U4.4 and alternating U4.4 to BV-2 cells. Increasing length of time does not impact survival chances in neat insect cells for the G7R and 3X virus, and generally not for the 4X virus. The G7R, 3X and 4X viruses showed increased survival in U4.4 to BV-2 cell passage, with the 4X showing increasing survival with increasing time, whilst the 3X showed no time bias and the G7R showed a negative correlation. For the WT at least, this suggests extending the time point at which we sample wild viral populations from insect saliva may provide a more accurate look at whether the mosquito is capable of transmitting virus or not.

3.3 Discussion

3.3.1 The role of stock variation in experimental results

We carried out several different passage experiments to determine why the G7R, 3X and 4X low-fidelity viruses were attenuated in mosquito and mouse infections. The first two experiments focused on an MOI of 0.01 for all viruses at both 48 and 72hr of insect cell infection. The third experiment focused on extending the time of infection to 6-days and varying the MOI. Between these experiments we experienced variation that can be attributed to selection of subpopulations during pipetting and stock variation. For example, in C636 to vero cell alternating passage, our results showed that some repeats of the 4X virus could be rescued whilst others could not, and in BV-2 to U4.4 cell alternating passage one repeat of each of the 3X and 4X viruses were lost. We also found that in the final experiment using a new viral stock, the WT was able to survive to passage five in some repeats for both C636 and U4.4 cells at 72hr at an MOI of 0.01, despite being lost after passage 1 or 2 in the first two experiments. Additionally, we saw difference in the SI between the same virus in the same cell type. For instance, the SI produced by the WT at passage one in BV-2 cells for neat BV-2 cell and BV-2 to U4.4 cell alternating passage shows a 1000-fold difference, from 1.4 x 10^2 to 9.4 x 10^5. We hypothesise that random

selection during sampling takes up a difference quasispecies for the infection, particularly when a low MOI of 0.01 is used. This has implications for experimental design, as suggests that using different stocks can produce influence the results to a large extent.

This stock effect is likely due to different variants selected for during stock generation. Kautz (2018) found that different electroporation pools of the 3X and 4X virus had different virulence due to minority variants randomly generated in each pool. Grubaugh (2017) showed that mosquitoes transmit unique WNV populations during each feeding episode. When collecting infected saliva from mosquitoes they noted that the genetic variability between saliva collections and viral populations, were just as different between feeding episodes of individual mosquitoes as from different mosquitoes, with different consensus sequences recovered between feeding of individual mosquitoes. They hypothesised this was because of genetic drift in the salivary glands, and superinfection exclusion preventing continuous movement between cells, leading to distinct and isolated subpopulations. Once one population was transmitted in saliva the process began again with a new population. Our work corroborates this, with some viruses surviving more passages in one experiment compared to the next. The results generated each time are different at a low MOI because of such stochastic generation of variants, fitting with genetic drift, whereby stochastic mutations in a small population are selected for leading to a different quasispecies. It is highly likely that even within a single stock, pipetting leaves room for selection of individual subpopulations of virus.

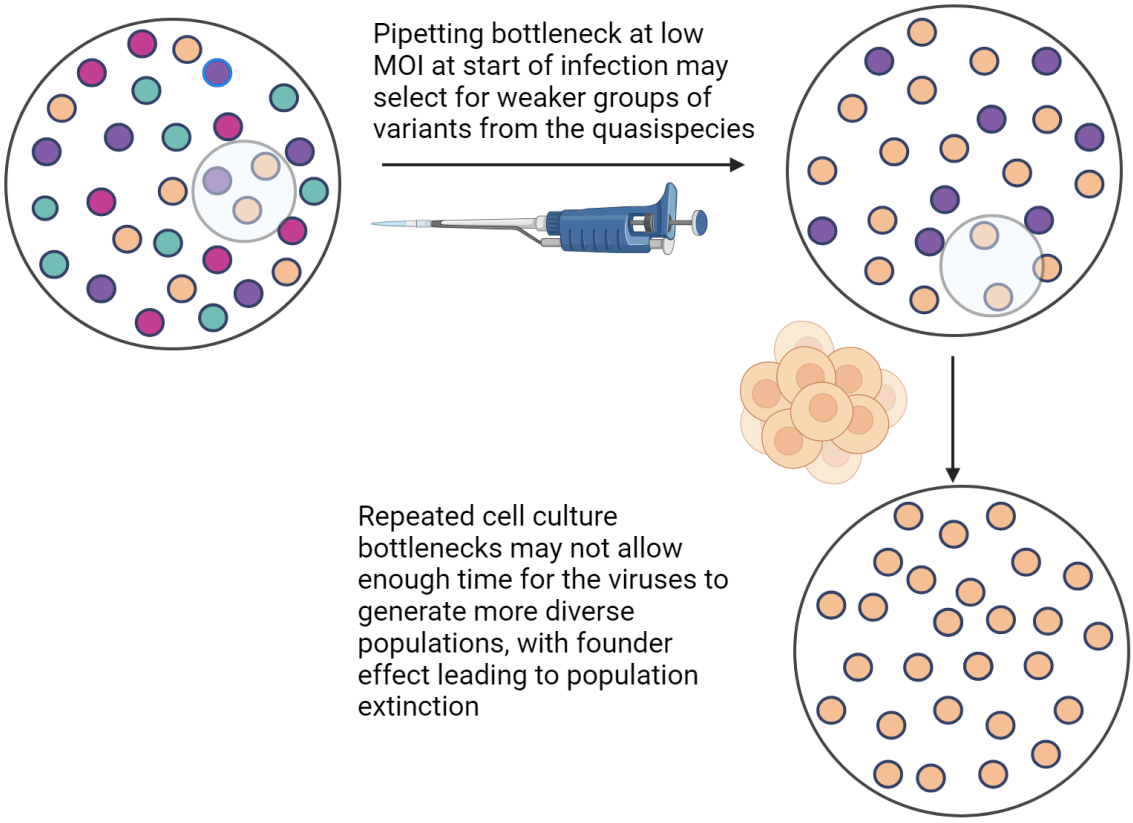


Figure 3.7 Founder effect, whereby random mutations are fixed into the population due to bottlenecks. Small numbers of variants are selected for during pipetting of low MOIs. These subpopulations are unable to diversity due to quick succession of multiple bottlenecks leading to population extinction. This effect could also be seen due to variable conditions within cell culture. Modified from Weaver *et al*., 2021 using Biorender.

As shown in figure seven this leads to the founder effect and can influence the course of infection by amplifying specific subpopulations, including those with deleterious mutations. We initially speculated pipetting may lead to selection of subpopulations and to try and prevent this we diluted our viral stocks so that a larger volume would be taken for the low MOI to include as many of the variants as possible. However, this appears difficult at a low MOI and is representative of when a mosquito feeds. This effect is then amplified between different viral stocks. Despite each stock generated in an identical manner, selection and amplification of different subsets of variants will allow for generation of a quasispecies covering different areas of sequence space, whilst maintaining the same consensus sequence. We suggest that future experiments do each repeat with a different stock of virus to account for this variation. This

should be considered with upmost importance for experiments to accurately see the results variation across three separate repeats.

3.3.2 The WT can traverse repeated bottlenecks in insect cells whilst the low-fidelity mutants cannot

Our work shows that, subject to stock variation, the fidelity mutants are unable to traverse repeated bottlenecks compared to the WT which had a higher survival percentage after five passages at a low MOI. This accounts for their attenuation in mosquito models, suggesting their altered diversity prevents them from traversing repeated bottlenecks in mosquitoes. Fidelity mutants are generally unable to survive as well as the WT, with the high-fidelity G64S poliovirus RdRp mutant having reduced ability to build a reservoir of beneficial mutations. This led to the WT adapting significantly faster in new environments and outcompeting the G64S in adverse growth conditions (Vignuzzi *et al*., 2006). Equally, an increase in the mutation rate in low-fidelity variants can lead to Mullers ratchet, whereby there is an accumulation of deleterious mutations that cannot be compensated for leading to population extinction (Muller *et al*., 1964). These mutations then have a low probability of reversion if the population size is below 10^4 (Weaver *et al*., 2021).

The 3X and 4X viruses were prone to extinction events within their repeats, showing they are less consistent in producing similar quasispecies due to their increased diversity. The low MOI means the populations were unlikely to reach above 10^4 pfu/ml in the insect cells, increasing the chances of Mullers ratchet. This is likely the case for all viruses, as can be seen in figure three for C636 to Vero whereby the WT produced titre just below 10^4 pfu/ml and the G7R below 10^3 pfu/ml, though remained a consistent factor for the fidelity mutants compared to the WT which showed stock variation.

It is likely that in the insect cells the low-fidelity viruses produce more defective genomes than the WT and these lead to Mullers ratchet because of founder effect through the repeated bottlenecks. A higher titre may be beneficial to overcome this. Given the viruses did not survive to the end of the five passages in insect cells we were unable to determine the genome copy number for these passages. Given the extinction events were not seen in neat BV-2 to vero cells, and the SI showed less than 10-fold variation across all viruses, it is unlikely the number of defective particles plays a role in the clearance of the low-

fidelity variants from the brains of mice (Warmbrod *et al*., 2019. In this case it may just be that the sequence space covered by the variants is unable to stretch to areas beneficial for immune evasion within the mouse brain, rather than producing too many DIPs and replicating to extinction. This may be why we see more extinction events in the immune competent cells without a drastically dissimilar SI, and when there is no immune response as in the vero cells the viruses are able to replicate undisturbed and produce different volumes of defective particles.

In half of the passages tested, on average the WT produced the smallest ratio of genome copy number to infectious particles compared to the low-fidelity mutants. This suggests that in neat vero, vero to C636 and U4.4 to BV-2, the WT is producing a smaller amount of defective particles. This is in comparison to the G7R and 3X viruses in neat vero with a SI of 5.3 x 10^3 and 1 x 10^5 compared to 5.6 x 10^1 for both the WT and the 4X virus. This suggests that a lower amount of defective particles is beneficial for replication in vero cells where there is no immune response. Interestingly, whilst there is less than 10-fold difference between the SI of the viruses in BV-2 cells, in alternating BV-2 to U4.4, the WT produces the highest number of defective particles. This may be a direct method of immune evasion and is interesting that the low-fidelity mutants show a lower SI here. Given both the 3X and 4X show extinction events in this passage, it is possible that they are generally less able to replicate in the immune competent cells and therefore produce lower genome copies overall. With the WT producing higher titres in all of the passages other than the BV-2 to U4.4 alternating passage, it is likely the WT produces different numbers of defective genomes in different cell types due to selection pressures on the virus, though the mechanism responsible for this regulation is unclear. This may work to aid infection through immune evasion or to increase/decrease infection rates where acute or persistent infection is required. Unfortunately, we cannot determine the SI for neat insect cell infection.

3.3.3 The higher titre produced by mammals is needed to traverse the midgut infection bottleneck

Studies have shown that as few as <5 PFU of VEEV subtype IE can infect its natural vector, *Culex melanoconion taeniopus*, leading to subsequent transmission (Scherer, Cupp, Lok and Brenner, 1971 and Cupp, Scherer and Ordonez, 1979). Weaver (1996) found that serum titres of VEEV in humans

exceeded those needed to infect a several epidemic mosquito species in an experimental setting, showing that the titres in mammals far exceed those needed for infection of a mosquito. We believe that the higher titre produced by the mammalian cells is required to establish infection in mosquitoes.

In our experiments, the mammalian cells produced higher titres of the viruses than the insect cells, with the highest in C636 cells seen following alternating vero to C636 cell passage. This high titre was not seen in C636 to vero or from neat vero, suggesting the high titre of the vero cells enables adaptation, and that without the higher titre of the mammalian cells, adaptation alone is not enough to sustain efficient replication in the insect cells.

Through the MOI passage experiments, we can clearly see that a higher titer of virus increases the chances of the viruses surviving to passage five in the insect cells, presumably by reducing the impact of the bottleneck between cell passages. We can determine this because we designed the experiments with a low MOI to replicate a natural bottleneck. When a mosquito ingests an infectious blood meal, the virus must initiate successful infection of the midgut epithelial cells, where it experiences its first population bottleneck. The impact of the initial midgut infection bottleneck is largely dependent on the starting titre ingested by the mosquito (Forrester *et al*., 2012). When culex mosquitoes were infected with a high dose or low dose of infectious 68U201 clones, all clones were present upon ingestion from the high dose and the large bottleneck was subsequently seen upon escape from the midgut. With the low dose, there was a major bottleneck upon ingestion to the midgut, with only two clones then starting infection. Similarly, 68U201 expressing GFP was fed to mosquitoes at a low (<5 log10) and high (>5 log10) dose with 11% of midgut cells showing clear infection in the low dose whilst 100% were infected at the high dose (Kenney *et al*., 2012). Smith (2008) found that in *A. taeniorhynchus* only a few midgut cells were susceptible to VEEV infection, constituting a bottleneck. However, when Forrester (2014) infected *Culex (Melanoconionn) taeniopus* mosquitoes, that have a larger number of susceptible midgut cells, the low dose viruses were still unable to traverse the bottleneck into the haemocoel (Forrester *et al*., 2014) and GFP expressing 68U201 showed no preference to regions of the midgut (Kenney *et al*., 2012). This reinforces that the titre ingested by the mosquito has a significant impact on the success of

the initial stages of infection and that the reason for this is the higher titres are better at traversing the initial bottlenecks within the mosquito, as we are seeing in our experiment.

It is worth noting, that studies have also shown that differences in titre ingested by the mosquitoes does not impact the titre expectorated during a blood feed to infect a mammal, though it does change between mosquito species, with *A. albopictus* saliva tittered at 0.2 to 1.1 log 10 pfu/ml whilst *A. taeniorhynchus* had higher median titres of 0.2 to 3.2 log 10 pfu/ml (Smith *et al*., 2005). Our experiment shows that despite low titres infecting the mammalian cells, they are still able to replicate VEEV to high titres. Forrester (2012) states that when mosquitoes transmitted VEEV to mice, they could not identify a consistent bottleneck, though didn't rule out the possibility. Our work in cell culture corroborates Warmbrod (2019) where the mutant viruses were unable to replicate in mosquitoes, suggesting that the bottlenecks are experienced strongly within mosquitoes and that the mammals may be key reservoir hosts in maintaining arboviral transmission, providing a high enough titre for mosquitoes to become infected successfully. This impresses the importance of the vertebrates as the reservoir species for building up viral titre, whilst the mosquitoes provide the harsher infection environment.

The higher titres in mammalian cells may be a result of the higher temperature of 37 degrees enhancing the replication kinetics of the viruses in mammalian cells, whilst the insect cells were maintained at 29 degrees. Weaver (1999) maintained their insect and mammalian passages at 32 degrees to prevent temperature from acting as a selection factor. However, this biases the results as this is not a temperature you would likely find in the mammals or mosquitoes in which these viruses replicate. Ziegler (2023) found that mosquitoes tend to prefer cooler resting places and were commonly found with an internal temperature 4 degrees lower than the ambient temperature. This corroborates Sauer (2022) who found that the microclimate of mosquito resting sites is lower than standardised meteorological data. They state this cooler temperature would prolong the EIP of mosquito-borne pathogens relative to data at weather stations, suggesting that cooler temperatures for the mosquito cells compared to mammalian in our experiment is an accurate representation of natural transmission temperatures. Our experimental design represents more accurately how the virus would replicate in nature.

3.3.4 Mammalian diversity and time dependent generation in mosquitoes

As shown in several studies, we hypothesised a higher titre of the clones would allow more efficient survival of the bottlenecks. In 2019, Warmbrod showed that feeding mosquitoes two doses of infectious clones of the G7R, 3X and 4X viruses (a range of 10^5 pfu/ml and a range of 10^6 pfu/ml) allowed dissemination to the legs and wings in the higher dose of 10^6 pfu/ml only. This dissemination correlated with more clones, suggesting a higher titre may be able to compensate for altered diversity. We hypothesised that the higher titre produced by mammalian cells allowed for more efficient infection of insect cells due to a more diverse population. The higher titre would increase the likelihood of beneficial variants able to traverse the initial bottlenecks in the mosquito. Jerzak (2005) found that WNV was more diverse in mosquitoes than in birds, but also stated that WNV infection of mosquitoes is chronic whilst in birds it is acute and often lethal. They contemplated the higher diversity in mosquitoes could be attributed to the longer infection period. The diversity of a VEEV population in the hemocoel of *C. taeniopeus* increased significantly by 8 days following the initial escape from the midgut (Patterson *et al*., 2018). This was the same following infection of the legs and wings. We therefore hypothesised that the lower MOI of 0.01 would show increased survival in insect cells if we gave the virus more time to replicate and produce higher titre and subsequent diversity. We designed an experiment to look at how viruses at a low MOI would replicate given more time. We looked at neat C636, U4.4 and U4.4-BV-2 cell passages as these were ones in which we saw consistent extinction events (figure 3.6).

In our experiment, the WT showed a clear positive trend of increasing survival at an MOI of 0.01 with increasing time in all cell passages tested. This is the same for the 3X and 4X virus in the alternating passages. For the 3X and 4X viruses, this suggests the longer time allows for more adaptation when the mammalian cell is present to produce higher titre. We suggest that the mammals produce more immediate diversity to traverse the initial bottleneck in mosquitoes, whilst mosquitoes require a longer period to generate diverse viral populations needed to establish infection and dissemination in the different tissues of the mosquito. Jerzak (2005) also stated that higher titres are produced in birds, so although the population is proportionally more diverse in mosquitoes, a similarly large pool of variants may be present in the birds, meaning that the mammals don't necessarily produce less diversity as seen

in other studies (Ciota *et al*., 2007). The mosquito requires a longer period than the mammal to generate the diversity that is needed for infection and dissemination. Without the extended period, the viruses wouldn't survive infection in mosquitoes. Mammals replicate the virus faster and to higher titres, providing more immediate diversity for the initial infection of the mosquito.

3.3.5 Purifying selection in insect infection puts strain on arbovirus transmission

Interestingly none of the fidelity mutants benefited from increased time in the neat insect cells by passage five, and the G7R virus actively showed a negative correlation between survival and increasing time in the U4.4 to BV-2 cell alternating passage. *Flavivirus* studies have shown that mosquito infection exerts strong purifying selection. In 2016, Lequime found that following a strong population bottleneck upon midgut infection, DENV intra-host diversity in the mosquito is shaped by stochastic events followed by strong purifying selection during expansion and diversification in the midgut. The RNAi response in mosquitoes is a driver of diversity, through purifying selection, selecting for rare variants in the quasispecies by targeting common sequences (Grubaugh and Ebel, 2016). Purifying selection may aid adaptation to multiple cell types by removing variants beneficial to only one cell type, preventing fixation on beneficial variants. This would also account for a fitness cost often seen in alternating passages.

We see greater titres in U4.4 cells both when infected directly from BV-2 cell adapted viruses or from BV-2 to U4.4 cell alternating passage than with C636 cells. Additionally, in the CPE assays from the passage experiments, we saw more repeats surviving further into the passage series in U4.4 cells than in the C636 cells. The WT shows a drop in specific infectivity from 6.6 x 10^9 at passage one to 1.5 x 10^6 at passage five in BV-2 to U4.4 cell alternating passage, a larger drop than in vero to C636 cell passage, suggesting that purifying selection is removing defective particles. All of the viruses follow a similar trend, with a decrease in SI over the five passages, particularly in immune competent cells.

Repeated passage in both U4.4 and C636 cells appears detrimental to the survival of the fidelity mutants, though we saw higher titres in U4.4 cells in the replication kinetics experiment (Figure 3.4) than in the C636 cells. The siRNA response of the U4.4 cells may be acting to purify out the defective genomes to increase the overall fitness of the population, allowing higher titres to be reached than in the C636 cells.

The 4X virus replicates to the highest titre in U4.4 cells of all the viruses following alternating BV-2 to U4.4 cell passage, suggesting that provided the right subpopulation of variants is selected for initially and there is a higher titre produced by the mammalian cells, the purifying selection of the U4.4 cells can help adaptation. In our work, following adaptation to BV-2 cells over five passages the WT replicates efficiently in U4.4 cells. This is lost however when passaged alternately between BV-2 and U4.4 cells, suggesting that repeated passage in U4.4 cells exerts a strong pressure on the virus that reduces its overall fitness. In previous work, all three fidelity mutants showed a significant drop in diversity compared to the WT when infecting U4.4 cells at an MOI of 0.1 (Warmbrod *et al*., 2019), though were only allowed 24hpi before testing. In our time-dependent diversity experiment we saw some higher percentage survival at an MOI of 0.1 for the G7R and 4X viruses and this may be because these viruses produce higher numbers of defective interfering particles which lowers their fitness and slows the infection. This would give the U4.4 cells more time to purify out the population before it replicates to extinction. Whilst the U4.4 cells may initially allow a higher chance of survival compared to the C636 cells by purifying out defective particles, this effect becomes too strong over a prolonged time, and the low-fidelity mutants cannot survive this, whilst the normal diversity of the WT allows it to sustain itself over repeated passage.

The immune response of the mammalian cells is more intense in BV-2 cells than the interferon-incompetent vero cells so we still see more extinction events, but when the correct subpopulation is replicating we get higher titres in the U4.4 cells. This is likely driven by the higher titre put into the U4.4 cells by the BV-2 cell replication, and so when U4.4 cells are the predominant cell line we see the 3X and 4X viruses unable to survive over repeated bottlenecks.

3.3.6 An increasing length of time may not benefit the low-fidelity mutants in insect infection

The WT clearly benefited from an increased period in the insect cells, with a higher percentage survival towards 6 days of infection. The fidelity mutants did not show the same pattern and saw a negative correlation between increasing time and survival in U4.4 to BV-2 cell alternating passage. Whilst the longer time may generate higher diversity, for low fidelity mutants this may increase their chances of replicating to extinction, despite the purifying effects of the RNAi response. Replication may then fixate

on deleterious mutations which lose overall fitness of the population or are targeted by the RNAi response leading to population extinction in a longer time frame. This may be why the low-fidelity mutants were attenuated in insect infection. The level of mutation in a viral population is highly controlled, and slight changes can have detrimental effects. It is likely that given the longer time points, the fidelity mutants produce too many deleterious genomes that the RNAi response cannot remove, and the population reaches a natural extinction. This provides a sufficient explanation for why the G7R, 3X and 4X viruses are attenuated in mosquito infection. With mosquito infection requiring longer infection times to generate enough diversity to overcome the bottlenecks between tissues, the fidelity mutants likely replicate to extinction.

3.7 Conclusion

To conclude, mammalian cells play a key role by producing high enough titres of virus to overcome the initial insect cell infection. This likely represents the role of mammalian cells in the arboviral life cycle, producing a high enough titre to be ingested by the mosquito to overcome the initial mosquito midgut infection bottleneck. I believe these high titres are beneficial because of the associated diversity, though sequencing of the different MOI stocks in this experiment is required to confirm they are associated with higher or lower levels of diversity. In mosquitoes, the infection is then a slow process due to the lower levels of replication, owing to the strong purifying selection of the insect cells and repeated strong bottlenecks, taking time for viral population diversity to build. During this time the low-fidelity variants are likely to accumulate deleterious variants due to their erroneous nature, despite the purifying response, and replicate to extinction following Mullers ratchet. This accounts for the attenuation seen in mosquito models of the fidelity variants. Interestingly with the WT we saw stock variation, which suggests at low MOIs the experiments are subject to strong selection forces during handling and repeats should use unique viral stocks. This work shows not only how mammals drive infection of mosquitoes, but also how changing the mutation rate of a virus impacts its ability to survive in vivo infection. Further work to confirm this would involve sequencing of viral populations within mosquitoes at different time points during infection and following blood meals of different MOIs.

Chapter 4 The altered fidelity of TC83_3X does not alter the RNAi response against it compared to parent TC83

4.1 Introduction

Venezuelan Equine Encephalitis Virus (VEEV) is constrained by the need to infect both a vertebrate and invertebrate host and is subject to both a vertebrate and invertebrate immune response. As mentioned in the introduction to this thesis, the RNAi is a key part of the mosquito innate immune response. There are three key aspects to this pathway, the small interfering RNA (siRNA), micro-RNA (miRNA) and PIWI-interacting RNA (piRNA), that have been studied extensively and have been shown critical to maintaining persistent infection of arboviruses (Myles, Morazzini and Adelmn 2009, Liu *et al*., 2019).

For invertebrates the most important anti-viral pathway is the RNAi pathway that is triggered by the presence of double-stranded RNA (dsRNA) during viral infection (Keene *et al*., 2004). RNA viruses naturally generate dsRNA intermediates during their replication, which provide a target for the RNAi (Adelman *et al*., 2002). These RNAi pathways have been extensively studied in *Drosophila* and have been shown to be critical in maintaining the persistent infection of arboviruses in mosquitoes (Myles, Morazzani and Adelmn 2009, Liu *et al*., 2019). In the siRNA pathway, 21nt length small RNAs are recognised and processed in the cytoplasm of mosquito cells. Exonuclease activity of the RISC complex is then used to cut complimentary RNA. siRNAs tend to show even distribution across the genome and are unbiased in the strands they map to. Whilst most studies of the siRNA pathway have been conducted in *Drosophila*, multiple studies have identified 21nt read lengths mapped to arboviruses in mosquitoes and mosquito cell lines (Sabin *et al*., 2013, Miesen *et al*., 2016).

The miRNA pathway uses Dicer-1, Loquacious and Argonaute-1 to generate 22nt miRNAs in the cytoplasm of cells, used to regulate posttranscriptional gene expression, and the piRNA pathway processes viral piRNAs of 24-30nt in length that associate with members of the PIWI clade of argonaute proteins including Piwi, Aubergine and AGO3. The piRNA pathway is involved in gene and transposon silencing in both the nucleus and cytoplasm and is Dicer independent. Secondary piRNAs are produced by the ping-pong pathway explained in the reviews below. piRNAs are reported to map predominantly

to the positive strand of the virus and U1/A10 bias are characteristic of piRNAs of the ping-pong pathway.

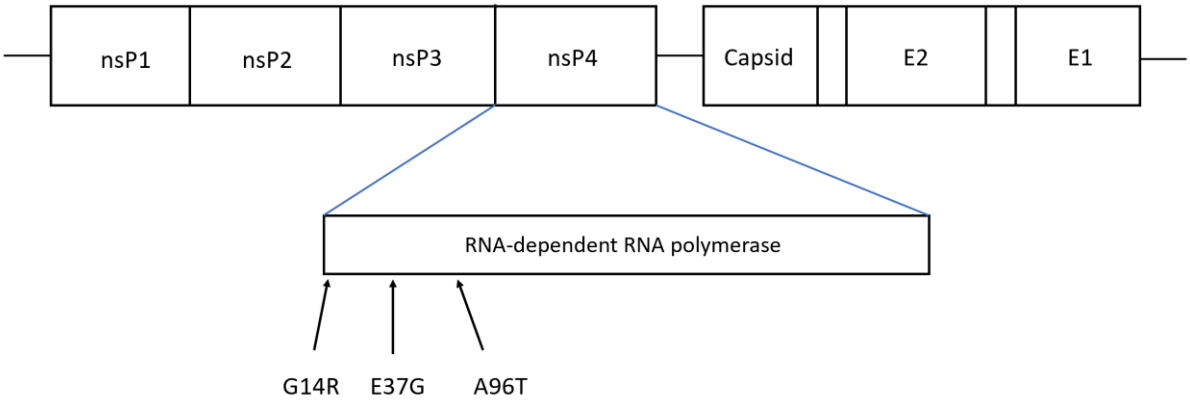


Figure 4.1 The genome of VEE-TC83 with the mutations found in the 3X virus strain. These three mutations include the G14R, E37G and the A96T and are all found within the viral RdRp, affecting fidelity. (Adapted from Kautz *et al*., 2018).

Recent work with VEEV has identified mutations that alter the mutation rate of the virus (Kautz *et al*., 2018). These mutations were generated in TC83, a vaccine strain of VEEV (Berge *et al*., 1961), to make the TC83_3X (3X) virus (Figure 4.1). To investigate how altering the mutation frequency alters the interaction between the virus and the immune response, we used the wild-type TC83 and the 3X virus, described in Kautz (2018). The 3X virus shows increased attenuation compared to TC83 while still inducing complete protection against lethal challenge in mice (Kautz *et al*., 2018). Given recent results showing similar mutations in the wild-type strain 68U201 result in significant attenuation in mosquito infections (Warmbrod *et al*., 2019), we wanted to identify if alteration of the mutation rate alters the virus-host interaction and particularly the siRNA response.

As with the 3X variant of VEEV strain 68U201, the 3X variant of TC83 was unable to successfully infect mosquitoes. Even when using titres 10,000 times higher than expected from equine viremia, the mosquitoes they used were unlikely to be infected. They identified no difference between parent TC83 and the low-fidelity 3X virus replication *in vitro* in mammalian cells and so hypothesised that the mosquito RNAi may be playing a key role in the attenuation of the 3X mutant. In the previous chapter, our results suggested that due to the altered diversity produced by the fidelity-mutants, their attenuation

would be because of inability to traverse bottlenecks in mosquito infection. We subsequently wanted to investigate the impact of the increased diversity on other aspects of infection control, particularly the siRNA response. To investigate how altering the mutation frequency alters the interaction between the virus and the immune response, the wild-type (WT) TC83 and the 3X were used to infect cell culture and the resulting samples were sequenced for their RNAi response.

4.2 Results

To determine if alterations in the mutation rate alter the RNAi response, we used U4.4 cells infected with TC83 or the 3X as wells as mock infected cells. As an additional control we used the C7/10 cell line which lacks a functional siRNA response, to ensure any changes we saw were due to the mutations. Following infection with TC83, the 3X or mock infection, total cellular RNA was harvested at 24 hours post infection (hpi) and underwent Illumina miRNA sequence. RNAs were then mapped to the genomes of TC83 and TC83_3X to understand read lengths and read distributions for the RNAi responses.

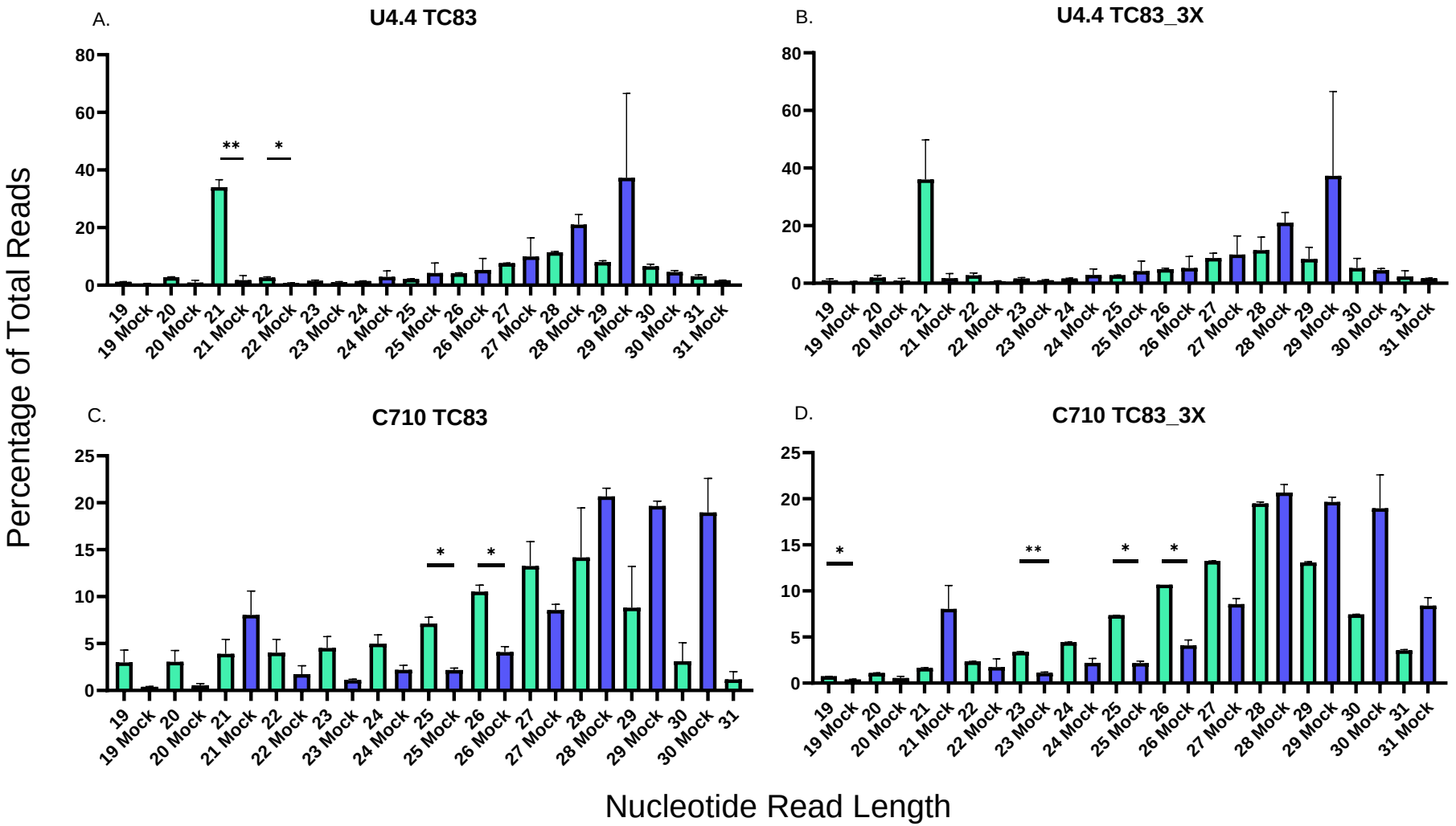

A.
U4.4 TC83
B.
U4.4 TC83_3X
C.
C710 TC83
D.
C710 TC83_3X
Percentage of Total Reads
Nucleotide Read Length

Figure 4.2 The percentage of total reads observed for each read length alongside the corresponding percentage mock infection mapped to the genome of either TC83 or TC83_3X. A. U4.4 Cells infected with TC83, B. U4.4 cells infected with TC83_3X, C. C7/10 cells infected with TC83, D. C7/10 cells infected with TC83_3X. Read count percentages were compared to the corresponding mock count percentages for significance using the unpaired Welch's T-Test. Total reads extracted from cells ranged from 15-37nt in length. Percentage analysis on this subset was performed using the total of all reads.

4.2.1 Relative Abundance of siRNA, miRNA and piRNA response after infection

We wanted to determine if the different mutational spectrum of the 3X virus altered the RNAi response of the cells. The percentages of all reads that corresponded to siRNA (21-22bp), miRNA (22bp) and piRNA (24-30) and their mock infections were plotted to show abundance of these reads following infection in U4.4 or C7/10 cells. In U4.4 cells infected with TC83, read lengths of 21 ($P = 0.0096$) and 22nt ($P = 0.0130$) had significant read count percentages in comparison to their mock. These read lengths are indicative of the siRNA and miRNA response and were not seen in the control C7/10 cells, as expected. Significance was unable to be determined in the 3X virus infection of U4.4 cells, despite distinct 21 and 22nt peaks during infection, due to large variation between the experimental repeats ($P = 0.1722$ and $P = 0.1510$ for 21 and 22nt respectively). This can be expected due to the low-fidelity nature of the 3X virus compared to TC83 and is unlikely to be a difference between the cellular response to the two viruses. This suggests that the altered mutational spectrum between the two viruses does not alter the siRNA response, as initially hypothesised. This makes it likely the observed attenuation is due to the altered diversity of the 3X virus rather than a change in the immune response, corroborating results for 68U201 in the previous chapter.

The same set of experiments were carried out in C7/10 cells as a comparison to the functional immune response in U4.4 cells. Interestingly, the C7/10 cells employed as a control for the siRNA response showed that a piRNA like response may be active in absence of the siRNA response. In C7/10 cells infected with TC83 read length percentages of 25 ($P = 0.0409$) and 26nt ($P = 0.0104$) were identified as significant in comparison to their mocks at 24hpi. In C7/10 cells infected with TC83_3X, read length percentages of 19 ($P = 0.0371$), 23 ($P = 0.0061$), 25 ($P = 0.0178$) and 26nt ($P = 0.0396$) were identified as significant in comparison to their mocks at 24hpi. No other reads were determined as significant in comparison to their mocks at 24hpi in the cellular fractions of U4.4 and C7/10 cells. I hypothesise this is the piRNA response acting when the siRNA response is no longer active. piRNA knockout cells and mosquitoes would be required to determine if this backup response was antiviral.

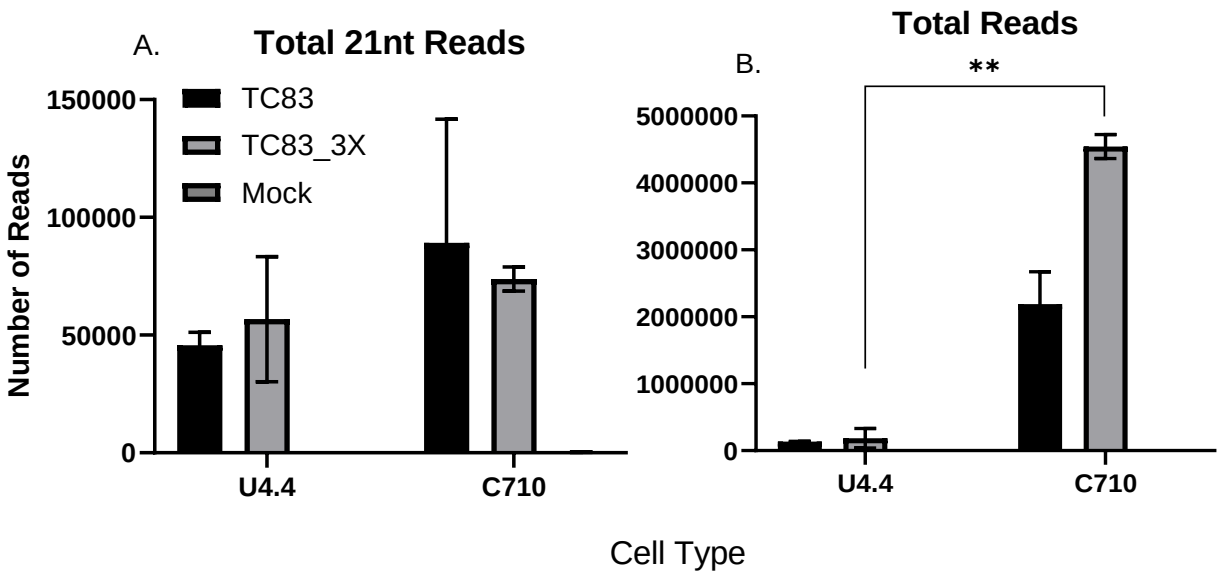


Figure 4.3 The raw read counts generated from sequence reports of the BAM files in R have been plotted for TC83, TC83_3X and mock infection in U4.4 and C7/10 cells. A. Total read counts for 21nt read lengths, B. total read counts for 15-37nt reads. *n = 2*. Results were analysed using an unpaired t-test with Welch's correction.

4.2.2 The total number of siRNA reads produced is higher in C7/10 cells

To gain perspective on the total number of siRNA reads being produced in each cell type with each virus, we plotted the total read counts from the two repeats from each infection. The 3X virus infection produced a greater number of 21nt reads than TC83 in U4.4 cells (P = 0.6587), though shows markedly higher variation between repeats. As previously mentioned, we hypothesised this would be due to the increased diversity spectrum of the 3X, which has been shown to produce significantly higher genetic diversity compared to the parental TC83 regardless of cell type. This graph also shows that despite a lack of siRNA response, C7/10 cells produce a greater amount of 21nt reads for both viruses at 24hpi, though this is not significant between virus types (P = 0.4479 and 0.5292 for TC83 and the 3X virus respectively). Interestingly, the 3X virus produces similar amounts of 21nt reads in C7/10 cells to in U4.4 cells whilst TC83 produced the higher volume of reads and greater variation between repeats.

We also plotted the volume of total reads analysed within this work (lengths 15-37). C7/10 cells produced markedly higher reads than U4.4 cells, though this was only significantly higher between cell types for the 3X virus (P = 0.0017). Interestingly the total reads produced in U4.4 compared to C7/10 was not significantly different for the TC83 (P = 0.1051). There is no significant difference between the total volume of reads produced by either virus in C7/10 cells (P = 0.0637) (fig 4.3 graph B).

4.2.3 Positive and Negative Strand Bias

Virus	Cell Type	Read Length (nt)	Positive Strand Count	Negative Strand Count	Percentage of total reads (%)	P-value
TC83	U4.4	21	22781	22851	34.06	0.9822
TC83	U4.4	22	1783	1786	2.67	0.9905
TC83_3X	U4.4	21	28236	28539	30.60	0.9836
TC83_3X	U4.4	22	2263	2297	2.40	0.9815
TC83	C7/10	25	78513	79264	7.21	0.9783
TC83	C7/10	26	115388	113569	10.47	0.9288
TC83_3X	C7/10	19	15979	17125	0.73	0.3780
TC83_3X	C7/10	23	77622	76186	3.39	0.7374
TC83_3X	C7/10	25	165127	169171	7.36	0.5802
TC83_3X	C7/10	26	246956	238109	10.68	0.4428

Table 4.1 The distribution of significant reads between the negative and positive strands of the genome of TC83 and TC83_3X in U4.4 and C7/10 cells. Counts and percentages are an average of two repeats for the cellular infections. Significance shown in the final column is determined from the raw counts of the positive and negative strand. Significance was determined via an unpaired T-test with the two repeats. 21 and 22nt reads for TC83_3X in U4.4 cells are shown here given limited difference from TC83.

Studies have shown that in some cases there is no bias for the positive or negative strand for siRNAs, mainly due to their generation from dsRNA replication intermediates (Scott *et al*., 2010). To assess if there was a difference between the read counts for the positive and negative strands of the genome for the reads previously identified as significant, an unpaired T-test was conducted (Table one). There was no significant difference between the reads mapped to the positive and negative strand for 21 (P = 0.9822) and 22nt (P = 0.9905) in U4.4 cells infected with TC83, or 21 (P = 0.9836) and 22nt (P = 0.9815) reads in U4.4 cells infected with the 3X virus. Similarly, there were no significant differences in read counts mapping to the positive or negative strand for TC83 for 25 (P = 0.9783) and 26 (P = 0.9288) or for the 3X virus for 19 (P = 0.3780), 23 (P = 0.7374), 25 (P = 0.5802) and 26nt (P = 0.4428) in C7/10 cells infected at 24hpi.

4.2.4 Distribution of 21 and 22nt Reads in U4.4 Cells

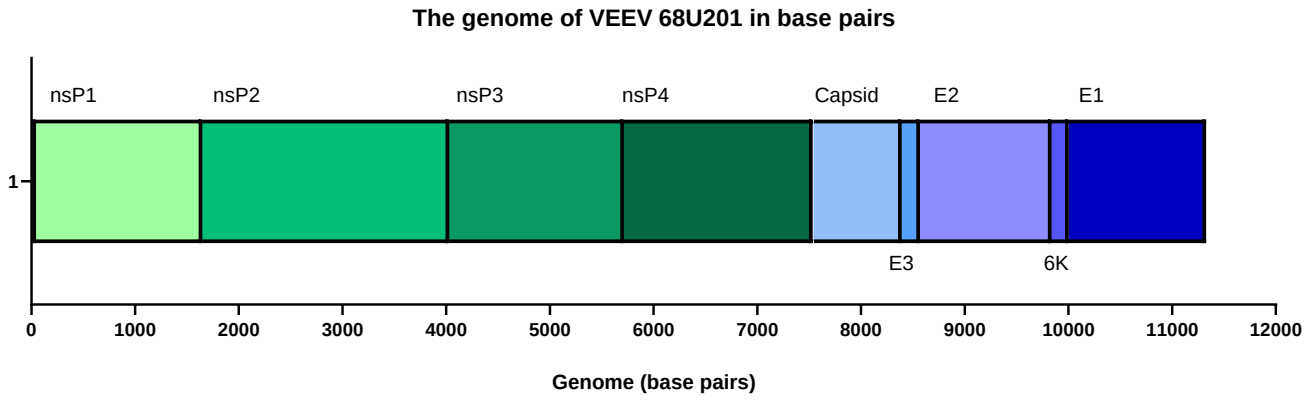


Fig 4.4 The genome of VEEV showing the positions of the genes along the genome. The position of each gene is as follows (base pairs): nsP1 (44-1648), nsP2 (1649-4030), nsP3 (4031-5716), nsP4 (5717-7537), Capsid (7546-7545), E3 (8398-8574), E2 (8575-9843), 6K (9844-10011) and the E1 (10012-11337).

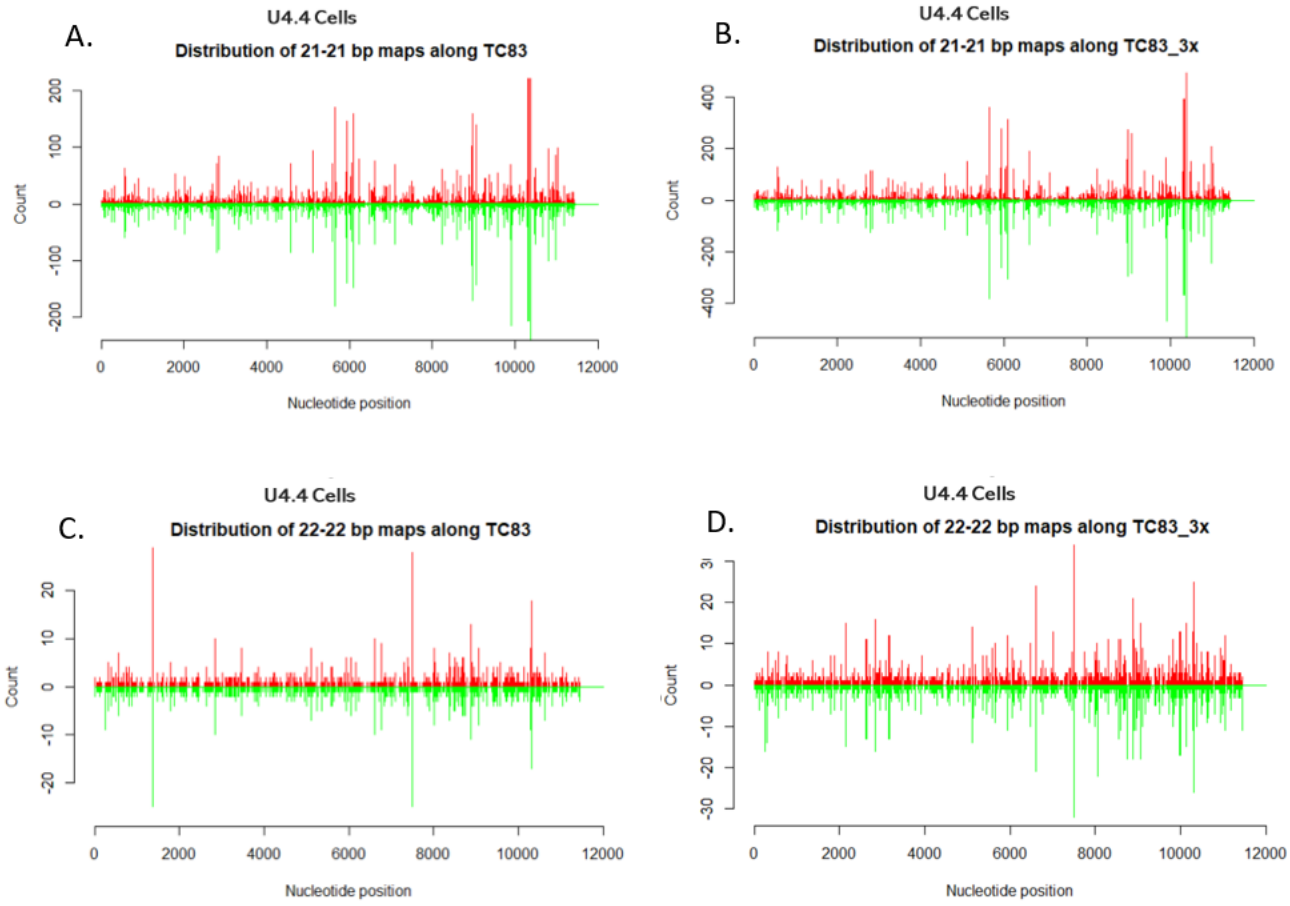


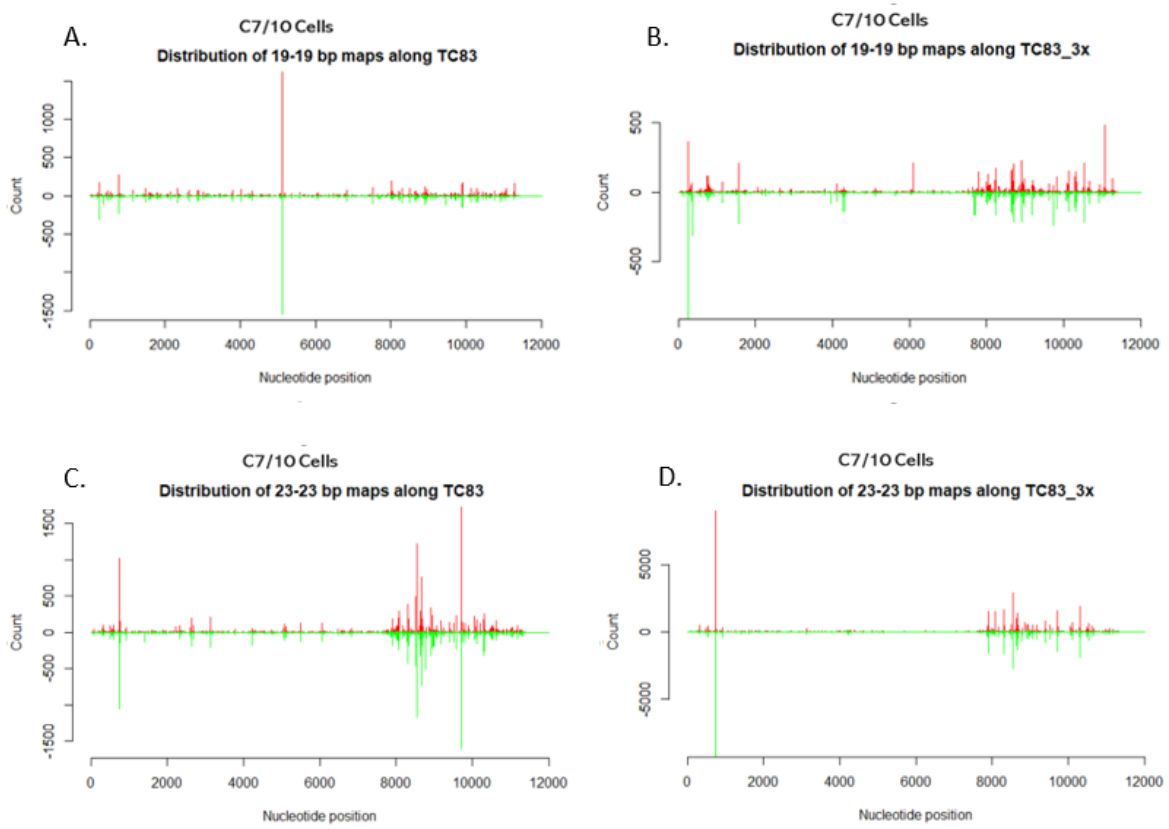
Figure 4.5 The distribution of 21 and 22nt along the viral genomes of TC83 or TC83_3X in U4.4 cells. 21nt reads along A. TC83 and B. TC83_3X and 22nt reads for A. TC83 and B. TC83_3X. *n = 1*. Only one repeat was shown as data could not be combined and there were limited visual differences between graphs.

To determine if there are specific regions of the genome associated with the reads identified during infection, we mapped the reads to their corresponding position in the genome on both the positive and negative strands. We found 21 and 22nt reads are evenly distributed along the genome of both viruses when infecting U4.4 cells. Both viruses show hotspots between bases 5000-6000bp, 9000bp and 10-11,000bp for 21nt reads, suggesting the response is targeting conserved regions in the viral nsP3 and nsP4, E2 and 6K. For 22nt reads both viruses have hotspots at 1000bp for TC83 and then similar increases at 8-9000 and 10-11,000bp, though these increases are minor. This suggests the siRNA or miRNA response in U4.4 cells is targeting the entire genome and is not impacted by the altered diversity

of the 3X virus. For both read lengths and viruses there is slightly more coverage over the structural genes, particularly for the 3X virus. This may be a result of the diversity produced within the viruses and sequencing followed by a Shannon entropy test is required to study the diversity hotspots along the viral genomes.

Aside from higher volume of reads mapped to the 3X virus, which could be attributed to its higher diversity and potentially higher volume of genome copies, there is limited difference in read distribution for 21 or 22nt reads along the genomes of the TC83 and the 3X virus. This suggests that regardless of diversity, there is not a difference in where the siRNA response targets each virus.

4.2.5 Distribution of 19, 23, 25 and 26nt Reads in C7/10 Cells



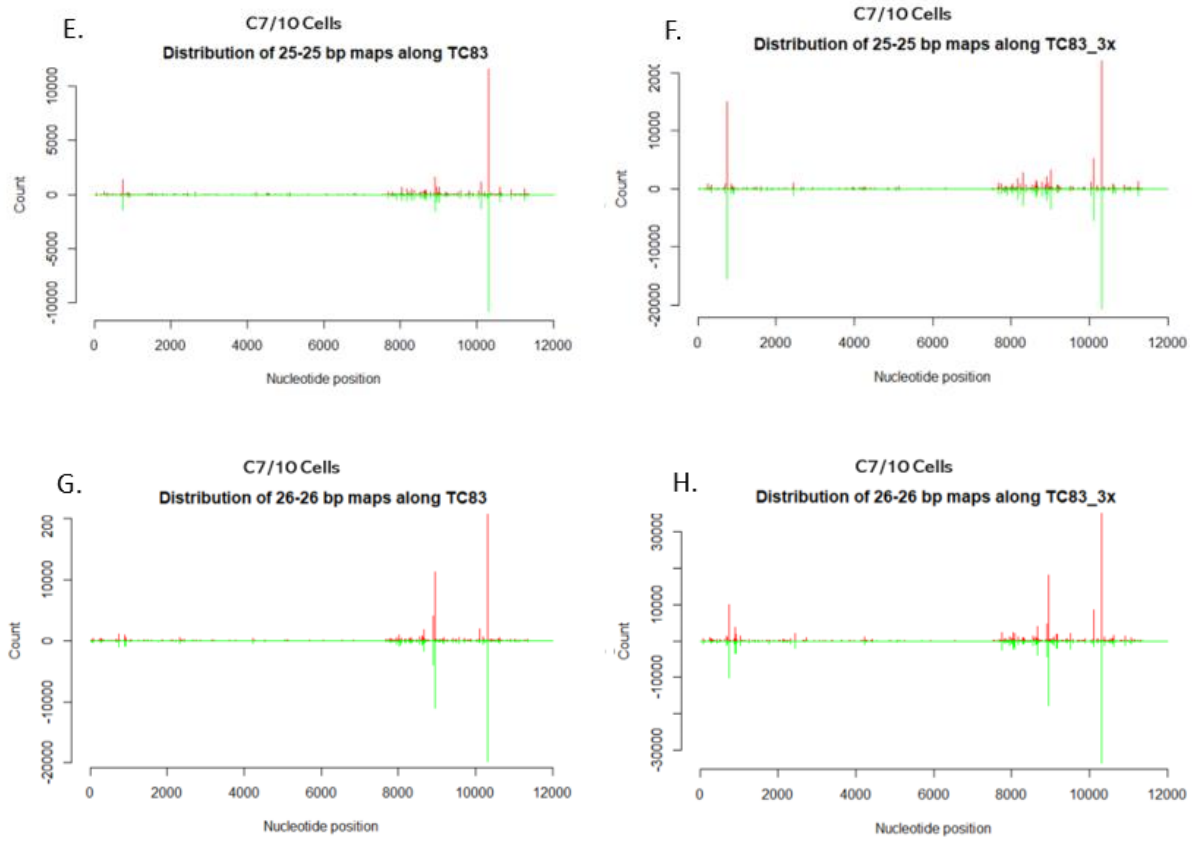


Figure 4.6 The distribution of 19, 23, 25 and 26nt reads along the viral genomes of TC83 and TC83_3X in C7/10 cells. 19nt reads mapped to A. TC83 and B. TC83_3X, 23nt reads mapped to C. TC83 and D. TC83_3X, 25nt mapped to E. TC83 and F. TC83_3X, 26nt mapped G. TC83 and H. TC83_3X. *n = 1*.

Given we saw significance for 25 and 26nt reads in C7/10 cells lacking an siRNA response, we hypothesized the C7/10 cells were producing a piRNA response to compensate and establish persistent infection. As with 21 and 22nt reads, both viruses infecting C7/10 cells show an increase in density of 19nt reads mapped to the structural genes, particularly the capsid, E3 and E2 proteins. This may be due to their importance in forming infectious virions and therefore may increase the need for the cells to target these. The capsid and E2 proteins are heavily involved in entry and exit from the cell and therefore may be more conserved hence targeted to a higher extent. For the 3X virus there is an increase at 500pb in the nsP1. 19nt are not indicative of a piRNA response and C7/10 cells lack an siRNA response, so it is possible this is an miRNA response against the virus or a fault of limited experimental repeats.

23nt reads were identified as significant in C7/10 cells infected with TC83_3X. 23nt reads mapped to TC83_3X show an increase in mapping to the virus at around 1000 in the nsP1 gene. There is also an increase in density from 8,000-11,000 in the structural genes. For comparison, TC83 shows more pronounced reads mapped at the same spots though with fewer counts. In U4.4 cells, the 23nt reads are distributed along the entire length of the genome for both viruses at 24hpi. There is an increase in density for both viruses near 1000bp in nsP1, similar to both viruses in C7/10 cells. These may be mapping to the 51nt and 19nt conserved sequence elements found at the 5' end and 3' end of the positive and negative strand respectively. These elements are limited in their mutational capacity and therefore may be targets for RNAi responses.

25nt reads, significant for both viruses compared to mocks in C7/10 cells show similarities in their distribution. There is in an increase near 1000bp, similar to that seen with 23nt reads and 19nt reads mapped to TC83_3X in C7/10 cells. This increase is larger for TC83_3X 25nt reads in C7/10 cells compared to TC83. Both viruses then show an increase in reads mapped to 8000-9000 and a strong increase just after 10,000. Suggesting the piRNA-like reads are targeting the E1 protein involved in cell entry and exit. Whilst this piRNA pathway is clearly active in C710 cells, there is not evidence to suggest it has antiviral activity against TC83 or the 3X virus, given their increase in titre in C710 cells. piRNA knockdown cell lines could be generated to study the potential antiviral activity.

26nt reads were identified as significant in comparison to mock infections for both viruses in C7/10 cells. The reads show clustering from 7000 to just after 10,000bp for both viruses. Again, indicating strong targeting of the piRNA-like reads to the structural genes, specifically the E2/3 and E1 glycoproteins involved in cell entry and exit. TC83_3X shows an increase in reads at 1000bp, not seen with TC83. This may be targeting conserved sequence elements mentioned earlier.

It is clear that both viruses are being targeted by RNAi responses. Further work is needed to identify the base coverage of the reads mapped to the viruses, as this may give further insight into the RNAi response producing them.

4.3 Discussion

The RNAi response is important to control of viral infections in mosquito cells and was hypothesized to be responsible for the attenuation of both TC83 and 3X viruses in mosquito cells. In the previous chapter we showed that the altered diversity of the 3X virus reduced its ability to traverse bottlenecks compared to the WT 68U201, alongside two other low-fidelity mutants. This is likely the reason for its observed attenuation in mosquito infection. Given the RNAi is the key antiviral response in insect cells, we wanted to rule out the role of the RNAi response in the attenuation of the TC83_3X virus. The vaccine strain TC83 and it's low-fidelity mutant the 3X, were used to infect U4.4 and C7/10 cells and the small RNAs produced were analysed to determine which of the RNAi pathways is involved in controlling the infection of the individual viruses. The results suggest that the siRNA pathway targets both TC83 and the 3X virus equally, suggesting that the altered fidelity of the 3X virus does not interfere with the RNAi response. Interestingly, a piRNA-like pathway appears to be a redundant pathway when the siRNA is inactive in C7/10 cells.

4.3.1 The altered mutation rate of TC83_3X does not change the siRNA response against it compared to parent TC83

There are many studies that identify read lengths of 21nt as characteristic of the siRNA response in mosquitoes. 21nt small RNAs were identified when *A. aegypti* was infected with Sindbis virus (SINV) and were isolated from *Culex* cell lines when infected with West Nile Virus (WNV) (Myles *et al*., 2008, Ruckert *et al*., 2019). In 2009, Brackney identified viral siRNAs of 21nt in length mapped to WNV in mosquito midguts following a 7- or 14-day extrinsic incubation period. Our work suggests that the siRNA response is the dominant antiviral pathway against both TC83 and the 3X virus as read lengths of both 21 and 22nt were identified as counts significant in comparison to their mocks at 24hpi in U4.4 cells. The significance was unable to be determined for the 3X virus at 24hpi, though there is a clear increase in 21nt reads in comparison to the mock infection. The high error bar prevents statistical analysis; however, we believe this variation in repeats is due to the increased diversity produced by the 3X virus and the results in the previous chapter suggest that further repeats would not lend more clarity due to the erroneous nature of the 3X virus. Furthermore, both 21 and 22nt reads in U4.4 cells for both

viruses show signatures of antiviral siRNAs, with no strand bias and even distribution along the genome leading us to conclude there is no difference in siRNA response between the two viruses.

*Alphavirus*es and *flavivirus*es synthesise the negative strand of the genome for a limited period, compared to synthesis of the positive strand genome that continues for much longer during infection. Some studies have provided evidence for siRNA bias towards the positive strand. A study to determine the impact of mutational diversity of WNV on the RNAi response infected *Culex* midguts and sequenced RNA after a 7- and 14-day EIP as previously stated. They found that WNV siRNAs derived from the positive strand at 74% in both EIPs tested (Brackney *et al*., 2009). Additionally, Myles (2008) injected *A. aegypti* mosquitoes with double subgenomic SINV (dsSINV) and dsSINV expressing the Flock House Virus B2 protein. Comparison of the resulting 21nt siRNAs derived from the positive and negative-sense strands of both viruses found a significant bias for accumulation from the positive strand.

However, positive strand bias does not appear to be conserved across arboviruses. Deep sequencing of viral siRNAs from various mosquito species and cell lines identified viral siRNAs from SINV and Dengue virus that are present in equal ratios of genome to anti-genome and distributed along the length of the genome (Myles *et al*., 2008, Scott *et al*., 2010). 54-60% of DENV siRNAs in Aag2 cells were positive sense at 5dpi, with DENV infected mosquitoes even closer to the 1:1 ratio with 55% mapping to the positive strand, leading the researchers to confirm siRNA reads are generated from double stranded replication intermediates during viral replication (Scott *et al*., 2010). This was corroborated in 2013 by Sabin when 21nt reads sequenced from Vesicular Stomatis virus-infected *Drosophila* cells were evenly distributed between the sense and antisense genome of the virus at a ratio of 1.2 to 1 and Rift Valley Fever virus (RVFV)-generated 21nt siRNAs were derived from the large and medium segments of the genome from both the positive and negative strands at a 1.3 to 1 ratio (Sabin *et al*., 2013). However, it is worth noting both latter are negative-sense viruses and RVFV is a segmented virus and so they may show slightly different distributions compared to the positively stranded, non-segmented viruses in this study. In our study, both 21 and 22nt reads are evenly distributed between both the positive and negative strands, indicating no bias. This suggests that these are generated from double stranded

DNA intermediates, characteristic of the siRNA response in U4.4 cells infected with both TC83 and TC83_3X.

Viral siRNAs mapped to the WNV genome showed asymmetric distribution along the genome with some regions highly targeted and others weakly or not targeted at all. The most intensely targeted region in this case was the capsid (Brackney *et al*., 2009). We saw distribution of 21nt reads mapped to the genome of both TC83 and the 3X virus show peaks in the 5000-6000, 9000 and 11,000bp. Interestingly, the 5000-6000bp peak in density suggests a high number of siRNAs targeting the nsP3 (4031-5716bp) and nsP4 (5717-7537bp) regions. This is interesting as the RdRp is fundamental to the replication of the virus within the infected cells. Targeting this gene could lead to increased attenuation or lower titre of virus and as such aid the mosquito cell in its immune response and may contribute to achievement of persistent infection in these cells, preventing the virus from replicating to titres detrimental to persistent infection.

Further hotspots include the E2 and 6K proteins. Previously, two mutations in the 6K were identified by Forrester (2011) and were shown to result in abnormally high diversity (Patterson *et al*., 2018). Furthermore, the E2 glycoprotein is involved in cell receptor binding and so provides a strong target for the cell. Finally, the 11,000 peak rests in the E1 glycoprotein, maintaining the same functions as E2. We see similar peaks for 22nt reads, and further peaks around the capsid gene. For DENV-2 small RNAs were evenly distributed along the entire length of the genome when infecting Aag2 cells (Miesen *et al*., 2016, Scott *et al*., 2010), though the latter found a higher portion of reads at 10,000bp. In mosquitoes, they identified several hostspots on the positive and negative strand that they attributed to secondary structures within the genome. In future this experiment should be repeated in mosquitoes and the resulting RNAi-produced reads can be mapped to secondary structures to identify any correlation.

Kautz (2018) identified diversity hotspots along the TC83 genome that are similar with the 3X virus. These include the E1 C10141U, C10371U, C10743G, C11009A, U11269C. Additionally, spots shared between TC83 and 3X virus include; nsP2 C2627A and A2634Del, E2 U9561G, 6K C9978A and E1 C11009A. Based on our distribution data, it appears the 21nt reads are targeting conserved regions of the viral genomes, such as around 6000bp in the nsP4 gene. The 21nt highly targeted region of 9000bp

and 10,000bp are slightly out of range of the diversity hotspots listed above. The diversity hotspots in the E1 gene were more prominent for TC83 than the 3X virus and may be reflected by the fact there are half the amount of 21nt reads mapping to this spot for the TC83 genome than the 3X virus. Though this appears to be the case for most positions and so cannot be attributed to be an effect of this particular diversity spot.

22nt reads are again evenly spaced along the entire genome of both viruses, with higher densities at 1,500, 7,500 and 10,500bp. These results suggest that the altered diversity of the 3X virus is not changing how the siRNA response targets the virus during infection at 24hpi. As with 21nt reads, the 22nt reads do not appear to map to areas of the genome considered diversity hotspots. This further suggests the siRNA response is targeting conserved regions of the genome. These regions include the 3' end of the nsP1 gene which contains a conserved stem loop, the section of the genome between the nsP4 gene and the start of the capsid gene, and the earlier half of the E1 gene. This suggests that the cells use the different read lengths of the siRNA response to target different regions of the virus.

Our results suggest that altering the fidelity from parent TC83 does not alter the cellular siRNA response to the virus. This corroborates that the attenuation seen with these viruses in mosquitoes and mosquito cells is due to an inability to traverse repeated bottlenecks, rather than its interaction with the RNAi response. This is corroborated as the 3X virus was attenuated in C636 cells in the previous chapter and Warmbrod (2019) found them attenuated in mammalian models. This suggests that the attenuation of the virus is a result of repeated bottlenecks within a multicellular and multi-host environment as we have previously shown.

4.3.2 The piRNA pathway is active in siRNA deficient cells

Whilst we determined the changed interaction with the siRNA response is not the reason for the attenuation in mosquito models, we identified a piRNA-like response as a back-up in siRNA deficient cells. Scott (2010) identified small RNAs in Aag2 cells like the siRNA response, whilst the C636 cells produced longer, positive strand reads likely to have been produced by another pathway. In our experiment when TC83 infected C7/10 cells, cells defective in the siRNA response, reads lengths

indicative of the piRNA response were identified. Counts of 25nt and 26nt read length were identified as significant compared to their mocks in cellular infection. The piRNA response results in reads of the lengths 24-32nt (Ruckert *et al*., 2019, Morazzani *et al*., 2012), suggesting that the C7/10 cells mount a piRNA response against TC83 when the siRNA pathway is defective.

As mentioned earlier, *alphavirus* replication of the positive strand of the genome occurs for longer than that of the negative, and so it could be expected that the piRNAs would derive more from the dominant strand. piRNAs generated from SINV-infected C636 cells mapped almost exclusively to the positive strand and WNV and SINV 28nt reads in C636 were also predominantly from the positive strand (Scott *et al*., 2010, Ruckert *et al*., 2019) and Miesen (2016) found 25-30nt length reads that mapped exclusively to the positive strand. However, for our work neither 25nt or 26nt reads show bias towards the positive strand of the genome. In C7/10 cells infected with TC83 and the 3X virus, the 19, 23, 25 and 26nt reads identified as positive in comparison to their mock showed equal distribution between the positive and negative strands of the genome. This suggests that the reads identified in C7/10 cells mapping to TC83 and the 3X are not piRNAs. It is interesting to note that whilst both *alphavirus*es and *flavivirus*es piRNAs tend to show extreme bias towards the sense strand of the viral genome, Bunyavirus piRNAs are produced at more equal ratio (Meissen *et al*., 2016). Though it is unknown if this is due to the antisense genome or if it is how the piRNAs interact with the RNA of specific viruses (Meissen *et al*., 2016). TC83 is a mutated *alphavirus* and therefore may not exhibit the same piRNA patterns as expected for *alphavirus*es. The results for Bunyaviruses indicate the piRNA response cannot be expected to follow the same patterns with each individual virus. We still hypothesise that the 25-26nt reads are a result of a piRNA-like pathway in the C7/10 cells against both viruses.

It is possible that the piRNA response differs between virus-cell combination and therefore we are not seeing positive strand bias. In some cases, it has been shown that piRNAs are biased to specific regions of the genome compared to siRNAs that have targets throughout the genome (Miesen *et al*., 2016). 25 and 26nt length reads both show density around the 1000bp region, encoding the VEEV nsP1, involved in initiating negative strand synthesis. Increased targeting in this region by the piRNA response may alter the rate at which the virus replicates its negative strand. This could mean that more negative strand

is synthesized more than normal, offering a potential explanation to the even distribution of reads between the negative and positive strand, rather than positive strand bias. There is a peak of reads for both viruses for 25 and 26nt from 8000-9000, part of which spans the capsid protein coding region. The capsid protein is involved in binding to RNA to facilitate virion assembly, whilst also inhibiting host cellular transcription. Targeting of the capsid with the piRNA response may reduce the titer of infectious virus within the cell. Additionally, these reads are spread between 8000 and 9000 and so are also targeting the E3 and E2 proteins. E2 is involved in receptor binding and so is an important region for the cell to target. The dense peak at 10,500 is also situated around the coding region of E1, another glycoprotein involved in receptor binding. These proteins appear to be targeted by the piRNA response in C7/10 cells infected with TC83. The identified peaks occur in both the positive and negative strand which could be expected given there is no strand bias, though offers little explanation.

Importantly, piRNAs show characteristic bias towards U1 on the primary piRNA and A10 of the secondary piRNA (Miesen *et al*., 2016). Primary piRNAs are antisense to the target strand whilst secondary piRNAs have been cleaved in the ping pong pathway at the 5' end (Ruckert *et al.,* 2019). C636 cells infected with SINV as a positive control for vpiRNAs and WNV individually showed peaks at 28nt, indicative of vpiRNAs. Whilst SINV showed the classic nucleotide bias, WNV had no nucleotide bias and the study states that they cannot rule these reads as vpiRNAs lacking nucleotide bias as has previously been seen for other *flavivirus*es in *Aedes* cells due to lack of experimental evidence. Further analysis into the nucleotide bias of these reads is needed to validate our conclusions.

The results suggest there is a piRNA-like pathway acting as a redundant pathway active in cells that lack an siRNA response. This may be a secondary mechanism to reduce the titre of the viruses to achieve persistence and may account for the higher titres in C7/10 cells if this pathway is not as potent as the siRNA response. Furthermore, future experiments with piRNA knockdown cells and mosquitoes are required to understand if the piRNA pathway plays an antiviral role against TC83 or the 3X virus.

This corroborates the hypothesis that piRNA-like small RNAs can mount an antiviral defense in mosquito cell lines that are defective in the siRNA response (Morazanni *et al*., 2012). Once the siRNA pathway has been knocked out, a redundant pathway becomes the dominant pathway in antiviral

defense. As previously mentioned, Ruckert (2019) studied WNV infection alone and with other viruses in a range of *Culex* mosquito midguts, salivary glands and cell lines. The WNV infections produced a strong 21nt siRNA response but showed no evidence of viral piRNAs. When infecting C636 cells, there was a peak at 28nt. However, the lack of base bias led the study to believe these may not be piRNAs. The evidence collected for this paper suggests that the siRNA response is the dominant RNAi pathway against both TC83 and the 3X virus, and that once this pathway is rendered inefficient, a redundant pathway that produces vpiRNA-like small RNAs becomes dominant. Given this, it is unlikely the piRNA response plays an important antiviral role in transmission of TC83 or the 3X virus, however it does suggest that any variants capable of avoiding the siRNA pathway may be targeted by the piRNA pathway. Further work with siRNA deficient mosquitoes would be beneficial to study this hypothesis *in vivo*.

4.4 Conclusion

To conclude, the siRNA response is active against both TC83 and the 3X, and the RNAi responses in the cell culture infection is not leading to the same attenuation seen in mosquito infection. This suggests there is a different reason for the attenuation seen in mosquitoes. Our previous chapter provided evidence that the altered diversity of the G7R, 3X and 4X variants of 68U201 prevented them from traversing multiple bottlenecks that are associated with arboviral infection. This work shows that the siRNA response is not altered against TC83 by changing its diversity as with the 3X virus. We previously hypothesized that in immune competent cells the siRNA response acts to purify the viral population, removing defective particles and allowing survival of the viral population in doing so. These results show that the siRNA response is active against the 3X and is likely involved in purifying selection in insect cells. We also see higher read counts in the C7/10 cells compared to the siRNA competent U4.4, suggesting that the U4.4 is removing more viruses than C7/10, as expected. In cells lacking the siRNA response, reads similar to the piRNA pathway are targeting both viruses. Despite their characteristic piRNA length, these reads do not show the characteristic strand or base bias of the piRNA pathway, however given both TC83 and the 3X virus are mutated *alphavirus*es with a differential mutation spectrum, it may alter the characteristics of these responses. These results suggest that a piRNA-like

response is a redundant pathway that acts second to the siRNA response. This may mean it can target variants that have evolved to evade the siRNA pathway, or that it does not play an active role in mosquito control of either of the two viruses tested.

Chapter 5 Exposure to the Agricultural Chemicals Glyphosate and Triticonazole May Alter the Vector Competence of *Aedes aegypti* to Chikungunya and Zika Virus

5.1 Introduction

Mosquitoes are important vectors for medically important arboviral pathogens. Mosquitoes are reared in habitats that contain still water, including stagnant streams, ponds or old tires that may be contaminated with agricultural or horticultural chemicals. The microbiota of mosquitoes has been shown to influence pathogen susceptibility and mosquito immune responses and is largely influenced by the environment in which they are reared. Several studies have focused on the impact of glyphosate on the bee microbiome and mosquito vector competence for malaria. It is likely exposure to agricultural chemicals may alter the mosquito microbiome and given its importance, impact their vector competence for medically important arboviruses. Our goal is to identify if treatment with a commonly used herbicide; glyphosate (GLY) and a fungicide; triticonazole (TRI), alters the vector competence of *A. aegypti* for Chikungunya Virus (CHIKV) or Zika Virus (ZIKV). We then aim to identify the mechanisms behind the changes in competence by looking into changes to the mosquito immune response.

5.1.1 Glyphosate and Triticonazole

GLY is the most widely used herbicide in the world, with 6.1 billion kgs applied between 2006-2016 (Benbrook *et al*., 2016). It is a non-selective broad-spectrum herbicide that competitively inhibits the EPSP synthase in the shikimate pathways (responsible for aromatic amino acid production) in plants, killing them, and has bacteriostatic properties (Steinrucken *et al*., 1980, Poppe *et al*., 2019). Since its introduction in 1974 GLY usage has dramatically increased due to low cost and genetically resistant crops (smith *et al*., 2021).

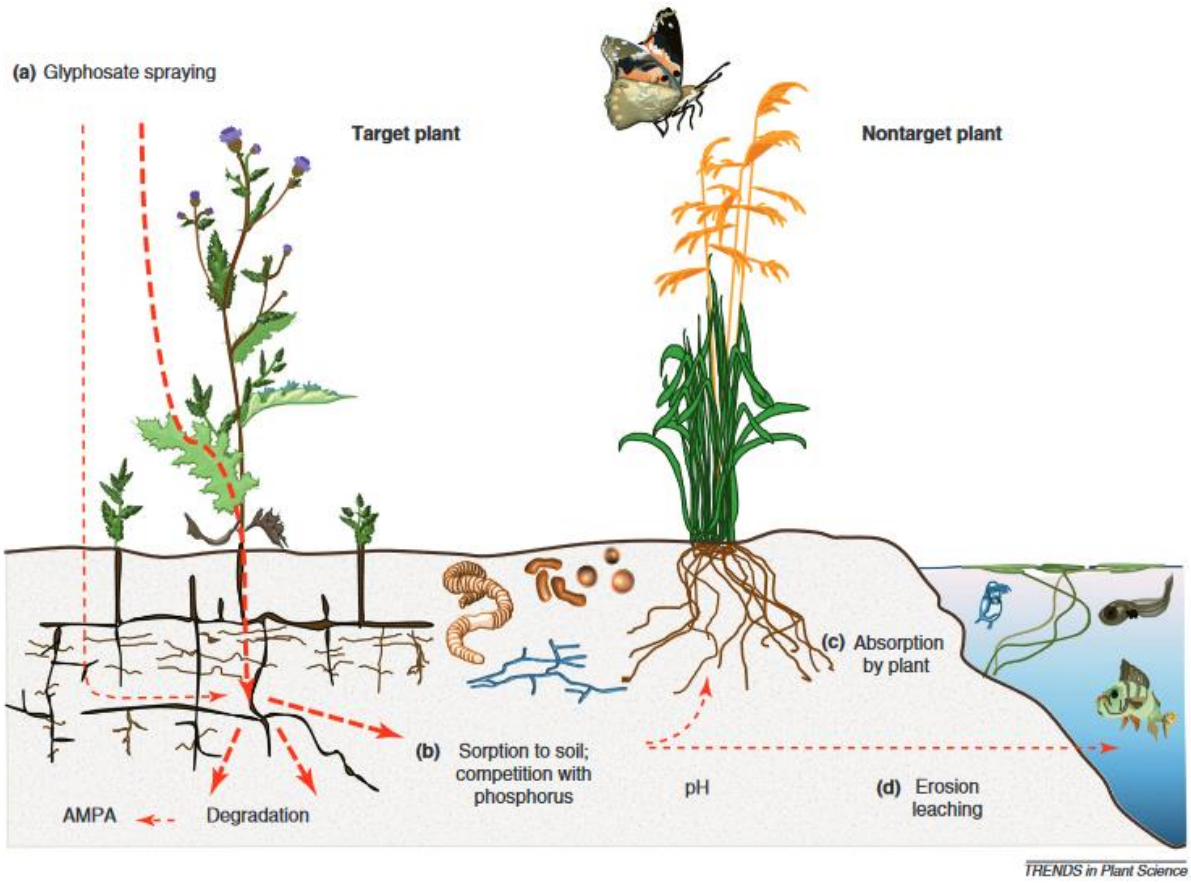


Figure 5.1 How GLY can accumulate in and be transported through an ecosystem. This can be through A. directly on soil and absorbed by plants to then be translocated throughout the plant including shoots and roots. B. Exuded from the roots into soil and then consumed by soil animals or microbes. C. residues taken up by nontarget plants, can alter the plant or end up in animal feeds. D. soil erosion and leaching of surface water transports GLY to aquatic systems. (Helander, Saloniemi and Saikkonen 2012).

In 2019, 40% of the volume of fungicides used on cereals were azoles (Jorgensen and Heick, 2021). TRI is an azole fungicide generally applied to cereals, turf and ornamentals in a preventative or curative manner. The fungicide works by inhibiting sterol synthesis thus preventing production of ergosterol, an essential component in fungal cell membranes (Borjesson *et al*., 2003). The US EPA has suggested that TRI may be genotoxic to non-target organisms and the European safety authority scientific report in 2005 stated it may pose a risk to aquatic organisms (Authority EFSA, 2005).

5.1.2 Exposure to agricultural chemicals has been shown to alter immune expression and microbiota.

GLY experiences widespread use and isolation of GLY from human urine is common, resulting from occupation/residential exposure, identified in multiple studies covered by (Niemann *et al*., 2015). Recently, GLY has been the focus of many studies on its effect on non-target species, altering the microbiome, causing immune dysregulation, and changing pathogen susceptibility. GYL has been shown to cause neurotoxicity and seizures in round worms, and cause immune dysregulation in, and alter the microbiota of honeybees. Motta (2022) exposed honeybees to different concentrations of GLY and a previously tested antibiotic and monitored immune changes and their effect on opportunistic pathogens. In one of the replicates, done in the fall season, they found GLY induced a 3.6-fold downregulation of the gene for an important antimicrobial peptide and a toll-like receptor 4 in the honeybee midguts. When the whole bodies were tested, they found a significant decrease in apidaecin antimicrobial peptide (AMP), defensin-2 and hymenoptaecin. The two opportunistic bee pathogens they tested did not show susceptibility to these AMPs and so were unlikely to be affected by these changes, however, important species of the gut microbiota did show susceptibility increasing the likelihood the gut microbiota would change following these perturbations of immune components.

TRI has not been studied as extensively as GLY, however Roman (2022) found soil microorganisms decreased following exposure to TRI. As TRI inhibits sterol synthesis and bacteria do not contain sterol in their membrane, they concluded this was due to indirect effects of TRI on the bacterial population due to functional and nutritional connections with fungi. To our knowledge, few studies have investigated the effects of TRI on the microbiome or immune response of multicellular organisms. We hypothesise that exposure to GLY or TRI will alter *A. aegypti* vector competence through changes to the microbiome and/or immune responses.

The mosquito immune system is key to antiviral defence. We hypothesise the chemicals will alter the innate immune gene expression or microbial composition of the mosquito gut, both of which may cause indirect changes to the other, leading to changes in the vector competence of *A. aegypti* for CHIKV or ZIKV. To determine if exposure to GLY or TRI affects the vector competence of *A. aegypti*, we placed *A. aegypti* eggs into H2O containing 0.5mg/L of GLY, TRI or control and reared the mosquitoes to adulthood. 0.5mg/L had previously been found not detrimental to mosquito lifespan (data not shown).

Once in adult stages, mosquitoes were fed GLY, TRI or control at 0.5mg/L in sugar water. We monitored mosquito life history traits in the form of pupal emergence, pupal weight, and adult mosquito survival. We then challenged mosquitoes with either CHIKV or ZIKV and determined infection percentages and dissemination rates. Changes to viral titre in each of the treatments was also determined in cell culture growth curves. Finally, we screened for immune gene expression in a range of mosquito and cell culture systems. We found that exposure to TRI reduces the vector competence of *A. aegypti* for CHIKV and ZIKV by reducing the percentage infected, dissemination rate and survival. GLY reduces the percentage of mosquitoes infected however increases the dissemination rate and survival, increasing the likelihood of infected mosquitoes transmitting virus during each transmission season. The immune gene results were largely inconclusive. Our work is important to understand how these commonly used agricultural chemicals may impact the spread of vector borne diseases as our climate moves towards hosting important disease-carrying mosquito species.

5.2 Results

5.2.1 Mosquito Life History Traits

5.2.3 Pupal Emergence

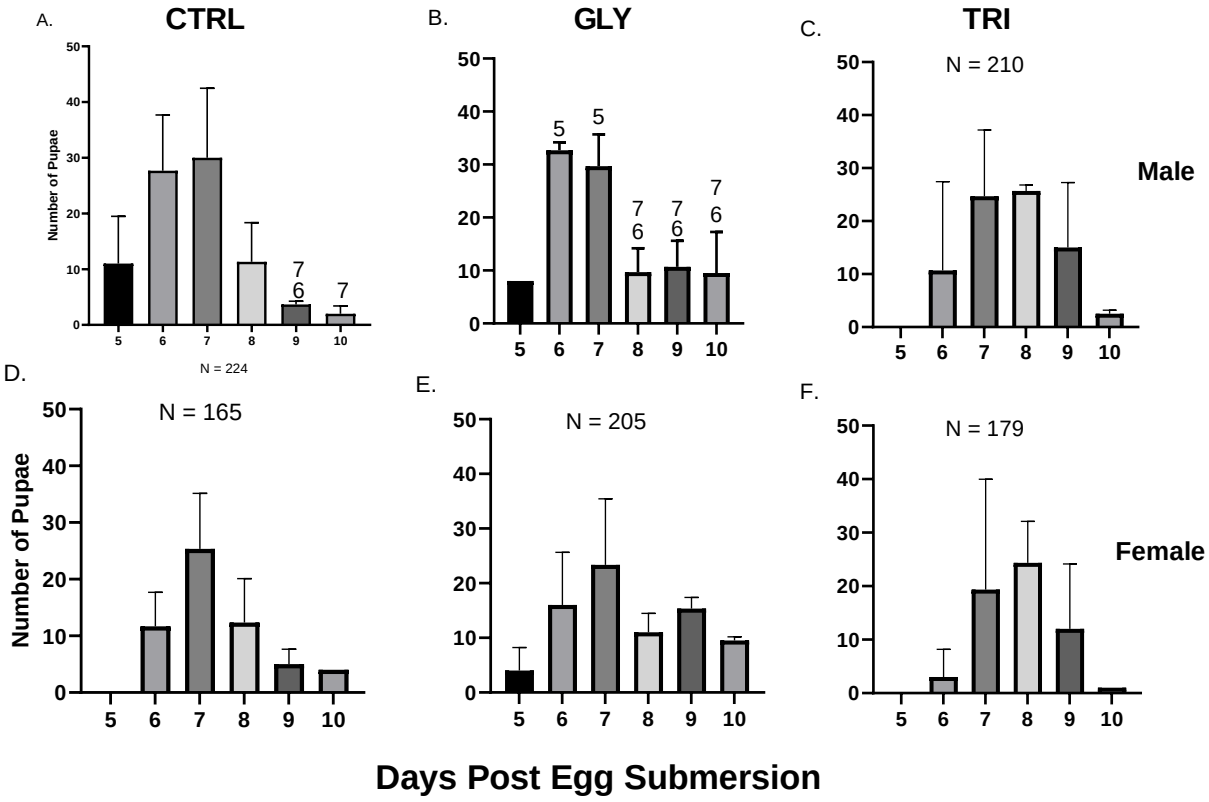


Figure 5.2 The emergence of pupae in each treatment measured by days post egg submersion DPES (day on which the eggs were first placed in the treatments or control). The graphs show the male pupae emergence in the treatments: A. CTRL, B. GLY and C. TRI, and the females in the treatments: D. CTRL, E. GLY and F. TRI. N on graph represents total pupal numbers, n = 3 experiments. Due to multiple significant differences on one graph, the significance is marked with a number representing the days post egg submersion (Graph B DPES 6 and 7 are marked with a 5, because the number of pupae emerging on those days were significantly higher those that of day five).

To determine if exposure to GLY or TRI altered the life history traits of *A. aegypti*, we monitored pupal emergence starting from the day the eggs were placed in their appropriate treatments. As seen in figure one, CTRL male show a steady emergence curve starting on day 5 and finishing on day ten with the peak emergence at day 6 and 7. Day 6 pupae were significantly higher than day 9 (P = 0.0446) and day 7 pupae were significantly higher than days 9 (P = 0.0264) and 10 (P = 0.0352). Control female show a similar curve but starting on day 6 and finishing on day 10 with day 7 the peak day. GLY males show

strong peaks at days 6 (significantly higher than days 5 (P = 0.0021), 8 (P = 0.0015), 9 (P = 0.0021) and 10 (P = 0.0033) and 7 (significantly higher than days 5 (P = 0.0053), 8 (P = 0.0043), 9 (P = 0.0061) and 10 (P = 0.0088) and similar levels for all other days from 5 to 10. GLY females emerge a day earlier than ctrl females and show a similar curve to control females, although a higher number of pupae emerged on days 9 and 10. TRI males show emergence a day later than the GLY or CTRL males, with peak days on day 7 and 8 but sharply declining by day 10. TRI females emerge similarly to control females and TRI males, starting day six, peaking days 7 and 8 and finishing on day 10 with a sharp decline, though the main peak is a day later for the TRI females. None of the females from any treatments showed any significant difference in the number of pupae emerging between days by a One-way ANOVA on Graphpad Prism.

5.2.4 Pupal Weight

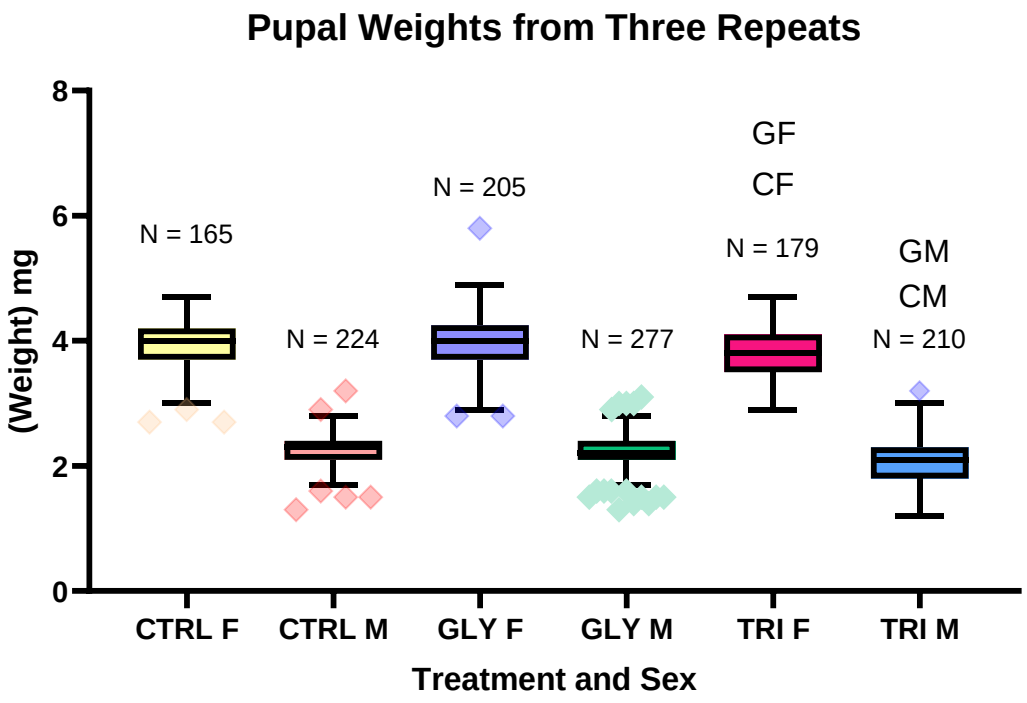


Figure 5.3 The weight (to the nearest mg) of male and female pupae following the CTRL, GLY or TRI treatments. Data was collected from 3 experimental repeats. N on graph represents total pupal numbers, n = 3 experiments. The weight of females and males from the treatments were compared separately, using a one-way ANOVA. Significant differences to TRI treated samples are shown using initials. GF (GLY female), CF (CTRL female), GM (GLY male) and CM (CTRL male).

Mosquito size can impact vector competence and capacity in several ways. Larger female mosquitoes can lay larger egg batches, increasing the numbers of mosquitoes during a viral outbreak and mosquito size has also been shown to impact vector competence, with smaller *Aedes triseriatus* more likely to become infected with La Crosse Virus (Hawley 1985, Armbruster and Hutchinson 2002, Grimstad and Walker 1991). To determine how treatment with 0.5mg/mL affected *A. aegypti* pupal size, we weighed the pupae to the nearest 0.1 of a mg upon emergence. GLY treated pupae show similar weight distribution as the control group for both males and females, suggesting they do not alter the vector competence of *A. aegypti*, as described above, by changing the size of the mosquito. TRI females and males weigh significantly less than both the control and GLY groups (males $P = <0.0001$ and $P = <0.0001$, females $P = 0.0022$ and $P = <0.0001$ for CTRL and GLY respectively). Though control and GLY treated males and females appear to have more outliers than TRI treated males suggesting they have more variation in pupal size. Aside from this there are no drastically visible differences. This suggests that TRI alters the size of both male and female mosquitoes and therefore may alter the vector competence this way.

5.2.5 The Effect of The Treatments on Viral Titre at a Cellular Level

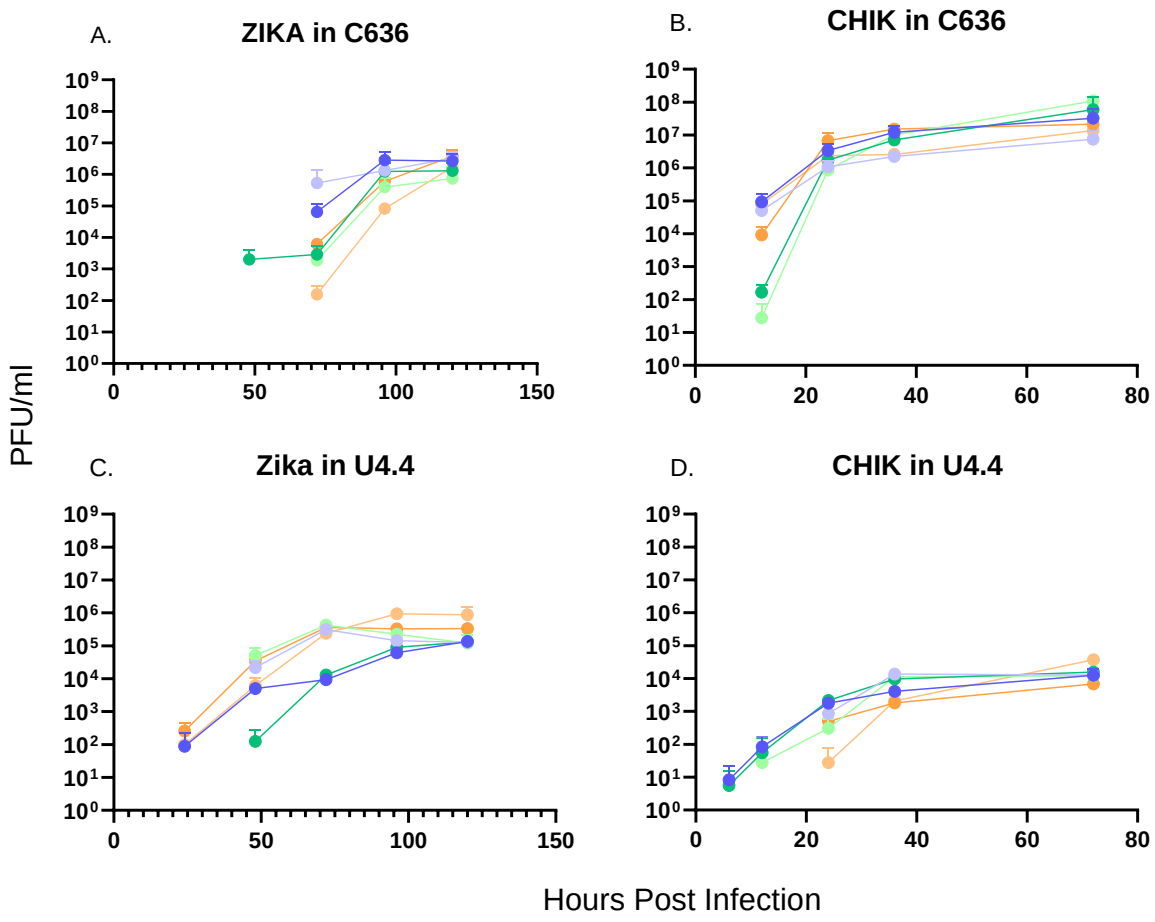


Figure 5.4 The pfu/mL of CHIKV and ZIKV in U4.4 and C636 cells following treatment with GLY or TRI at 0.5mg/L. Graph A and C show ZIKV PFU/mL across five timepoints in C636 and U4.4 cells. Graphs B and D show CHIKV PFU/mL across five timepoints in C636 and U4.4 cells. n = 3. Results were analysed by a one-way ANOVA in GraphPad Prism. Points are where the virus was detectable by plaque assay, missing points indicate no quantifiable virus by plaque assay.

To understand how the treatments may affect viral infection of mosquitoes at the cellular level, U4.4 and C636 cells were passaged three times in each treatment (0.5mg/mL) or control. On the day of infection cells were counted and infected with either CHIKV or ZIKV at an MOI of 1 or 0.01. Supernatant was harvested at five time points, 0, 6, 24, 36 and 72hpi and 0, 24, 48, 72, 96 and 120hpi for CHIKV and ZIKV respectively with three repeats for each. Samples were plaque assayed to determine viral titre from each time point. No significant differences were determined between treatments via a one-way ANOVA.

For C636 cells infected with ZIKV, at 72hpi the CTRL MOI 0.01 shows increased titres whilst the other treatments and MOIs produce lower amount of infectious virus. By 96hpi both CTRL MOIs resulted in high titres matched only by GLY MOI 1. ZIKV titres from both TRI MOIs and GLY MOI 0.01 are slower to reach higher levels. Interestingly, by 120HPI the TRI cells infected with ZIKV at an MOI of 1 has produced the highest titre closely followed by both CTRL MOIs, the MOI of 1 having slightly dropped in titre on average, perhaps due to having infected most of the cells at an earlier time point. ZIKV produced in TRI MOI 0.01 and both GLY MOIs are clustered at a lower titre.

For CHIKV, at 24 and 36hpi TRI MOI 1 followed by CTRL MOI 1 produce higher titres than the other MOIs and treatments. By 72hpi GLY treated cells infected with CHIK at an MOI of 0.01 produce the highest titre followed by GLY MOI 1, suggesting that GLY treatment is increasing the titre of infectious virus produced by cells. CHIKV produced by CTRL and TRI treated cells at both MOIs are clustered at a lower titre. These results suggest that in C636 cells, treatment with GLY increases infectious CHIKV viral production, particularly at a lower MOI, whilst higher titres leading to higher amount of virus at earlier time points regardless of treatment. This effect with the GLY is not seen in the C636 cells with ZIKV and could be virus specific. However, the C636 cell CHIKV experiment was done later than the other experiments with a new stock of GLY and there is a possibility this change in stock has resulted in the difference compared to the other curves. We see CHIKV titres highest for both GLY MOIs compared to CTRL or TRI. This was not repeated with ZIKV, though again was a new stock of GLY. This suggests that the GLY stock perhaps degrades faster than we expected, and this may need further work to validate the GLY cell culture experiments.

In U4.4 cells, both MOIs of 1 of ZIKV are later to increase in titre for the CTRL and GLY treated cells than for the MOIs of 0.01 or either MOI for TRI cells at 72hpi, suggesting that the fungicide is increasing viral titre at earlier time points and that lower MOIs are reaching higher titres faster than the high MOIs. For CHIKV, at 36hpi the virus follows the same trend as described with the ZIKV, except the TRI MOI 1 takes longer whilst the GLY MOI 1 reaches higher titres faster along with the MOIs of 0.01, suggesting each virus may be differentially affected by the treatments. At 120hr viral titres are highest for TRI treated cells infected with ZIKV and an MOI of 0.01. This is the same for CHIKV at 72hpi in U4.4 cells. These results suggest that smaller starting titres of virus lead to faster production of infectious virus and that a lower titre of TRI MOI 0.01 produces the highest amount of virus over the course of the infection. Given the extreme results in the U4.4 cells and not C636 cells, we thought treatment with TRI may be is modulating the siRNA response, not actively present in the C636 cells.

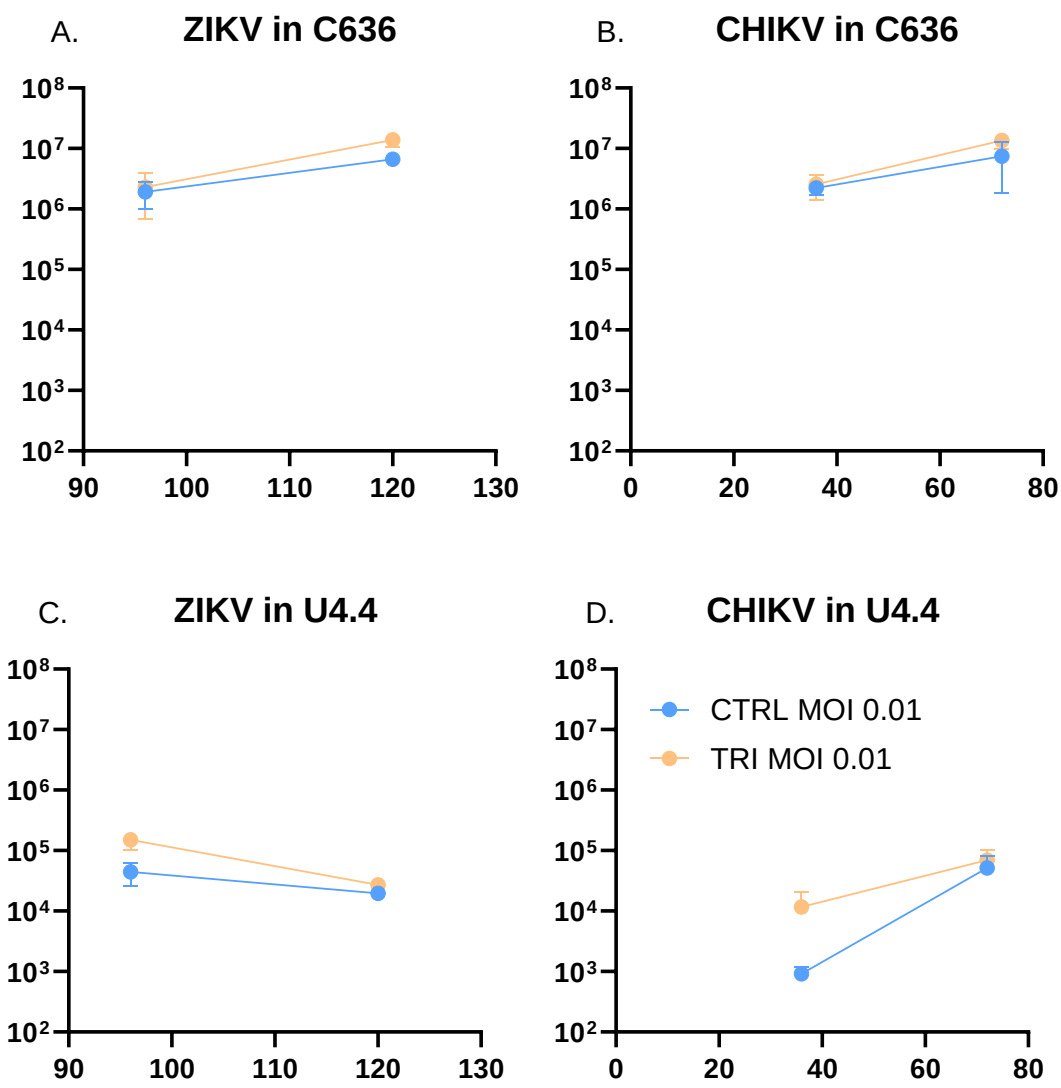


Figure 5.5 The pfu/mL of CHIKV and ZIKV following infection of C636 and U4.4 cells following treatment with CTRL or TRI. ZIKV samples were harvested at 96 and 120hpi in A. C636 and C. U4.4 cells. CHIKV samples were harvested at 36 and 72hpi in B. C636 and E. U4.4 cells. *n* = 3 independent replicates.

To determine if the reason for the increase in both CHIKV and ZIKV titre for U4.4 cells when infected with an MOI of 0.01 was due to modulation of the siRNA response, we repeated the last two time points at the MOI of 0.01 as these were where the main differences were seen. Samples were plaque assayed at each time point. We then monitored changes the siRNA immune gene expression. In this second set of experiments, the TRI MOI 0.01 produces higher titre of virus at all time points, though few are much

higher than that of the control MOI 0.01. For C636 both ZIKV and CHIKV end up with higher titre in the TRI treated cells than CTRL. This was not seen in the first set of experiments for ZIKV as both CTRL MOIs and TRI MOI of 1 were higher. These results suggest that the siRNA response is not responsible for the difference seen in the first experiment between the c636 and u4.4 TRI. This suggests that it could be the way the TRI is interacting with either the virus or the cells themselves that is the reason for the increase in TRI of MOI 0.01 titre. This increase in viral titre produced for both viruses in TRI treated cells when infected at a lower MOI suggest that the TRI could be impacting how the virus is entering cells. It is worth looking into the effects TRI has on cells, using fluorescent microscopy to look at the chance of pore forming mechanisms or modulation a phosphatase involved in receptor binding. This latest experiment was also using CHIKV from a new plasmid rescue so may account for the differences, and furthers the conclusions in chapter 3 that individual stocks of virus need to be used for each repeat. One-way ANOVA was unable to be carried out due to lack of values.

5.2.6 Immune gene results from the 36/72 and 96/120hr CHIKV and ZIKV (siRNA DCR2 and AGO)

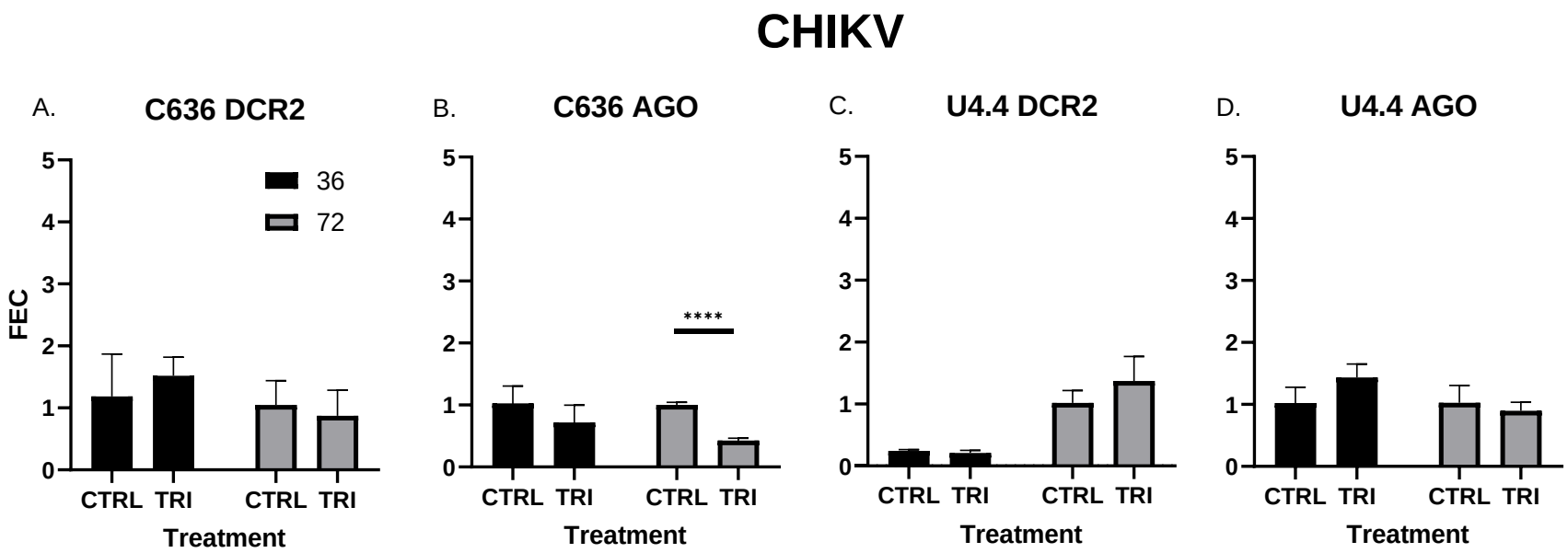


Figure 5.6 The fold expression change (FEC) of DCR2 and AGO in CTRL or TRI treated cells following cell infection with CHIKV at an MOI of 0.01. C636 cells were treated either with control or TRI and FEC was recorded of A. DCR2 and B. AGO. U4.4 cells were treated with control or TRI and FEC was recorded for C. DCR2 and D. AGO. Expression data was normalised to the RPS17 housekeeping gene as per the double delta Cq method. Time points 36 and 72hr were chosen for this experiment. N = 3. Significant difference was determined by an unpaired T-test with Welch's correction.

To determine whether the difference in titre at MOI of 0.01 for the latter time points in the growth curve was a result of the siRNA response, we used qPCR for each sample from the new growth curve set (Figure 5.6). At 36hpi in C636s when CHIKV is at a lower titre, there is no difference in DCR2 expression between CTRL or TRI treated cells. At the same time point in U4.4 cells, when viral titres for both treatment and control are low, DCR2 is downregulated 6-fold on average in the TRI treated cells compared to the control (very hard to see this with the scales on the graphs as presented). Whilst the titres are similar for the two, the titre from TRI treated cells is higher than the control and this could be a result of the TRI modulating the siRNA response early in infection. AGO shows similar expression in both sets of cells at 36hpi, with slight upregulation in U4.4 cells. At 72hpi DCR2 is minorly upregulated for TRI treated cells compared to the control. Given the titres are very close for both at this point, it is unlikely that a change in the immune response is having an impact. At 72hpi AGO is significantly downregulated compared to control expression in C636 ($P = <0.0001$), whilst similar to the control in U4.4. Interestingly, the siRNA response is not active in C636 cells so why AGO is significantly downregulated for this is uncertain. At this timepoint in C636 cells MOI 0.01 in TRI treated cells is at its highest, though unlikely significant compared to the control. The effect of the treatments on the housekeeping gene itself was not looked into, however RSP17, RPL32, Actin and GADPH were tested initially, with RPS17 consistently working well across the samples tested.

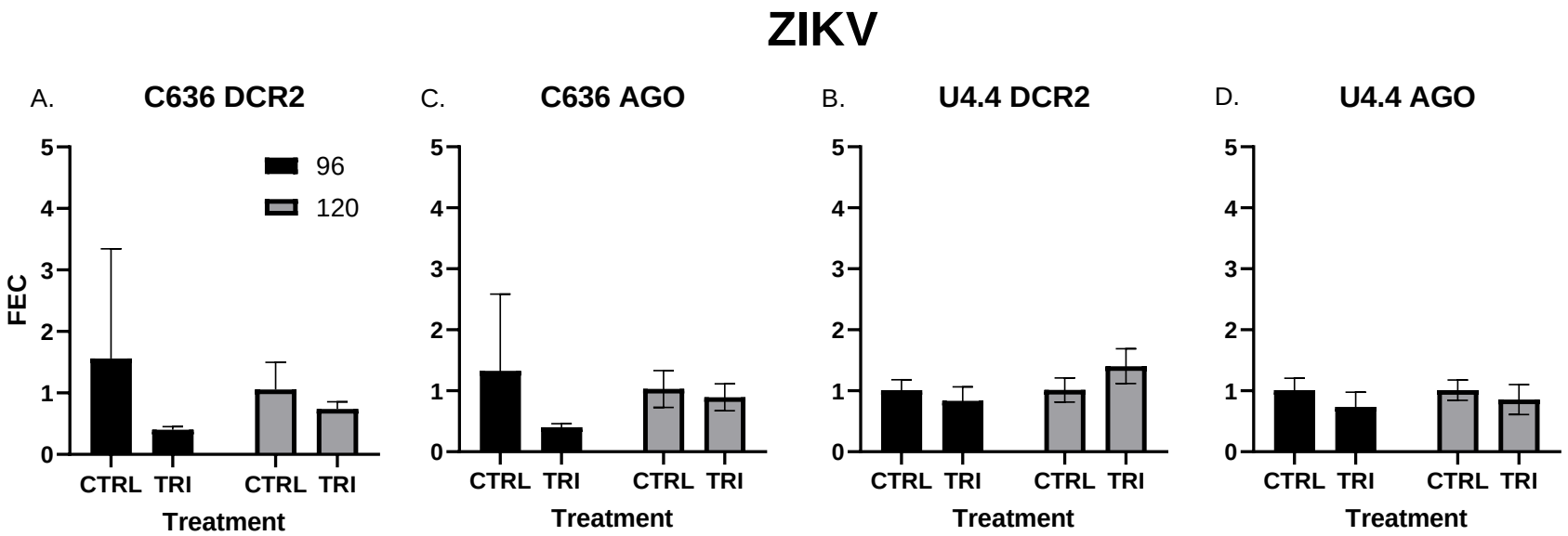


Figure 5.7 The fold expression change (FEC) of DCR2 and AGO in CTRL or TRI treated cells following cell infection with ZIKV at an MOI of 0.01. C636 cells were treated either with control or TRI and FEC was recorded of A. DCR2 and B. AGO. U4.4 cells were treated with control or TRI and FEC was recorded for C. DCR2 and D. AGO. Expression data was normalised to the RPS17 housekeeping gene as per the double delta Cq method. Time points 96 and 120hr were chosen for this experiment. N = 3. Significant difference was determined by an unpaired T-test with Welch's correction.

For ZIKV infection of C636 cells, DCR2 is reduced in TRI treated cells compared to the control at 96hpi. ZIKV titres match control titres 96hr. DCR2 expression is similar for both in U4.4 cells at 96hpi. AGO is also downregulated in TRI treated C636 cells compared to the control at 96hpi whilst showing similar levels of expression in U4.4 cells. This is interesting as to why there is a change in AGO given the titres in these cells at this time point are the same for both control and TRI treated cells and C636 cells do not have a functioning siRNA response.

At 120hr DCR2 is slightly reduced in c636 but increased in u4.4. In C636 cells, TRI treated cells produce a much higher titre of virus than control cells at 120hpi. It is unlikely this slight reduction in DCR2 is the reason given C636 cells lack an siRNA response, and it is a minor difference. Interestingly, at 120hpi ZIKV titre is reduced for both treatments in this set of infections in U4.4 cells, contrary to the first run. This suggests the differences seen in these experiments are due to chance, rather than an effect of the treatments. DCR2 is expressed at slightly higher levels in TRI than CTRL though this is unlikely to have made a difference as the change is minor. For AGO, 120hpi levels are similar for both control and TRI in both types of cells.

To conclude, the results show that alterations to the siRNA response are unlikely to be the reason for the increase in titre we see with the TRI treated cells infected with CHIKV or ZIKV at an MOI of 0.01. The second set of experiments show that this effect is not limited to U4.4 cells and is also seen in C636 cells with no siRNA response. Further miRNA sequencing of the cell cultures, as described in chapter 4, can be done to identify exact changes to the RNAi responses to the viruses that may result from treatments.

5.2.7 Exposure to GLY or TRI Has Limited Effect on the Vector Competence of *A. aegypti* for CHIKV and ZIKV

An important aim of this study was to look at whether exposure to GLY or TRI increased or decreased the vectoral capacity of *A. aegypti* for important arboviruses. To study this, we fed female *A. aegypti* a blood meal containing 1 x 10^5 pfu/ml CHIKV or 1 x 10^6 pfu/ml ZIKV and incubated engorged females for 14 days. Following the incubation, mosquitoes were harvested, and the bodies were tested for presence of infectious virus by cytopathic effect assays (CPE).

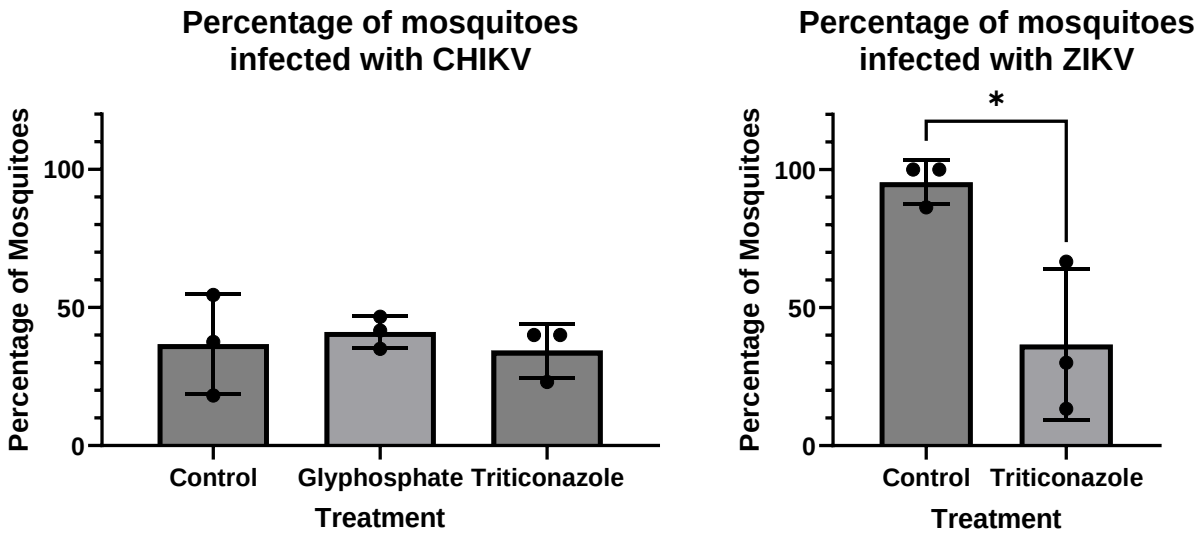


Figure 5.8 The percentage of *A. aegypti* infected with CHIKV or ZIKV after a 14-day incubation following treatment with CTRL, GLY or TRI. Graph A. The percentage of CHIKV infected and uninfected mosquitoes. B. The percentage of ZIKV infected and uninfected mosquitoes. N = 3. Significant difference was determined by unpaired T-test with Welch's correction.

A total of 31, 27 and 25 female *A. aegypti* fed on a CHIKV-spiked blood meal for CTRL, GLY and TRI respectively (graph A). There were no significant differences in the percentage of mosquitoes infected from the control vs GLY (P = 0.7124), control vs TRI (P = 0.8513) or GLY vs TRI (P = 0.3625) groups. The two treatments and control average around 40% of the mosquitoes infected, with GLY consistently producing slightly higher infection rates. Due to feeding difficulties, only small numbers of mosquitoes were analysed in these experiments and a larger number of mosquitoes is required for more accurate statistical analysis. Whilst there are no significant differences between the treatments, these results suggest GLY may increase the percentage of mosquitoes infected compared to the control, and with a larger number of mosquitoes used in future experiments, the significance of this increase with GLY may become more apparent.

Interestingly, treatment with GLY and TRI reduces the variation between repeats compared to the control. A total of 70 and 45 female *A. aegypti* fed on a ZIKV spiked blood meal. A significantly higher percentage of mosquitoes were infected with ZIKV in the control group compared to the TRI treated

group (P = 0.0231). This suggests that treatment with TRI drastically reduces the percentage of mosquitoes infected with ZIKV. It is worth noting, the cell culture results with increased TRI are at very early stages of infection. We saw an initial reduction in DCR2 which may have led to the increased titre seen in the cell culture, but then by 72hpi DCR2 expression was high. This may remain high during mosquito infection to clear the virus leading to reduced infection rates in TRI mosquitoes. Future studies should also look into the direct impact of TRI on the virus, as this may be a reason we see reduced infection rates. To conclude, treatment with TRI shows no significant differences in infection rates for CHIKV compared to the control, but significantly reduces the percentage of *A. aegypti* infected with ZIKV. GLY treatment was not studied for ZIKV infection, however shows a slight increase in infection rates for CHIKV and requires further experiments to ascertain higher numbers of mosquitoes to truly determine the significance of this. Overall, there were no significant differences in infection rates across the treatments for CHIKV.

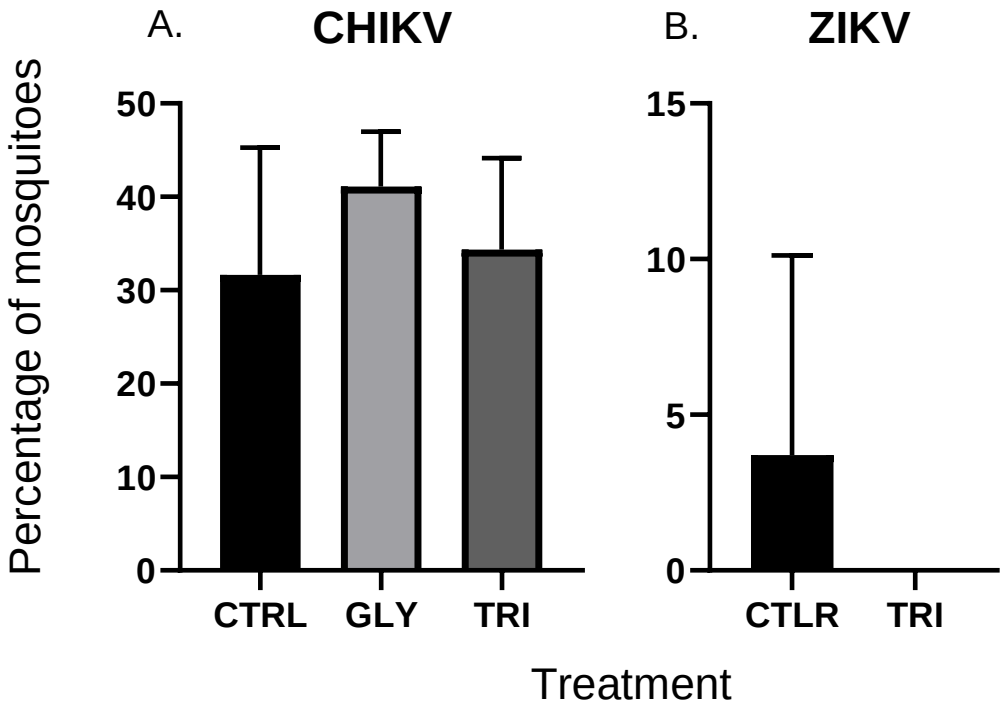


Figure 5.9 The percentage of mosquitoes with dissemination of A. CHIKV and B. ZIKV, following treatment with CTRL, GLY or TRI. *n* = 3 repeats. Dissemination was determined using a combination of CPE assays. Results were tested for significance using an unpaired T-test with Welch's correction.

To determine if exposure to agricultural chemicals increased or reduced the dissemination rates of CHIKV and ZIKV in *A. aegypti*, we performed CPE assays on the heads and legs of infected mosquitoes. For CHIKV, GLY shows the highest percentage of infected mosquitoes showing dissemination at just under 50%, though there is no significant difference between this and the control (P = 0.3569), whilst TRI treated mosquitoes show only slightly more dissemination than the control though (P = 0.79343). There was no significant difference between control and TRI treated mosquitoes for dissemination (P = 0.4229), and dissemination rates were low for both averaging 5% of the infected control mosquitoes. None of the ZIKV infected mosquitoes in the TRI groups showed disseminated infection. This suggests that treatment with TRI has limited impact on the dissemination rate of CHIKV and only slight reduction for ZIKV, suggesting it does not alter the vector competence of the viruses by modulation of the dissemination rate. GLY exposure may increase the dissemination rate of CHIKV in infected mosquitoes and more work is required to see if this effect is seen for *flavivirus*es as well as *alphavirus*es. It is worth noting this strain of *A. aegypti* already has shown in these experiments a low vector competence for ZIKV and this experiment could benefit from being left for 21 days to see if this increases the chances of dissemination.

5.2.8 Survival Probability of treated or control mosquitoes exposed to CHIKV or ZIKV

To determine if exposure to agricultural chemicals altered mosquito survival following exposure to CHIKV or ZIKV, we monitored the deaths over the 14-day incubation period following infectious blood meal. Throughout this time, mosquitoes were fed either control or treated sugar feeds every three days.

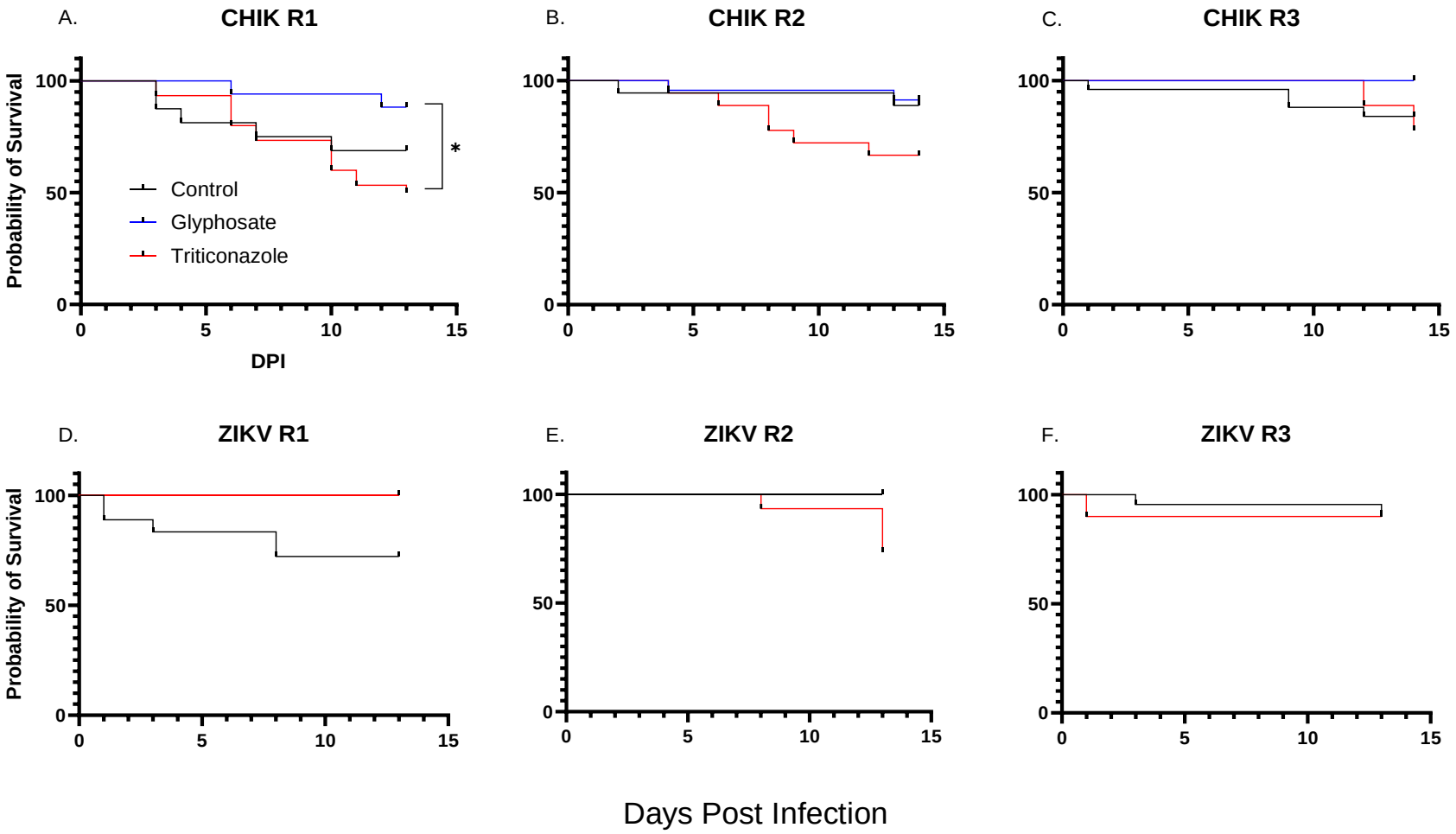

A.
CHIK R1
B.
CHIK R2
C.
CHIK R3
D.
ZIKV R1
E.
ZIKV R2
F.
ZIKV R3
Probability of Survival
100
50
0
0
5
10
15
DPI
Control
Glyphosate
Triticonazole
*
Days Post Infection

Figure 5.10 The survival probabilities of *A. aegypti* over 14 days following exposure to CHIKV or ZIKV at 5 x 10^5 or 1 x 10^6 pfu/mL respectively. A, B and C show repeat 1-3 of CHIKV infected mosquitoes. D, E and F show repeat 1-3 of ZIKV infected mosquitoes. Survival analysis was performed on prism. (R refers to repeat).

There is an overall trend of decreasing survival probability when mosquitoes are treated with TRI and exposed to CHIKV, though this is not statistically significant. Treatment with GLY appears to improve changes of survival compared to control and TRI and repeat 1 has a significant difference in survival between the GLY and TRI treated mosquitoes infected with CHIKV (P=0.0107). There are no significant differences between any of the other treatments for the other two repeats.

ZIKV exposed mosquitoes do not follow the same trend across the three repeats of decreasing TRI survival. Repeat one showed reduced survival for the control group (P = 0.0298). Repeat 2 shows significantly reduced survival for the TRI group, matching the CHIKV results (P = 0.0094). Repeat three shows no significant difference between the control or TRI group. There is more variation in this experiment regarding survival probability than in the CHIKV experiment. This could be experimental variation or may suggest that the titre of the virus in the mosquito has an impact on the mosquito survival rate. It would be interesting to see if we had left the ZIKV infected mosquitoes for longer if this would have impacted their infection and survival rates.

5.2.9 The effects of exposure of GLY or TRI on mosquito immune gene expression

To determine if the treatments were altering the immune responses in the mosquitoes, we extracted RNA from three infected, three uninfected and three control blood-fed mosquitoes from each repeat. When then performed qPCR with immune genes from three innate pathways including the main antiviral pathway, the RNAi. Dome and the Protein Inhibitor of Activated STAT (PIAS) of the JAK-STAT pathway, MyD88 and Cactus (CACT) of the Toll pathway and Dicer-2 (DCR2) and Argonaute (AGO) of the siRNA pathway.

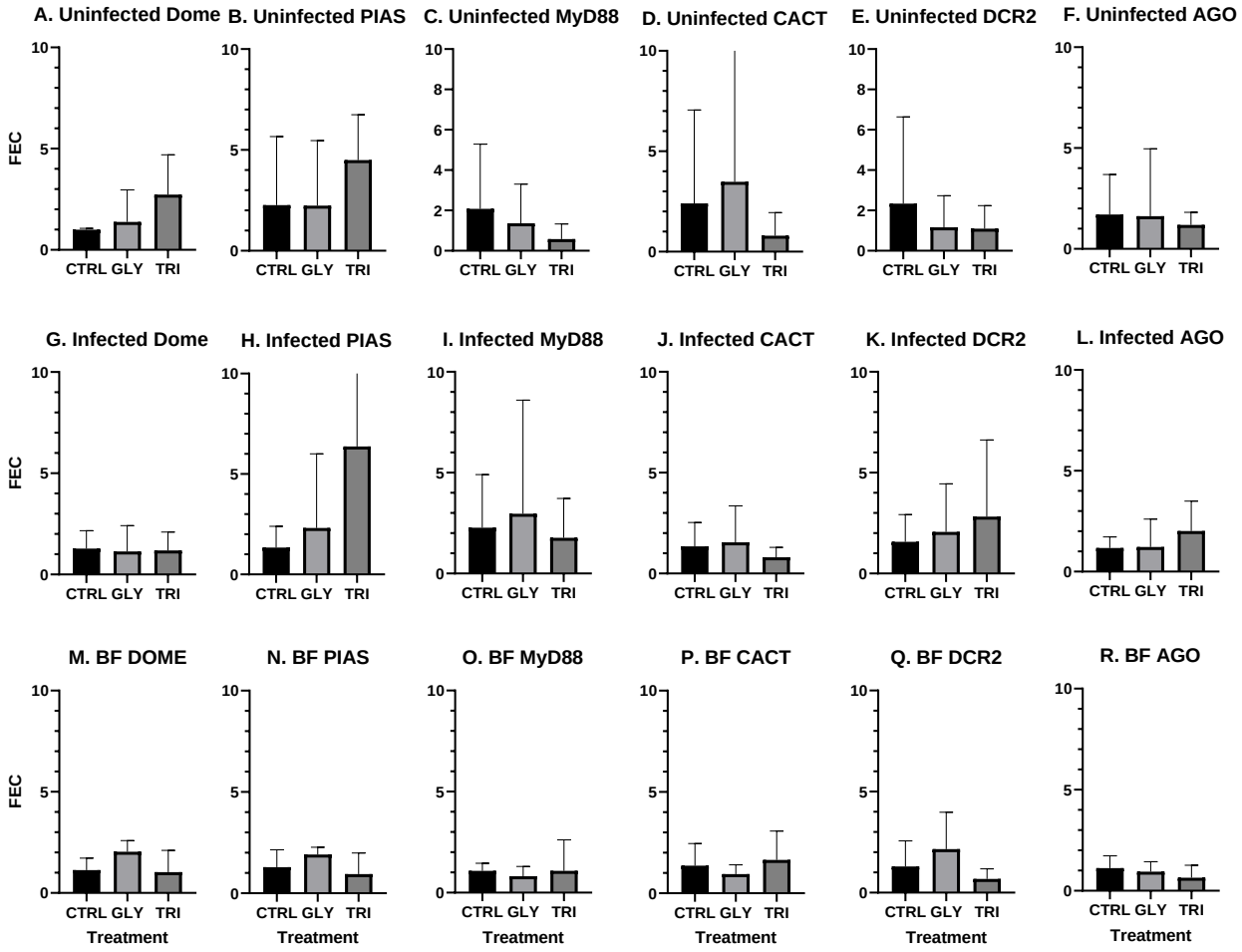


Figure 5.11 The fold expression change of the treatments compared to the control for three pairs of mosquito immune genes for uninfected, infected and control blood fed (BF) mosquitoes. Graphs A-F show mosquitoes that were exposed to CHIKV and remained uninfected, G-L show mosquitoes infected with CHIKV and M-R show mosquitoes fed a control blood feed. n = 9 from (n = 3 per repeat). For statistical analysis, the double delta Cq values were compared by an unpaired T-test using Welch's correction.

In uninfected mosquitoes both GLY and TRI upregulate Dome by 2- and 4-fold respectively, however infected mosquitoes show equal expression of Dome in CTRL, GLY and TRI treated mosquitoes. This suggests that the JAK-STAT pathway is upregulated in event of active infection, but once infection is established expression levels are that of the control. With CHIKV infection, on average 40% of the mosquitoes were infected in all groups.

This suggests that the JAK-STAT pathway may be the reason for clearing virus in infected mosquitoes. As for PIAS, the negative regulator of the jak-stat pathway, in both infected and uninfected mosquitoes this negative regulator is upregulated by TRI. This suggests TRI could be altering the negative regulation of the pathway, though unless by allowing the viral population to replicate to Mullers ratchet, this is not impacting infection with CHIKV. The uninfected mosquitoes show slight increase between GLY and control whilst the infected mosquitoes do show an increase in PIAS for GLY treatment. A control blood feed seems enough to upregulate the pathway in GLY mosquitoes.

For the Toll pathway, both uninfected and infected GLY treated mosquitoes show upregulation of MyD88 and CACT compared to the control, though to a lesser extent in the infected mosquitoes. This suggests that GLY increases activation of the pathway. This could be a result of up/downregulation of different populations of bacteria within the mosquito microbiome, as has been a reported effect of GLY on honeybees. These could be generating PAMPs sensed by the pathway. Unlike the JAK-STAT pathway, a neat blood feed slightly downregulates the pathway in GLY treated mosquitoes. TRI appears to downregulate expression of MyD88 and CACT slightly in both infected and uninfected mosquitoes. Downregulation of this could lead to overgrowth of the viral population again leading to Mullers ratchet.

For the main antiviral pathway, the RNAi, we looked at DCR2 and AGO involved in the siRNA branch of the pathway. GLY increases expression of DCR2 in both uninfected and infected mosquitoes, though to a lesser extent in the infected. This suggests that this is playing a role in clearing viral infection as expected. TRI shows upregulation of DCR2 and AGO in infected mosquitoes suggesting presence of virus activates the response more so in TRI treated mosquitoes. Interestingly, DCR2 is downregulated in the uninfected mosquitoes and AGO is upregulated. The downregulation of DCR2 may coincide with changes in ISV populations in the mosquito virome as a response to the CHIKV infection and treatment, as the CHIKV infection is reduced the ISV population declines, leading to less stimulation of DCR2. Sequencing of the mosquito microbiome would be required to identify these changes. The AGO upregulation may be a result of the RISC complex dealing with the dsRNAs previously produced by DCR2 when the mosquitoes were infected- a delay in the response perhaps. The reason for GLY still

showing high AGO expression in uninfected may be a result of GLY treated mosquitoes taking longer to clear infection, hence why we see slightly higher infection percentages in the GLY treated mosquitoes.

Between groups (i.e Infected vs Uninfected, Uninfected vs Blood fed) the only significant differences were detected within the TRI treated mosquitoes, mainly between the infected mosquitoes and the control blood fed mosquitoes. Dome, AGO and PIAS all showed significantly different FEC between the infected mosquitoes and the control blood fed mosquitoes. Dome showed significant higher FEC in blood fed mosquitoes compared to infected ($P = 0.0409$), whilst AGO and PIAS showed significantly higher FEC in the infected mosquitoes compared to the blood fed ($P = 0.0313$ and $P = 0.007$ respectively). The only other significant difference was the uninfected mosquitoes having a higher FEC than the blood fed mosquitoes for PIAS ($P = 0.0026$). Aside from Dome, both AGO and PIAS show downregulation in TRI treated mosquitoes given a non-infectious blood feed.

To conclude, the main differences between the control and the treated are in the uninfected mosquitoes for the Toll and RNAi pathways. This suggests that these treatments are upregulating these pathways to lead to enhanced clearing of viral infection. Though these FECs were not significantly different and could be a result of experimental variation. TRI treated mosquitoes were the only ones to have differences depending on their feed/infection status with some genes showing different FEC between feeds.

5.2.10 How exposure of larvae to treatments alters expression of immune genes in newly emerged mosquitoes

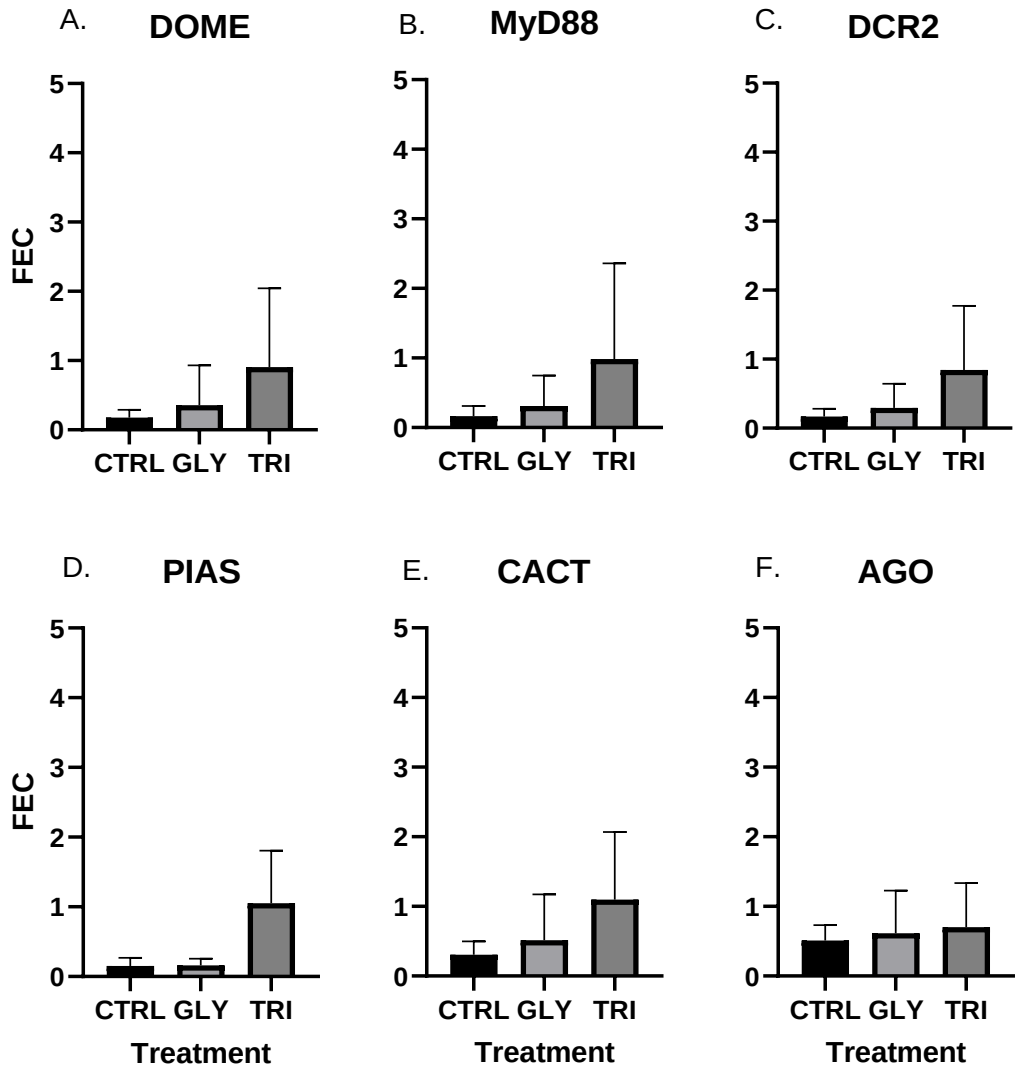


Figure 5.12 The fold expression change of six mosquito immune genes following exposure to GLY or TRI as larvae. Results were analysed as previously described. N = 9 (3 repeats).

Given mosquitoes will largely encounter agricultural chemicals when in their early stages in still water contaminated by surface run off, we wanted to see how exposure to GLY or TRI affected immune gene expression in larval stages of mosquito. We reared larvae in treatments and harvested adult mosquitoes upon emergence. This allowed us to see how the immune gene expression was altered at the larvae stages.

A trend across all the immune genes is that treatment with TRI increases expression of the genes. This is most prominent with PIAS of the JAK-STAT pathway and is least noticeable with AGO, though there are no significant differences. It is worth noting the TRI repeats have the highest variability in expression changes. GLY treatment also appears to increase expression compared to the control in all genes other than PIAS of the JAK-STAT pathway. These results suggest that in the early stages of mosquito development, exposure to GLY and TRI increases the expression of key regulators of immune genes, though this is only a small increase and not significant. Mosquito larvae have a higher abundance of microbiota and so this may be through alteration of this. However, given this is not shown in the adult mosquitoes, its significance in infection is not clear. It would be interesting to see the effects treatment with GLY and TRI has on vertical transmission and see if treated larvae are more capable of clearing persistent viral infection.

5.3 Discussion

GLY and TRI are commonly used herbicides and fungicides across the globe, both agriculturally and horticulturally. Multiple studies have focused on the effects of GLY on the immune system and microbiome of bees, making them more susceptible to certain pathogens and immune changes associated with changes in the microbiome. The gut microbiome of mosquitoes is picked up mainly from two avenues: vertical transmission from female mosquitoes to offspring or consumption of the water, nectar and other environmental food sources in which they are reared (Scolari *et al*., 2019, strand *et al*., 2018). Mosquitoes, such as those that carry medically important arboviruses, are naturally found in still water, typically streams and puddles that may contain agricultural runoff. *Aedes* is an urbanised mosquito, with most of their larval habitats in standing water in areas around house such as pots or old tires. With GLY and TRI being used in both agricultural and horticultural settings, it is likely remnants of them will enter mosquito habitats and interact with the aquatic stages of the mosquito life cycle. Following on from the bee immune gene/microbiome research, we hypothesised that exposure to either of these chemicals may alter the vector competence of *A. aegypti* to CHIKV or ZIKV. We performed a series of experiments in cell culture and live mosquitoes to monitor changes to mosquito life history traits and infection capacity.

5.3.1 Life history traits

First, we wanted to assess if exposure of mosquito larvae to either of the chemicals would alter the life history traits of *A. aegypti*. We reared mosquitoes from eggs in 0.5mg/L of control, GLY or TRI treated dH2O and recorded the number of pupae that emerged each day following the submersion of eggs. Both CTRL and TRI followed a smooth curve of emergence, with the second and third days having the highest rate of emergence. This was the same for both sexes, with the exception that the males first emerged the same day as the females, rather than a day before as expected with the CTRL. In cell culture we observed slower cell growth in TRI treated media and this may mean that TRI is slowing development time slightly (observation so data not shown). A potential reason for the slower developmental growth of TRI treated mosquitoes and cells may be with TRI activating ROS which can damage cells; however, we would expect to see more cell death in this case. TRI has also been shown to have potential to disrupt the endocrine system in human cells which may be the reason for the slightly slower development (Roman *et al*., 2022). If this is the case, TRI may also affect egg development as steroid hormones promote yolk synthesis in mosquitoes (Strand *et al*., 2016). Further experiments are needed to determine if TRI slows cell growth. Though the results suggest TRI has limited impact on the development time of male or female mosquitoes and is unlikely to alter vector competence by this means.

For many mosquito species, larger females lay more eggs, and egg batch size of the first gonotrophic cycle increase with pupal mass and wing length for *Aedes albopictus* and *Aedes geniculatus* (Hawley 1985, Armbruster and Hutchinson 2002). Should one of the treatments alter the size of the mosquito, it is possible the treatment could contribute to larger populations of mosquitoes during peak seasons, through an increased volume of eggs. This in turn would provide a larger population of mosquitoes capable of transmitting viruses they are competent for. GLY pupae showed no weight differences compared to the control, matching data where treatment with GLY did not alter the size of *Anopheles* or *C. pipens* (Smith *et al*., 2021, Bataillard *et al*., 2020). On the other hand, TRI treated pupae of both sexes are significantly smaller than both the control and GLY treated group. Whilst larger mosquitoes lay larger egg batches and can ingest larger infected blood meals, Grimstad and Walker (1991) showed that smaller *Aedes triseriatus* were more likely to become infected and disseminated with La Crosse virus

than larger mosquitoes, though this may be mosquito species dependent. However, this did not seem to increase the likelihood of infection in our experiment, suggesting minimal impact on vector competence or capacity of either of the treatments through changes in mosquito size.

5.3.2 Mosquito infection and dissemination rates

Several studies have looked at whether exposure to GLY increases the vector competence of different organisms. Motta (2018) found that GLY increased the mortality of Honeybees exposed to the opportunistic pathogen *Serratia marcescens*, while Smith (2021) found that malarial oocyst burden increased with increasing GLY dose in mosquitoes. Contrarily, Bataillard (2020) showed no difference in *Culex pipens*, so this effect appears to be vector-pathogen specific. To our knowledge, this has not been tested with CHIKV or ZIKV in *A. aegypti*. To understand if exposure to GLY would alter the vector competence/capacity of *A. aegypti* for medically important arboviruses, we reared mosquitoes in treatments as previously described and fed females a blood meal spiked with 1 x 10^5 pfu/ml CHIKV or 1 x 10^6 pfu/ml ZIKV. At 14dpi we tested the bodies and heads and legs for virus infection and dissemination.

For CHIKV, in CTRL, GLY and TRI treated groups on average the percentage of infected mosquitoes is between 30-40% for all groups. The control group shows more variability between experiments, with 30-50% infected across the three repeats, whilst GLY and TRI treated groups are more consistent at 40 and 30% infected respectively. There is a slightly higher infection rate on average for the GLY treated group compared to the control and TRI groups, however limited numbers of mosquitoes prevent us from identifying the significance of this. Given the initial cell culture results and mosquito infection preliminary results, only TRI was taken further with ZIKV mosquito infections. The CTRL groups show near 100% ZIKV infection, significantly higher than the percentage of uninfected CTRL mosquitoes. Here, exposure to TRI has drastically reduced the number of mosquitoes infected, lowering the vector competence of *A. aegypti* for ZIKV. This is contrary to the cell culture results, where treatment with TRI appeared to increase the titre of both CHIKV and ZIKV produced during infection across multiple experiments, and as eluded to earlier, may be a result of the earlier time points sampled in the cell culture treatments. A potential explanation for the effect of TRI on ZIKV infection compared to a limited

difference seen with CHIKV, is that TRI alters the microbiome of the mosquitoes, removing specific fungi that may directly or indirectly affect the course of ZIKV infection. It would be interesting to sequence these samples and identify any changes in the microbiota associated with TRI treatment and the reduction in infection. Equally, TRI could be directly impacting the virus, and separate experiments tittering the virus following direct exposure to TRI would be needed to determine this.

For CHIKV, GLY treated mosquitoes on average show a slightly higher percentage of dissemination amongst the infected mosquitoes, though there is no significant difference between treatments and the control. TRI treated mosquitoes show no dissemination of ZIKV compared to only a small percentage in the control, showing a slight reduction in dissemination. This suggests that exposure to GLY or TRI does not significantly alter the vector competence of *A. aegypti* for CHIKV by increasing or reducing dissemination rates, though again larger sample sizes are needed to confirm this.

To see if exposure to GLY or TRI would alter the survival of *A. aegypti* following an infectious blood meal, we monitored the survival rates of the cohorts of mosquitoes. There is a trend of decreasing probability of survival for the TRI treated mosquitoes compared to the CTRL and GLY exposed groups following a blood feed spiked with CHIKV. Coupled with the lower infection percentage and unchanging dissemination rates, this leads to a shorter lifespan of the infected mosquitoes, with fewer opportunities for pathogen transmission and fewer opportunities for the uninfected mosquitoes to take infected blood meals. There is not the same trend of decreasing survival for the TRI treated mosquitoes exposed to ZIKV. A possible explanation for this is that there is a negative correlation between increasing viral load and decreasing mosquito survival. Should this experiment have been left to 21 days for the ZIKV we may see increased mortality resulting from increased dissemination. The emergence rates of TRI male and female pupae was no different to the control and with the reduction or lack of change in the infection and dissemination rates, this suggests that treatment with TRI reduces both the vector competence for CHIKV. Based on the survival rates of TRI treated mosquitoes fed a ZIKV-spiked blood meal, we cannot conclude that vector capacity is reduced due to lowered survival with further experiments needed to study this, however we can conclude that TRI reduces the vector competence of *A. aegypti* for ZIKV due to reduced infection and dissemination.

On the other hand, GLY appears to show increased survival of CHIKV-fed mosquitoes compared to the CTRL and TRI treated groups. This is in line with GLY data for other mosquitoes, where *A. gambiae* treated with a low dose of GLY had significantly improved survival (smith *et al*., 2021) whilst Bataillard (2020) showed that *C. pipens* had no changes to survival rates. Several studies have shown that treatment with GLY shortens the development time of *Culex quinquefasciatus*, *Anopheles* and *C. pipens* mosquitoes (Bataillard *et al*., 2020, Kibuthu *et al*., 2016). Whilst we did not find GLY reduced development time, GLY pupae seemed to show less of a decline in pupal emergence after the peak days compared to the control and TRI, which both dropped off sharply. This suggested that GLY treated larvae/pupae have an increased likelihood of surviving to adulthood. Coupled with the increased survival proportions of adult mosquitoes, this extends the transmission period of the moquitoes by providing them more opportunities to take infected blood meals, incubate and transmit arboviruses. Coupled with the increased dissemination rates, this suggests treatment with GLY may increase the vector capacity of *A. aegypti*, despite lowering infection rates (or at least compensate for the lowered infection rates). It is worth noting Kibuthu (2016) found that both GLY and CTRL laid fewer eggs in GLY treated water. This may counteract the effects of extended lifespan by reducing the reproductive rate of the mosquitoes. Additionally, a caveat of the experiment is that larvae were selected at random, suggesting larvae of different ages could be selected in each treatment, altering the emergence curve.

To conclude, the results suggest that treatment with GLY or TRI does not alter the vector competence of *A. aegypti* for CHIKV, however treatment with TRI reduces the capacity and competence of *A. aegypti* to transmit ZIKV. There are significantly fewer mosquitoes infected in the TRI treated group than in the control. No difference was seen with dissemination rates of TRI treated mosquitoes compared to the control. For CHIKV exposed mosquitoes TRI reduces the probability of survival but does not alter infection or dissemination rates. Based on these results, GLY does not alter the vector competence of *A. aegypti* for CHIKV. Slight increases in infection and dissemination rate were seen in the GLY treated group, along with increased chance of survival, however these were not significant compared to the control and are on small numbers of mosquitoes, requiring further experiments to determine the true significance of these findings.

5.3.3 Mosquito Immune Gene Discussion

The gut microbiota modulates mosquito responses to disease, through physical barriers in midgut epithelial cells, activation of immune signaling pathways and release of antipathogenic components. Mosquito microbiota has been shown to stimulate the mosquito immune system, with the fungi *Beauveria bassiana* activating the Toll and JAK-STAT pathways leading to restricted DENV infection (Dong *et al*., 2012). We hypothesized that treatment with GLY or TRI may alter the mosquito microbiota, leading to changes in immune gene expression that would alter vector competence. To investigate this, we used RNA extraction and qPCR to monitor fold-expression change of regulator genes in key mosquito immune pathways following mosquito treatment with CTRL, GLY or TRI. We looked at expression of genes from 3 immune pathways in mosquitoes that had received a CHIKV spiked blood meal but were uninfected at the end of the 14 days, mosquitoes that were infected, and mosquitoes that had received an uninfected blood meal, with a blood meal itself has been shown to stimulate changes in the microbiota. Further to this, we looked at fold-expression-change in cell culture and freshly emerged treated mosquitoes.

5.3.4 Immune Gene Changes in CHIKV Exposed Mosquitoes

5.3.5 JAK-STAT-

The JAK-STAT pathway has been shown to reduce viral infection and has also been shown to be modulated by changes in the immune microbiota, as mentioned in the previous pathway. To look for changes in the regulation of this pathway, we tested the cytokine receptor Dome, and the negative regulator of the system, PIAS. Infected mosquitoes showed similar expression of Dome for all treatments and control whilst Dome is upregulated compared to the control in the uninfected mosquitoes. This suggests that overexpression of Dome in the treatment groups, particularly TRI, helps clear the viral infection in the uninfected mosquitoes. Interestingly, blood feeding increases both Dome and PIAS expression slightly in the GLY group compared to the control, suggesting that in the GLY treatment, blood feeds may help prime the immune response for subsequent viral infection with CHIKV. Given this increased expression following a blood meal is only seen with GLY and not TRI, it suggests that

GLY may be altering immune gene expression by changing the mosquito microbiota. Shi (2022) found *A. aegypti* receiving a blood meal showed transiently higher ISV diversity compared to mosquitoes fed sucrose at 7dpi, though this had disappeared by 21dpi. They state earlier timepoints may be key to understanding this, with digestion of the blood meal in 2-3 days returning the immune system to homeostasis by day 21. It suggests that GLY is perturbing the microbiota leading to changes in expression of immune genes in response and sequencing would be required to confirm this and the limited differences in infection rates suggest this is not drastically impacting vector competence. Interestingly, In the TRI treated mosquitoes, Dome has a higher FEC in blood fed mosquitoes compared to the control, whilst PIAS shows higher FEC for both infected and uninfected mosquitoes compared to the control blood fed mosquitoes. This suggests that in the infected and uninfected cohorts there is more negative regulation of the pathway compared to the control blood feed, suggesting that exposure to the virus negatively impacts the JAK-STAT pathway in TRI treated mosquitoes compared to blood fed, though these were not significant compared to the control of each cohort and so are unlikely to impact infection itself.

Both TRI and GLY groups show upregulation of PIAS in infected groups whilst TRI shows this in uninfected also. The increase in PIAS expression in the TRI treated infected mosquitoes suggests that TRI leads to increased negative regulation of the JAK-STAT pathway and may therefore account for increased difficulty in the mosquitoes maintaining persistent infection, hence the lower infection percentages. In 2009, Souza-Neto showed that silencing of PIAS led to a reduction in DENV-2 infection, whilst silencing of Dome or Hop increased the viral load by 2.3 and 3-fold respectively at early stages of infection. Following the increase in negative regulation seen with TRI treated mosquitoes, CHIKV may be able to replicate to higher titres. As infection is heavily controlled in mosquitoes, this increase in replication may lead to Mullers ratchet, accounting for the reduction in the percentage of mosquitoes infected. This corroborates the need for balance during infection as stressed in the main thesis introduction.

The results suggest that for GLY, a blood feed is enough to stimulate expression of the JAK-STAT pathway genes, enabling quicker response of the pathway to a mosquito blood meal which may help

reduce the infectivity. It appears that the -presence of TRI is increasing the activity of the JAK-STAT pathway which may account for the reduced infection percentages in the mosquitoes of CHIKV. This increased activation of the pathway may be because of TRI perturbing the mosquito fungi, leading to changes in composition that stimulate the immune response. Despite this, there is limited difference between the groups and the results are largely inconclusive, whilst there are slight differences between feed/infection status, though it is unlikely to impact infection given no difference to the controls of each group.

5.3.6 Toll Pathway

The Toll pathway is important for viral infection, mediating antiviral activity against DENV (Xi *et al*., 2008). We wanted to see if exposure to either GLY or TRI would stimulate this response against CHIKV. We monitored expression of Myd88 and the negative regulator CACTUS. For the TRI treated mosquitoes, Myd88 and CACT show decreased expression in both infected and uninfected mosquitoes, whilst matching control levels following a blood feed. This suggests that in TRI treated mosquitoes, there is reduced expression of the Toll pathway following CHIKV infection. Xi (2008) found that silencing of the CACT gene reduced DENV infection in the midgut by 4-fold, though silencing of MyD88 increased infection. Interestingly, we are seeing both decrease in expression, but infection reduced compared to the control. TRIs reduction of Toll could be why we see higher CHIKV titres in cell culture infection. In mosquitoes, the viral population may then over replicate and lead to population extinction, hence why we see lower mosquito infection rates than in the control.

With TRI downregulating the Toll pathway and its increased negative regulation of the JAK-STAT pathway in mosquitoes, it may be that these allow the viral population to replicate to high titres as we see in cell culture. The viral population may then over replicate and lead to population extinction via Mullers ratchet, hence why we see fewer mosquitoes infected compared to the control. It is likely GLY upregulates the Toll pathway following the presence of a CHIKV-spiked blood meal and so may account for the lower number of mosquitoes infected in the GLY group compared to the control.

Unlike the JAK-STAT, GLY does not show activation of the pathway following a blood meal. This suggests that if microbiome changes following exposure to GLY and a blood meal are the reason for activation of the JAK-STAT pathway, the microbiota changes here are not responsible for activating the Toll pathway. Interestingly, there are changes in the expression of both genes in the uninfected mosquitoes, suggesting that this pathway plays an active role in clearing CHIKV infection, with MyD88 increased also in infected mosquitoes. However, with the variation between repeats and lack of significance it is likely that this is not the only factor at play. To conclude on the Toll pathway, GLY may stimulate this pathway for the clearance of CHIKV whilst TRI appears to reduce expression of the pathway.

5.3.7 siRNA response

The siRNA pathway is the key antiviral response in mosquitoes and have shown to be active against multiple arboviruses, including CHIKV (Dong *et al*., 2022) With the immune gene expression results, GLY shows upregulation of DCR2 compared to the control following both a control blood meal and in uninfected but exposed mosquitoes. This suggests that GLY treated mosquitoes can utilise their siRNA response to clear the virus better than in the control mosquitoes. This may be a result of GLY altering the microbiome, leading to changes in the virome that upregulate the siRNA response. Perhaps priming of the response by insect-specific viruses (ISVs). The continued expression following infection clearance may still be because of exposure to the arbovirus or may be that the microbiome of the mosquito is still dysregulated following GLY exposure. Sequencing the mosquito colony would identify any ISVs and see if their population dynamics change following GLY treatment. Given changes to the ISV community that have been noted following arboviral infection this may account for the reason. As mentioned previously, it took until day 21 for immune homeostasis to be reached and ISV populations to return to normal so this may account for differing immune stimulation (Shi *et al*., 2022). The continued expression of DCR2 in uninfected may be a result of GLY increasing ISV populations and the response activated against these, with AGO not yet active in the RISC complex.

On the other hand, TRI infected mosquitoes show a slight downregulation of DCR2 in uninfected mosquitoes whilst AGO shows upregulation. This could be that by this point of infection DCR2 has

finished cutting up the viral RNA and the RISC complex is playing the more important role at this stage of infection, suggesting that perhaps in TRI treated mosquitoes, the viral infection is cleared at a later stage than in the control, and that at 14 days, we are still seeing the end half of the response. Interestingly, when comparisons were drawn between groups, we see upregulation AGO in CHIKV infected mosquitoes vs control blood fed mosquitoes. This suggests that the RISC complex is perhaps more active and processing the small RNAs generated by DCR2 at a faster pace, which can be expected when virus is present. This could be the reason we see slightly lower rates of dissemination in TRI treated mosquitoes compared to GLY, though this was not significant and was similar to control mosquitoes so is unlikely to be making a difference.

Following an initial increase in titre in the cell culture experiments in U4.4 cells, an effect that was then lost in C636 cells which lack an efficient siRNA response (Brackney *et al*., 2010), we hypothesised treatment with TRI was altering the siRNA response. Interestingly on this second time round we did not see as stark a difference in the amount of virus produced from the CTRL and TRI treated U4.4 cells for either virus. The titre produced in C636 TRI treated cells was also higher than that of the control C636 cells which was unexpected. Whilst multiple repeats were done for each of these experiments, all of which showed little variation, this shows that between rounds of experiments there is room for variation in results. This could be because of using new viral stocks for the separate experiments. These results suggests that the initial differences in titres we saw from the cell cultures are not because of alterations to the siRNA response. AGO is significantly higher in the infected mosquitoes than in the control blood fed group, which would be expected due to viral stimulation of the siRNA response.

To confirm this, we analysed the fold-expression change for DCR2 and AGO, both of which have been shown to have antiviral activity against arboviruses. ZIKV is the exception, with no antiviral activity shown from AGO so this would be interesting to compare (Varjak *et al*., 2017). There is a 6-fold reduction in expression of DCR2 in U4.4 cells at 36hr in TRI treated cells infected with CHIKV. This is unlikely to be having an effect given CHIKV titres is only minimally above the CTRL titre at both time points. AGO shows no expression changes worth noting in U4.4 cells, however shows significantly lower expression compared to the control in C636 cells at 72hpi. CHIKV titre is higher on average than

the control at this time point, which suggests that reducing AGO expression may be playing a role in this. However, given the lack of efficient siRNA response in C636 cells, it would be through another means. Aside from this, there are no expression changes of importance across the time points, suggesting that treatment of U4.4 and C636 cells with TRI does not impact the siRNA response during CHIKV infection enough to account for differences in titre produced from TRI and CTRL treated cells.

For ZIKV, DCR2 and AGO both show minutely reduced expression in TRI treated C636 cells compared to control, whilst there is only slight downregulation of AGO in U4.4 cells. This slight downregulation may be due to the lack of antiviral activity of AGO against ZIKV. With larger error bars for DCR2 and AGO in C636 cells, it is likely this variation is natural during infection and experiments. As concluded with CHIKV, it is unlikely that differences in titre of ZIKV between CTRL and TRI treated cells is because of modulation of the siRNA response. In future, it will be worth looking into the direct effects TRI has on cells, using fluorescent microscopy to look at the chance of pore forming mechanisms, allowing more virus to enter cells, an effect that we may not see with the MOI of 1 due to such large volumes of virus used for all treatments and control. Given the variation between repeats, the importance of this cannot be determined. Given DCR2 and AGO are not effector proteins we are unlikely to see drastic fold differences in expression even if there are changes. RNAseq may be a better way to proceed with these experiments in future. The results suggest that TRI inconsistently leads to a higher titre of virus in early cell infection, though the mechanism of this is uncertain.

5.3.8 Larval immune gene changes in response to GLY or TRI exposure

All immune genes for the three pathways show upregulation with TRI. This is the same for GLY except for PIAS of the JAK-STAT pathway, though TRI produces a higher fold change. These changes are small and non-significant, and given these trends are not observed consistently in the adult mosquitoes and the larval survival is not altered between the treatments and control, the effect of this on vector competence/capacity is unclear.

5.3.9 Immune gene conclusion

Together, the immune gene expression results are largely inconclusive. We do see that compared to the control group both GLY and TRI lead to higher expression of the siRNA system to clear CIHKV infection which would be expected, though this does not appear to play a major role in the differing titres seen in cell culture infection. GLY also appears to activate the Toll pathway to help clear infection, whilst papers have stated that Toll does not have antiviral activity against CHIKV, this could be a result of changes to the microbiome following GLY exposure. TRI appears to increase negative regulation of the JAK-STAT pathway, which may enable the viral population to increase replication and lead to Mullers ratchet, eventually reducing the number of mosquitoes infected. Additionally, GLY shows upregulation of several immune genes following a blood meal suggesting it may provide better priming of the immune response again viral infection. Further work will need to be done to sequence the microbiome of the mosquito following exposure to treatments and virus, to see if changes in this led to changes in immune expression or pathogen infectivity.

5.4 Conclusion

To conclude, treatment with TRI appears to reduce the vector competence of *A. aegypti* for ZIKV. There are significantly less mosquitoes infected in the TRI treated group than in the control. Additionally, TRI does not increase the dissemination rate for CHIKV and reduces it for ZIKV. TRI reduces the size of the mosquitoes, suggesting that they cannot take larger blood meals which would reduce the midgut bottleneck of the virus nor are they likely to lay larger egg batches, reducing their population potential for transmission. Finally, TRI decreases the survival probability of exposed mosquitoes that have fed on blood meals spiked with CHIKV, reducing the transmission period of those mosquitoes. This trend is not seen with ZIKV; however, it may be that decreasing survival is associated with increasing viral load, and that we needed to leave the ZIKV infected mosquitoes for longer to determine this effect. Treatment with GLY and TRI does not significantly alter the vector competence of *A. aegypti* for CHIKV. Further experiments are needed with higher mosquito numbers, given GLY slightly increases both the infection and dissemination percentage for CHIKV. Further to this, GLY exposed mosquitoes show an increased probability of survival and extended emergence meaning they are likely to have an increased length of transmission period, making them more efficient disease vectors compared to control mosquitoes.

Finally, it is unlikely any changes in competence can be attributed to changes in immune gene expression for the 3 pathways studied. Further experiments with sequencing would be required to determine any direct changes to the microbiome and the effects this may have. Our results also show that the cell culture work with treatments does not carry over to *in vivo* work, likely because they cannot replicate the complex environment within the mosquito. Our work confirms there are multiple factors involved in influencing these infections and experiments, and that the best that can be done is to represent the specific infection conditions at any given time and that there is likely to be variation between different experiments at different times.

Whilst multiple studies have been done on GLY and its effect on vector competence/capacity of multiple organisms, none have been done on the effects of TRI and mosquitoes. Our work suggests that TRI may reduce mosquito-borne disease burden in the areas it is used and that more studies should be done on this. Further studies need to be done to see if GLY alters the vector capacity/competence of *A. aegypti* for *flavivirus*es.

Chapter 6 Exposure of *A. aegypti* to Negev Virus Strain M30957 does not Directly Increase the Vector Competence for Medically Important Arboviruses

6.1 Introduction

Insect specific viruses (ISVs) are viruses that cannot infect vertebrate cells, whilst replicating to high titres in their invertebrate counterparts. Much of the focus has centred around ISVs such as Cell Fusing Agent Virus (CFAV) and Palm Creek Virus (PCV) however, ISVs have been identified in many arboviral families including *Flaviviridae*, *Togaviridae*, *Reoviridae* and *Bunyaviriales*, amongst others (Bolling *et al*., 2015). Some of these viruses, such as CFAV are closely related to arboviruses such as *flavivirus*es, whilst others are closer to plant viruses (Cammisa-Parks *et al*., 1992, Vasilakis *et al*., 2013). A full list of ISVs and their families as reviewed in the literature is described in Carvalho and Long (2021). In 2013 a new taxon of ISV was formed called Negevirus. Negevirus are positive-sense, non-segmented ssRNA viruses distantly related to plant viruses (Vasilakis *et al*., 2013). These viruses replicated to high titres in the insects though could not replicate in or produce cytopathic effect (CPE) in the vertebrate cells tested. Negeviruses show wide geographic spread across the Americas, Africa and Asia in multiple different mosquito species and sandflies showing a wide host range. (Vasilakis *et al*., 2013). The Negevirus taxon contains 6 families of virus including Negev virus (NEGV). With a wide host and geographical range, these Negeviruses may play a role in infection with medically important arboviruses. This study sought to understand how NEGV virus may influence arboviral infection in the mosquito.

Several studies with ISVs have investigated their ability to alter *alphavirus* infection through either superinfection exclusion or priming of the immune system. Patterson (2021) coinfected mosquito cell lines with NEGV (strain M30957) and the *alphavirus*es VEE-TC83 or CHIKV. Regardless of the MOI (1 or 5) of the infecting NEGV or the time point at which it was reduced, VEEV titres were reduced by 5-7-fold at all times compared to the CTRL infection.

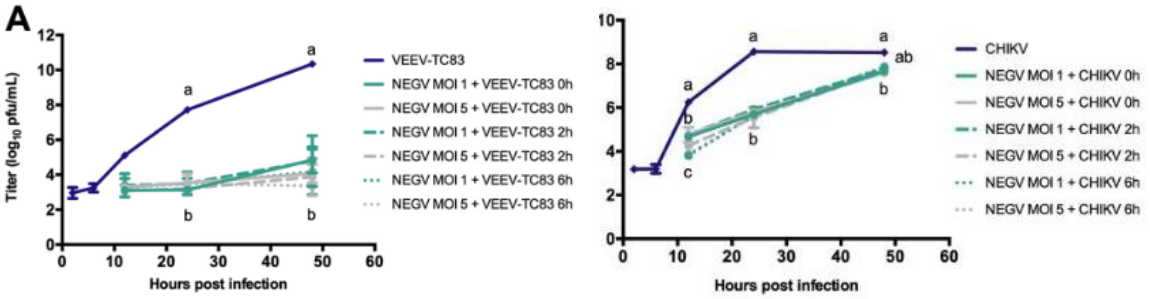


Figure 6.1 Replication kinetics of VEEV-TC83 and CHIKV in C7/10 cells following wild-type NEGV infection at different MOIs. C7/10 cells were infected with VEEV-TC83 (A) and CHIKV (B) 0, 2 and 6hpi after the cells had been infected with NEGV at an MOI of 1 or 5. VEEV-TC83 and CHIKV were inoculated at an MOI of 0.1. Letters indicate significant differences ($P < 0.0001$). Patterson *et al*., 2021.

This effect was *alphavirus* dependent. CHIKV showed a far lower reduction in titre, with 0.65-0.93 log10 pfu/ml reduction at 48hpi. CHIKV titres also varied depending on the strain of Negevirus used, with NEGV reducing the titres the least (0.65 log10) and PIUV-Lutzomyia the most (5.3 log10), suggesting the interactions are virus specific (Patterson *et al*., 2021). Both NEGV and PIUV-Lutzomyia also reduced titres of VEEV-IC strain, Mayaro Virus, Onyong'nyong Virus and Semliki Forest Virus. This study shows Negeviruses may play an important role in modulating *alphavirus* infection in mosquitoes, though these viruses have not yet been tested *in vivo*. We wanted to determine if mosquito coinfection altered the course of infection with a medically important *alphavirus* and *flavivirus*, thereby changing the vector capacity of *A. aegypti*.

6.2 Results

6.2.2 Optimising mosquito infection with an insect specific virus by the oral route

Many studies using ISVs use intrathoracic injection to bypass the midgut barrier and infect the bodies of the mosquitoes. Intrathoracic injection involves injection a solution of virus directly into the haemocoel via the thorax of the mosquito. Given the midgut infection and escape barriers are two of the most important bottlenecks in arboviral infection (Forrester *et al*., 2012), we wanted to optimise an oral infection strategy. Previous attempts at infecting these mosquitoes with NEGV were largely unsuccessful regardless of the titre. Given the transmission routes of ISVs and NEGV are not yet

understood, it is difficult to achieve infection in lab reared mosquitoes that are not already colonised with the ISV. To optimise the oral feeding route for NEGV, in a series of preliminary experiments we provided cohorts of mosquitoes with estimated 10^8 pfu/ml NEGV in either spiked sugar or blood feeds in different combinations on day 0 and day 2. Mosquitoes were monitored for their survival rates and bodies were tested by qPCR after 10 days. A caveat of this is we used infected blood meals which obviously does not replicate how mosquitoes would get this in the wild given NEGV doesn't infected mammals.

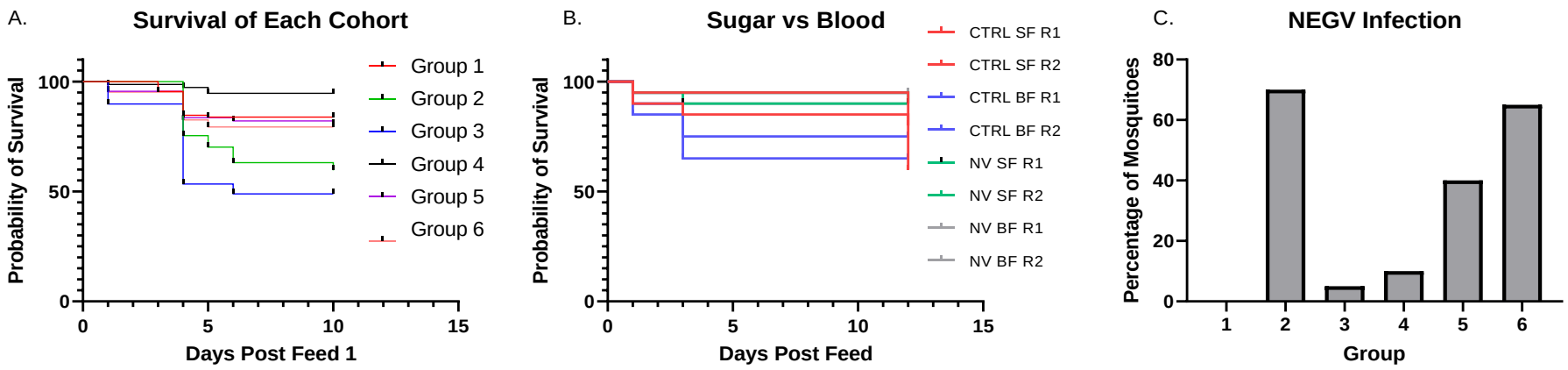


Figure 6.2 The results of optimising a NEGV oral feeding route in the laboratory. A. Survival proportions of mosquitoes from each feed cohort, as listed in (D). (B) Survival proportions of mosquitoes exposed to blood or sugar spiked feeds. This was done to determine if sugar or blood feeds had an impact on the survival of the mosquito, irrespective of NEGV. (C) Percentage of mosquitoes positive for NEGV determined by qPCR against NEGV, $n = 20$. CTRL data not shown (C).

Group	Day 0 Feed	Day 2 Feed
1	SW and ISV	SW and ISV
2	BM and ISV	SW and ISV
3	BM and ISV	BM and ISV
4	SW and ISV	BM and ISV
5	BM and ISV	SW
6	SW	BM and ISV

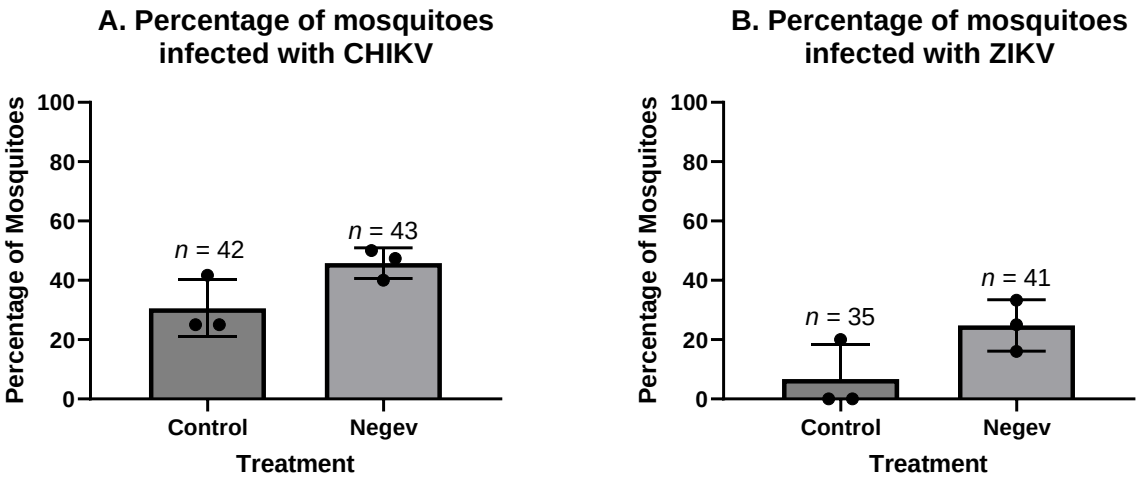
Table 6.1 The different feeds given to six cohorts of mosquitoes on day zero and day two. For group one, at day 0 mosquitoes were fed a sugar meal containing NEGV (ISV) and this was repeated on day 2. For group 2, on day 0 mosquitoes were fed a blood meal containing NEGV and a sugar meal containing NEGV on day 2. Group 3 were fed two blood meals, both containing NEGV, on day 0 and day 2. Group 4 were fed a sugar meal containing NEGV on day 0 followed by a blood meal containing NEGV on day 2. Group 5 were fed a blood meal containing NEGV on day 0 and an uninfectious sugar meal on day 2. Group 6 were fed an uninfectious sugar meal on day zero and a blood meal with NEGV on day 2. Due to time constraints, no other groups were tested.

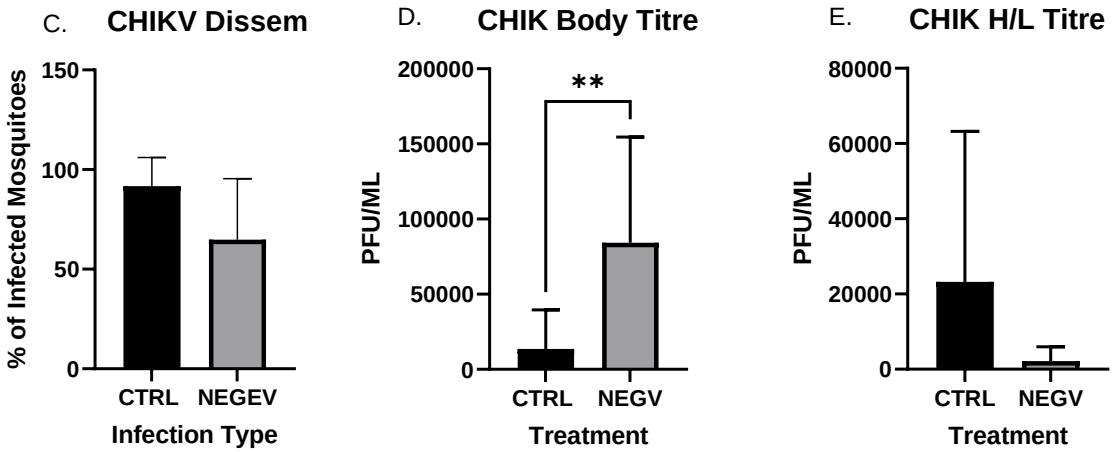
To see if different combinations of feed with NEGV altered the survival of the mosquitoes, we monitored the survival of the mosquito cohorts following feeding. The results indicate that group 2 and group 3, both fed an initial blood meal spiked with NEGV followed by a spiked sugar meal or blood meal respectively, have the lowest survival of the groups (graph A). The group with the highest survival was fed a sugar meal initially. Though the other groups are clustered closely in survival proportions, and these had a mix of initial feeds. To confirm that the presence of NEGV in a blood feed, or the blood feed itself wasn't having an impact on mosquito survival, we fed cohorts of mosquitoes either a CTRL blood or sugar feed or a NEGV spiked blood or sugar feed. These results show that the neat blood feeds do appear to reduce the survival of the mosquitoes slightly, but that the presence of NEGV does not impact this.

We then tested the bodies of the mosquitoes for presence of NEGV after 10 days (graph C). Group 2 and group 6 show the highest percentages of mosquitoes infected with NEGV, with group 2 showing significantly higher infection ($P = >0.0001$) compared to all groups except group 6. Group 6 then is significantly higher ($P = >0.0001$) than all groups other than group 5. It is worth noting that the Cq values for these mosquitoes are higher than we would expect for active infection, though lower than the negatives. Given Negeviruses replicate to high titres in insect cells it is unlikely this is due to lower levels of replication in mosquitoes. This suggests that if the mosquitoes receive a spike blood meal, they are more likely to get infected with NEGV than with a spiked sugar meal. The results also suggest that receiving a spike sugar meal first reduces the likelihood of infection. To counteract this group 3 receiving two blood meals showed one of the lowest percentage infections. Given the groups with the highest percentage of NEGV infection and the groups with the lowest were all fed a mixture of NEGV-spiked or negative blood and sugar meals, we cannot conclude on a specific feed regimen. Further repeats are needed to confirm if one set of feeds increases the likelihood of NEGV infection in our *A. aegypti*. To conclude, feeding results for NEGV are largely inconclusive, with no real patterns identified. Further work will be required to determine the best route for infection within a laboratory.

6.2.1 Exposure to NEGV increases the titre of arboviruses in the bodies of *A. aegypti* but reduces dissemination

To determine if mosquito exposure to NEGV (strain M3097) altered the vector capacity of *A. aegypti* for medically important arboviruses, we reared mosquitoes in cohorts and fed females a spiked sugar meal with estimated 10^8/9 pfu/ml NEGV. Engorged females were separated and 24-36hr post-sugar feed were fed a spiked blood meal of 1 x 10^5 or 1 x 10^6 pfu/ml CHIKV or ZIKV respectively. Blood fed mosquitoes were incubated for 14 days at 27 degrees before the bodies and heads/legs were tested for presence of infectious virus using a CPE assay. A qPCR assay was used to determine if the colony was already infected with NEGV, further sequencing should be required to validate this.


A. Percentage of mosquitoes infected with CHIKV
Percentage of Mosquitoes
100
80
60
40
20
0
n = 42
n = 43
Control
Negev
Treatment
B. Percentage of mosquitoes infected with ZIKV
Percentage of Mosquitoes
100
80
60
40
20
0
n = 35
n = 41
Control
Negev
Treatment


C. CHIKV Dissem
% of Infected Mosquitoes
150
100
50
0
CTRL
NEGEV
Infection Type
D. CHIK Body Titre
PFU/ML
200000
150000
100000
50000
0
**
CTRL
NEGV
Treatment
E. CHIK H/L Titre
PFU/ML
80000
60000
40000
20000
0
CTRL
NEGV
Treatment

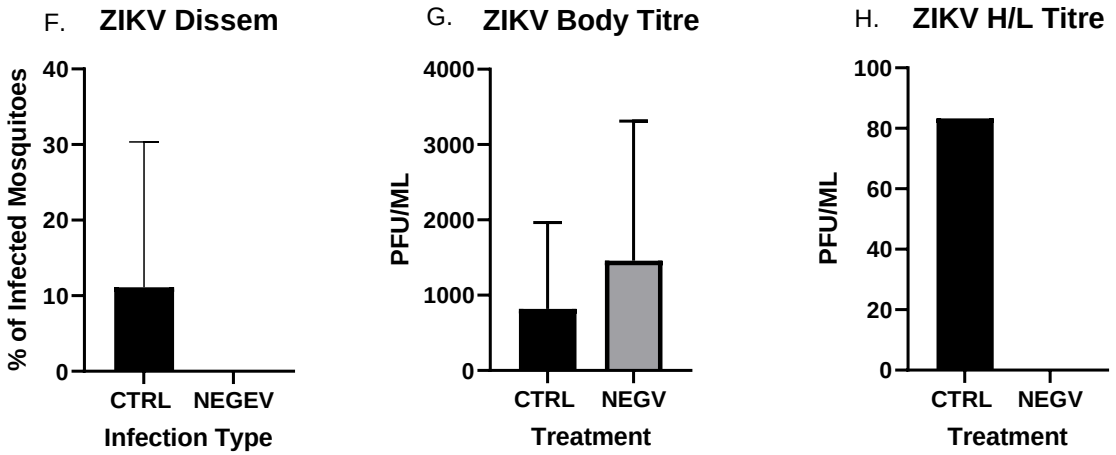

F. ZIKV Dissem
% of Infected Mosquitoes
40
30
20
10
0
CTRL
NEGEV
Infection Type
G. ZIKV Body Titre
PFU/ML
4000
3000
2000
1000
0
CTRL
NEGV
Treatment
H. ZIKV H/L Titre
PFU/ML
100
80
60
40
20
0
CTRL
NEGV
Treatment

Figure 6.3 The results of the CHIKV and ZIKV infection of *A. aegypti* following infection with NEGV. A. The percentage of mosquitoes infected with CHIKV measured with CPE and plaque assays from mosquito bodies, (B) the percentage of mosquitoes infected with ZIKV measured with CPE and plaque assays from the bodies (C) the dissemination rates of CHIKV by CPE and plaque assay, (D) titre of CHIKV in the mosquito bodies by plaque assay, (E) titre of CHIKV in the mosquito heads and legs by plaque assay, (F) the dissemination rates of ZIKV by CPE and plaque assay, (G) titre of ZIKV in the bodies by plaque assay, (H) titre of ZIKV in the heads and legs by plaque assay. n = 3 experimental repeats.

A total of 42 and 43 Mosquitoes were fed a CHIKV spiked blood meal for the CTRL and NEGV cohorts respectively. Based on the numbers currently tested, following a CHIKV blood meal, there was no significant difference between the percentage of infected mosquitoes in the control or Negv exposed groups (P = 0.0732) as determined by an unpaired T-test with Welch's correction. Greater mosquito numbers will be required to determine the true significance of this, given the NEGV treated group do show an increase in the percentage of the population infected compared to the control.

A total of 35 and 41 Mosquitoes were fed a ZIKV-spiked blood meal for the CTRL and NEGV cohorts respectively. For ZIKV the number of mosquitoes infected is higher in the NEGV infected group, though again this is not significant (P = 0.0955). The results suggest that infection with NEGV increases the number of mosquitoes infected for both CHIKV and ZIKV following an infected blood meal. This effect is seen for arboviruses across two families, both alpha- and *flavivirus*es. This is contrary to the cell culture infections previously mentioned, which showed that NEGV reduced *alphavirus* replication. However, those experiments were strain dependent. The NEGV strain used was shown to have a limited titre decrease for CHIKV so we may see different results using the PIUV-Lutzomyia strain. It is also possible that we are using a strain of NEGV that is too distantly related to alpha- or *flavivirus*es to activate the immune system against them.

For CHIKV infected mosquitoes, the titres of CHIKV in the bodies is significantly higher for NEGV infected mosquitoes than for CTRL (P = 0.0039). The number of infected mosquitoes showing dissemination is lower for NEGV infected mosquitoes (P = 0.2674) and the titres of the heads and legs

is far higher for the CTRL, though not significant (P = 0.1543). The same is seen for ZIKV, with higher body titres compared to the CTRL (P =0.5723). Additionally, only one CTRL mosquito and no NEGV exposed mosquitoes showed dissemination (P = 0.4226) with ZIKV so statistical analysis on the pfu/ml could not be done. These results suggest that whilst both CHIKV and ZIKV can replicate to much higher titres in NEGV infected mosquitoes, the viral quasispecies this generates is less able to traverse bottlenecks to achieve disseminated infection. These results indicate that whilst NEGV infection seems to increase the number of mosquitoes infected, likely by increasing the titre of CHIKV and ZIKV in the bodies of the mosquitoes, these mosquitoes show reduced ability of either virus to disseminate throughout the mosquito. This suggests that for this strain of *A. aegypti*, NEGV likely reduces the vector competence compared to the CTRL for both an alpha and *flavivirus*. Further work should qPCR for NEGV in the H/L and see if our theory runs true. I believe that because NEGV is so unrelated to CHIKV, its primed the immune system against NEGV, reducing the pressure of the RNAi on the other two viruses. To test this would initially require a simple cell culture experiment using RNAi proficient and deficient insect cells. Coinfection of CHIKV and NEGV, with NEGV infection preceding that of CHIKV, in RNAi deficient cells such as C637 or C710 cells should see similar titres. Coinfection of CHIKV and NEGV in RNAi proficient cells such as U4.4 cells should see diminished titres of NEGV and increased titres CHIKV, as this study has shown in mosquitoes. C636 or C710 and U4.4 cells can also be used to show that the RNAi response is active against NEGV, either by reduced titre in the U4.4 cells or using miRNA sequencing to show siRNA reads map to NEGV.

6.3 Discussion

Patterson (2021) found that in cell culture different Negeviruses could reduce the replication of certain *alphavirus*es. We wanted to see if this was replicated *in vivo* using *A. aegypti* and NEGV. We exposed mosquitoes to a high concentration of NEGV (estimated 10^8-9) the day before providing them with a blood meal spiked with CHIKV or ZIKV. The exact titre of NEGV was unable to be determined due to inability to plaque the ISV.

6.3.1 How NEGV altered infection rates of *A. aegypti* for CHIKV and ZIKV

We found that whilst there was always a higher percentage of uninfected mosquitoes, NEGV exposure increased the percentage of mosquitoes infected in each cohort compared to the CTRL and that this occurred for both the alpha- and *flavivirus*. The titres for CHIKV were much higher in the bodies of the NEGV exposed mosquitoes than for the CTRL, suggesting that the NEGV exposure allows the CHIKV to replicate to higher titres. This could be because of immune priming against NEGV. As NEGV is closely related to plant viruses rather than *alphavirus*es, it may be that the immune system is primed against NEGV and thus reduces the response against the CHIKV. In this way the NEGV could be acting to benefit the CHIKV quasispecies by distracting the mosquito RNAi. As described on page 169, this could be confirmed using a cell culture experiment where RNAi proficient and deficient cells are used to identify an RNAi response against NEGV and show it leads to an increase in CHIKV titre. Similarly, with ZIKV there is a larger number of mosquitoes infected than in the control group. This suggests that for both viruses, the introduction of NEGV is increasing the likelihood of mosquitoes being infected with arboviruses of two different families. This is the opposite of what was seen in the Patterson (2021). However, in that paper the main difference was seen with VEEV, with limited difference in coinfection titre for CHIKV. Bolling *et al* (2012) found that in *C. quinquefaceatus* there was a significantly lower dissemination rate of WNV at 7dpi in the CxFV infected colonies compared to the CTRL, whilst there were no differences at 14dpi. This suggests that the ISV may have an inhibitory effect early on in infection, but this is lost later. This could account for the reductive effects seen in cell culture in the Patterson paper at 48hpi but the increase we see in mosquitoes at the end of a 14-day incubation period. Additionally, Patterson (2021) used VEEV-TC83 which is a low-fidelity variant, so this may have altered its survival with the strain of Negevirus used in relation to what would happen in wild mosquito populations, though a reduction effect was seen with VEEV-IC strain.

The NEGV exposed mosquitoes show a lower dissemination rate of CHIKV and ZIKV compared to the CTRL mosquitoes. Those that are disseminated with CHIKV show a lower dissemination titre, with 2 x 10^4 on average and 1 x 10^4 in CTRL and NEGV respectively. In the NEGV exposed group, there was no dissemination with ZIKV in any repeat, though only one showed dissemination in the CTRL group. This suggests that whilst the NEGV exposure increases the titre in the mosquito bodies of both

arboviruses it does not lead to a quasispecies capable of traversing the bottleneck into the heads and legs. This could be for several reasons; 1. The CHIKV/ZIKV quasispecies has adapted to replicate in cells where the immune system is targeting NEGV and if NEGV cannot disseminate outside of the mosquito body then the arboviruses cannot survive the bottleneck into the immune competent heads and legs. Vasikalis (2013) infected *A. aegypti* and *A. albopictus* with NEGV strain E0239 and showed dose dependent infection and dissemination. At 10^9 pfu/ml there was 91% midguts infected and 80% showed dissemination. At 10^5 pfu/ml 8% of midguts were infected and none showed dissemination. Based on this, our dose may have been lower than we estimated and the NEGV may not have been able to disseminate. If the increased CHIKV and ZIKV replication is dependent on NEGV compared to the CTRL mosquitoes, then the inability of NEGV to disseminate may have reduced the ability of CHIKV to replicate and disseminate in the heads and legs. Or 2. NEGV is disrupting the mosquito microbiome, and this is leading to changes in abundance that enable CHIKV and ZIKV replication in the bodies but not in the heads/legs. It is worth noting that Shi (2022) didn't find infectious ZIKV in the heads until 21dpi, so there is a chance we did not leave it long enough to observe dissemination. Even then only 5 mosquitoes showed dissemination (Shi *et al*., 2022).

In 2019, Baidaliuk found that infection with Cell Fusing Agent Virus (CFAV) reduced the dissemination of DENV and ZIKV. Upon spiked-blood feeding of CTRL and CFAV infected mosquitoes, there was no change in the rate of mosquito infection, however there was reduced dissemination and dissemination titre in the heads for both *flavivirus*es. They could not identify the mechanism for this, however DENV body titre was predictive of the head titre in the CTRL group. CFAV infection removed this positive correlation. A caveat of this experiment is CFAV infection was achieved with intrathoracic injection which does not accurately mimic natural ISV infection. During vector competence studies on uninfected *C. pipens* and a naturally CXFV-infected *C. pipens* with WNV, they found there was no significant difference in infection rates between the two groups at 14 days (Bolling *et al*., 2012). Upon second repeat they found WNV dissemination rate was significantly higher for the *C. pipiens* not infected with CxFV than for the CxFV-infected group, though not significant. At 14dpi there was no significance again. It seems that early on ISV reduces WNV replication but by 14 days this effect is gone. Though it

is worth noting the negative dissemination rate was only noticed during early mosquito infection, whereas we are finding it at the end of our infection period. Furthermore, a study infecting *A. aegypti* with a Thailand strain of CFAV found this reduced the dissemination of DENV and ZIKV (Baidaliuk *et al*., 2019). These examples are using *flavivirus*-like ISVs, whilst our experiment would suggest that dissimilar viruses can also alter the course of infection. The Thailand CFAV experiment has been used as an example of why choosing the correct vector-ISV pair is important. Whilst this remains undisputed, their reasoning is that in cell culture, the original CFAV isolated from *A. aegypti* enhanced replication of DENV (Zhang *et al*., 2017), whilst the Thai strain reduced it. However, the cell culture paper using Aa20 cells found that although replication of both CFAV and DENV was enhanced up to day 3, it was then inhibited by day 5. This supports the reports that the Thai strain of CFAV reduces dissemination. It could be that in mosquitoes the complimentary effect of the ISV increases replication over a longer period due to the time it takes to rebuild diversity and overcome bottlenecks in mosquitoes that is not present in cell culture, but then inhibits replication after a certain point, hence the lower dissemination rates and titres that we see. Though if this occurred with the CHIKV/ZIKV and NEGV we would expect a lower body titre.

Other studies have identified positive correlations between ISV and arboviral infection, with mosquitoes positive for WNV 4X more likely to be infected with CxFV than in the spatiotemporally WNV negative pools and that CxFV (Newman *et al*., 2011). Furthermore, WNV and CxFV were more prevalent in *Culex* during epidemic years than in both interepidemic periods studied. Though they noted that infection rate of mosquitoes with CxFV was more associated with environmental factors than coinfection with WNV.

6.3.2 NEGV may alter the mosquito microbiome, preventing dissemination

The changes in body and dissemination titre could be a result of changes to the mosquito microbiome in the bodies and heads/legs. Shi (2022) compared the bacteriome and virome of *A. aegypti* and *C. quinquefaceatus* fed with sucrose/water, blood or blood spiked with ZIKV or WNV. The results were virus-mosquito specific. In *Culex*, they found that an increase in diversity for one ISV was associated with an absence of WNV dissemination. They found that only mosquitoes without, or with low levels

of Wenzhou sobemo-like virus 3 (WSLV3) genome copy number, were capable of having disseminated WNV infection determined by plaque assay positive heads. This suggested that presence of WSLV3 in the heads prevents successful dissemination. It's possible that NEGV is modulating the microbiome resulting in growth of titre and diversity of other ISVs in the heads that prevent dissemination of CHIKV/ZIKV to the heads/legs. To determine this, we would be required to sequence the bodies and the heads/legs of our samples.

Shi (2022) also found that in blood-fed *A. aegypti*, at 7dpi there was transiently higher ISV diversity compared to sugar fed mosquitoes and that this was lost by 21dpi. They suggest that the blood meal has stimulated this change and that then a sugar fed diet brings ISV diversity back to homeostasis by day 21. It could be that the NEGV coupled with the blood feed is causing disruption for the first few weeks, enabling CHIKV and ZIKV to replicate to higher titres and then this effect cannot traverse the bottleneck into dissemination infection and so is lost. Given NEGV cannot replicate in vertebrates, mosquitoes are unlikely to acquire NEGV via an infected blood-feed so these results may not represent effects of arboviral infection in natural mosquito populations.

Together, the results show that prior exposure to NEGV increases the likelihood of mosquito bodies to become infected from an infectious blood meal but decreases the likelihood of the virus disseminated throughout the mosquito as is needed for transmission, thus reducing the vector competence in these vector-ISV pairs.

6.3.3 Immune priming against NEGV and not CHIKV

We initially hypothesised that a way in which ISVs would alter arbovirus infection would be through immune priming. Vargas (2020) exposed adult mosquitoes to an inactivated DENV protein and found them to be more resistant to DENV infection. Further to this, they exposed mosquito larvae to the inactive protein and found the subsequent adults to also be more resistant. They measured the viral DENV load detected in the excreta of mosquitoes and by day 14 found that there were significantly lower viral genome copy numbers from the primed mosquitoes compared to the unprimed (confirmed by plaque assays on the heads at 21dpi). Serrato-Salas (2018) challenged adult mosquitoes with DENV-

2, inactive DENV-2 or mocks, and challenged them with DENV-2 7 days later. Inactivated virus primed mosquitoes consistently had less viral presence in midgut tissue and whole carcasses. Nhumirim and palm creek virus (PCV), both insect-specific *flavivirus*es, decreased *flavivirus* replication and transmission in cells and mosquitoes, but could not reduce replication of *alphavirus*es (Kenney *et al*., 2014, Romo *et al*., 2018, Hobson-Peters *et al*., 2013). Vargas (2020) and Serrato-Salas (2018) show pervious exposure to proteins of the same virus increases protection against a second challenge. As NEGV is more closely related to plant viruses than either of the flavi- or *alphavirus* used in this experiment it is unlikely it is reducing dissemination titre in the same means as these examples. There is limited information about innate immune responses of mosquitoes against ISVs they are persistently infected with, however it has been shown that ISVs are targeted by the RNAi response, as are arboviruses. This raises the question: Could NEGV be distracting the immune system, enabling CHIKV and ZIKV to replicate to higher titres in the same compartments as NEGV?

6.3.4 Can a lack of siRNA response in cell culture lead to superinfection exclusion?

Several studies have shown the siRNA response is active in mosquito cells persistently infected with ISVs, though this is dependent on mosquito species and ISV (Goertz *et al*., 2019, Franzke *et al*., 2018). The cell experiments that showed NEGV could reduce *alphavirus* replication were done in C7/10 cells that lack the prominent siRNA response. It's possible increased replication of NEGV *in vitro* where the siRNA response is unable to CTRL replication leads to superinfection exclusion of the *alphavirus*es, hence the observed decrease in replication of VEEV and CHIKV. There was limited effect on CHIKV titer when inoculated 0, 2 and 6hr after NEGV, suggesting that it is not time dependent in cells and therefore the reduction was unlikely to be the result of immune priming against CHIKV and so superinfection exclusion may explain this. On the other hand, once infecting the mosquito the siRNA response may focus on the NEGV introduced first and become distracted from the then infecting CHIKV and ZIKV. If our infecting titer was too low for NEGV to achieve dissemination, then the siRNA response in the heads/legs increases the size of the bottleneck the CHIKV and ZIKV quasispecies faces upon dissemination, hence the reduction. The two arboviruses may have a quasispecies that is unable to reach the areas of sequence space that will allow them to adapt quickly to the siRNA response. It would

be interesting to create low-fidelity mutants of both viruses and see if these would be able to disseminate better. Additionally, an experiment to test this theory would be to infect C636 cells with CHIKV/ZIKV and then passage into U4.4 or C636 cells at an MOI of 0.01 to represent the initial dissemination bottleneck. You can then determine if the siRNA response in the U4.4 cells decreases the likelihood of them traversing the bottleneck compared to in the C636 cells. Finally, you can do an experiment with NEGV and CHIKV/ZIKV in both U4.4 and C636 cells and see if replication is higher for CHIKV in the U4.4 cells, compared to C636 cells and CTRL U4.4 cells which would suggest that the NEGV is reducing the siRNA response. There is currently no evidence to support that one virus can distract the siRNA response from another, however further work as described above is needed to determine this.

6.3.5 Optimising an oral infection route for NEGV

For one part of our experiments, we planned to develop a colony persistently infected with NEGV, infect with CHIKV and collect eggs and then expose the second generation of mosquitoes to CHIKV again and see if there were differences in protection against CHIKV in the CTRL or the NEGV colony. It was important that we got a colony persistently infected with NEGV to mimic the natural environment. Most studies, including ours, that have wanted to look at how infection with ISVs alters mosquito responses to arboviruses have done the ISV infections just before the second virus in question. This may overstimulate the immune system and cause a larger reaction than would occur if these mosquitoes were persistently infected in the wild. If novel exposure to a virus will activate the immune system, then it is not a system that replicates the environment well, and so a persistent infection was required to witness the proper effects of NEGV on the mosquito.

To achieve this persistent infection, we trialled multiple different feeding routes of spiked blood or sugar feeds in different combinations. The three groups that showed the highest percentage of mosquitoes infected were all given a NEGV-spiked blood meal on at least one of the days. All three groups also received a sugar meal, but whether this was infected or not did not seem to make a difference. Pesko (2009) found that both *A. aegypti* and *A. albopictus* had significantly lower rates of infection when fed a blood meal containing 20% sugar on a cotton bud compared to a normal blood meal via membrane feeding. Though this was attributed to discontinuous feeding from the cotton rather than an effect of the

sugar. Additionally, group three that was fed two infected blood meals had a low infection percentage. More repeats would need to be carried out on this work to understand the optimal feeding strategy for NEGV in lab grown colonies.

The addition of NEGV to a blood or sugar feed does not increase the mortality of the mosquitoes. As mentioned earlier, perhaps a higher titre of NEGV would have led to more success, though this is unlikely to stimulate the titre of NEGV mosquitoes may pick up in the wild. Despite our intentions and previous proven efficiency of infecting *A. aegypti* with NEGV at high concentrations, Vasikalis (2013) acknowledges that titres of 10^9 are unlikely to be circulating in natural sources and that if mosquitoes did pick NEGV up from plant nectar it was more likely to be at 10^5. Previous experiments by Vasikalis (2013) also suggest the success of infection is virus and mosquito strain specific. Our study has used a strain of NEGV that has not originated from our species of mosquitoes, with it originally identified in *Culex* and *A. albopictus*. Given oral route is so difficult to achieve, and we know vertical transmission occurs, these viruses were picked up by mosquitoes so long ago that they were different viruses when they established the initial infection and have coevolved together for so long that they wouldn't work going into new mosquitoes. We know NEGV is closely related to plants and that NEGV-like viruses have been found in aphids (Qi *et al*., 2021). Perhaps these are where they originated, and they have changed over the course of infection and evolution with the mosquito.

To study how ISVs can be used as a CTRL strategy for arboviruses, it is essential to develop methods to successfully infect novel mosquito species with ISVs of choice. This will also have to be monitored over time to ensure the reductive effect of the ISV on the arbovirus is not due to initial exposure of the mosquito, and that it is maintained in the long run. Finally, if ISVs have co-evolved with the specific mosquito strain/species they were isolated from then it may not be possible to infect lab strains persistently. One method to achieve this could be to adapt an ISV to a mosquito by repeated infection and harvesting until you eventually have a strain that can persistently infect that strain of mosquito. Whilst the CHIK immune work was unable to be completed due to time constraints, this raises an important question of how we attempt to infect mosquito lab colonies with novel ISVs to replicate natural systems.

6.3.6 Intrathoracic injection bypasses the main bottleneck in mosquitoes, altering the course of infection

ISVs are likely transmitted in wild populations of mosquitoes by vertical transmission. Bolling (2012) identified circulating strains of ISVs in mosquitoes in 2006-2007 and decided to test a colony they set up in 2005 from wild-caught mosquitoes for ISVs. The colony and the progeny were persistently infected with these ISVs, showing vertical transmission naturally occurs. Multiple studies have reported problems in infecting their lab colonies with different ISVs and instead have used intrathoracic injection to bypass the midgut infection barrier. Working with ZIKV and DENV-1 isolated cell fusing agent virus (CFAV) from a colony of mosquitoes in Thailand, Baidaliuk (2019) infected their mosquitoes intrathoracically with CFAV and then blood-fed with ZIKV and DENV-1 two days later. Hall-Mendelin (2016) studied the effects of PCV infection in Australian mosquitoes exposed to *alphavirus*es. This study used a different strain of mosquitoes to the strain the virus was isolated from (as did we) and following failure to infect *Culex annulirostris* with PCV by oral infection, they used intrathoracic injection and had 100%, 95% and 100% infection of *Cx. annulirostris*, *A. vigilax* and *A. aegypti* respectively. Of these infected mosquitoes, none of the 1038 progeny were infected with the ISV, showing vertical transmission did not occur. Given Bolling (2012) has shown vertical transmission is likely a way persistent infection is achieved in natural populations, these results show that intrathoracic injection is altering the course of infection and is likely a poor way to study ISV caused changes to vector competence. As has been shown in multiple studies, midgut infection and escape are massive barriers to viral infection in mosquitoes and intrathoracic injection bypasses this. This could drastically alter the course of infection. This also reinforces that the midgut infection escape is a strong barrier for oral infection and that it is perhaps necessary for complete dissemination to occur in the mosquitoes. To conclude, intrathoracic injection does not accurately represent natural infection and it appears the midgut infection/escape barrier is a strong barrier to infection with ISVs. The data indicates vertical transmission is likely how ISVs are maintained in nature and natural ISV-vector pairs should be used for vector competence studies. Developing an infection route that allows vertical transmission to occur is key for use of ISVs as arboviral CTRL strategies.

6.4 Conclusion

Our work shows that exposure to NEGV 24hr before infection with CHIKV or ZIKV increases the percentage of mosquitoes infected with the arboviruses. This is likely due to an increase in titre of the arboviruses in the bodies of the NEGV exposed mosquitoes. However, this does not increase the vector competence as both dissemination and dissemination titre are reduced in the NEGV exposed mosquitoes. It is unclear how NEGV exposure is altering the arboviral infection, though it is unlikely to be via immune priming giving the different background of the viruses. It could be due to targeting by the RNAi response which then cannot target CHIKV with the same intensity, modulation of the mosquito microbiome by changing ISV diversity to prevents dissemination, or by generating a CHIKV/ZIKV quasispecies adapted to replicate in co-culture with NEGV, unable to disseminate in the absence of NEGV. This work suggests that the best way to study the tripartite interactions of ISVs, mosquitoes and arboviruses is using mosquito-ISV pairs that have co-evolved together. Intrathoracic injection does not accurately represent infection and exposing a mosquito to a novel virus before-hand just gives a brief representation of what may happen when a mosquito encounters a new virus that it is not already infected with. Mosquito lab colonies should be screened upon start up to identify any ISVs that they are infected with, and when look at transmission risks in each area, wild vector-ISV pairs should be identified and colonised for vector competence studies. Finally, to use novel ISV-mosquito pairs for arboviral CTRL strategies, a method of laboratory infection that achieves vertical transmission within the colony is key.

Chapter 7 Discussion

7.1 Introduction

The successful transmission of vector-borne diseases such as arboviruses relied on a delicate balance of factors that shape the competence of their mosquito vectors. Our work here has given fascinating insight into the interplay between the virus and mosquito, showing that low-fidelity viruses are unable to survive the barriers within a mosquito, regardless of the mosquito innate immune pathway, the RNAi. Moreover, the application of agricultural chemicals such as glyphosate and triticonazole reduce the vector competence of A. aegypti for both ZIKV and CHIKV, whilst the same effect is seen for the ISV NEGV. These discoveries have identified key factors that shape mosquito vector competence and help us to understand the implications of these in the greater picture of vector-borne disease research.

7.2 What is the role of the RNAi in affecting viral diversity and vector competence

7.2.1 How does RNAi impact viral diversity?

RNAi plays a crucial role in influencing viral diversity by targeting viral RNA for degradation. Targeting of specific sequences within the virus can lead to selective pressure which can shape the diversity of the viral population in its natural environment. Variation is introduced into the viral population via a number of different methods, including insertion/deletion, recombination and reassortment. Regardless of how the variation occurs, the wealth of variants encompassed within the mutant spectra give RNA viruses the capacity to change cell tropism or host range, or to overcome internal and external selective constraints placed on them such as the host immune system or RNAi (Domingo *et al*., 2012). Brackney (2009) found that in *Culex quinquefaciatus,* hotspots targeted by the RNAi along the WNV genome were more likely to contain point mutations compared to cold spots along the genome, reinforcing the selective pressure of the RNAi. WNV produces subgenomic *flavivirus* RNA that acts as a competing substrate for DCR2 in insect cells, allowing continued replication of WNV via distraction of the siRNA response (Schnettler *et al*., 2012). When generating viral siRNAs against SFV infecting U4.4 cells, the siRNAs generated against mutational hotspots of the viral genome were less effective in the RNAi

response than those generated against cold spots in the genome (Siu *et al*., 2011). Additionally, generation of DIPs to evade the RNAi response enhances the diversity within the population by producing genomes different to the parent virus. This evidence supports the generation of viral diversity as a means of coping with the RNAi response. This interplay between RNAi and viruses is a fascinating example of co-evolution and the impact on viral diversification.

7.2.2 How does RNAi impact vector competence?

RNAi plays a crucial role in determining vector competence. In this case, vector competence refers to the ability of mosquito vectors to become infected with and subsequently transmit an arbovirus. When a mosquito first takes an infectious blood meal, RNAi may be triggered in response to viral dsRNA replication intermediates. This leads to cutting of viral RNA and subsequent limitation of replication. If a vectors RNAi response is robust enough, it may reduce the likelihood of successful transmission to a new host. The Carb109M transgenic mosquito line was created to express a 587nt inverted repeat RNA (IR-RNA) from the DENV-2 genome following a blood meal (Franz *et al*., 2014). The IR-RNA was processed into 21nt small RNAs corresponding to the section of DENV-2 genome and these Carb109M mosquitoes were refractory to DENV-2 genotype infection. Equally, if the RNAi response is suboptimal or a virus has evolved mechanisms to evade the RNAi, transmission efficiency may increase. This suggests it is likely the RNAi plays an important role in the achievement of persistence in mosquito infection, preventing the viral population from over-replicating and leading to acute infection in the mosquito. We developed a cell culture model that replicates mosquito infection for VEEV 68U201, requiring an extended infection period within the insect cells. We hypothesised this is because the viral population takes 6-8 days to regenerate enough diversity to traverse the next bottleneck. It is likely that during this time the RNAi response is purifying DIPs within the population.

Given low-fidelity mutants impact the vector competence of a mosquito, with the mutants showing attenuation in mosquito infection, we looked at differences in the RNAi response against parent and low-fidelity viral strains. We saw no difference in the siRNA response between TC83 and TC83_3X nor was there a difference in survival of the 68U201 3X, 4X or G7R viruses between siRNA efficient/deficient cells. This suggests that lowering the fidelity of the viruses does not alter the RNAi

response against them. To further understand the impact of external factors on the RNAi and vector competence, we looked at how DCR2 and AGO were expressed following treatment with GLY or TRI. There were no significant differences between expression of DCR2 and AGO between control, GLY and TRI treated mosquitoes in control blood fed, CHIKV infected or CHIKV exposed but uninfected mosquitoes. The only notable difference was in TRI treated mosquitoes that showed AGO was upregulated in CHIKV infected mosquitoes compared to those that were control blood fed. It is likely the RISC complex is more active in infected mosquitoes compared to solely blood fed as it will be continuously processing the small RNAs produced by DCR2. Changing the diversity of the virus does not seem to change the number of siRNAs produced by DCR2, but TRI does seem to increase the activity of the RISC complex slightly, though the impact of this is unclear.

Together, our results indicate that the RNAi response shows limited change when we presented mosquitoes or mosquito cells with two commonly used agricultural chemicals and low-fidelity mutants, despite these factors reducing mosquito vector competence and changing viral survival probability respectively. The RNAi response is largely responsible for achieving persistent infection in mosquitoes, providing the right conditions for a successful lifelong infection.

7.2.3 What does this mean for the understanding and use of RNAi?

Based on our results, this suggests that the RNAi response is inherently robust and experiences little variation in its action regardless of the circumstance. This highlights its importance as a key antiviral response in the mosquito. Most studies on RNAi and siRNA have been done on *Drosophila melanogaster* and there are limited differences between the processes and proteins compared to mosquitoes, despite *Drosophila* and mosquito lineages diverged 260 millions years ago (Arensburger *et al*., 2010). This shows that despite different lineages and a huge amount of time, this response has remained robust and important in its viral control and suggests that limited factors will affect it, as we have already seen. This also raises the importance of other factors affecting vector competence. Despite the RNAi response not changing, viruses with lower fidelity are still attenuated in mosquito infection due to inability to traverse repeated bottlenecks. This shows the importance of studying every aspect of vector competence, as despite the importance of RNAi, it is not the sole factor affecting it.

This conclusion of the robustness of the RNAi response shows it should be one of the main focuses of vector control. If we could introduce an RNAi knockdown mosquito into the population, then it would no longer be an efficient vector given it may lead to over replication of the virus leading either to viral extinction or mosquito mortality. Further experiments with knockdown mosquitoes would be needed to confirm this expectation. RNAi has already shown promise as an effective control strategy in mosquitoes. Transgenic lines of *A. aegypti* were generated to express multiple copies of miRNA specific to one or 4 serotypes of DENV and were able to reduce DENV replication for the 4 serotypes compared to in the opposing non-transgenic line (Liu *et al*., 2021). Our work has highlighted the robustness of the RNAi response under several different conditions and show that viral diversity itself is an important factor in vector competence. This has highlighted the need to understand all aspects of vector competence and a focus on the use of RNAi as a control measure.

7.2.3 The role of internal and external factors on mosquito vector competence

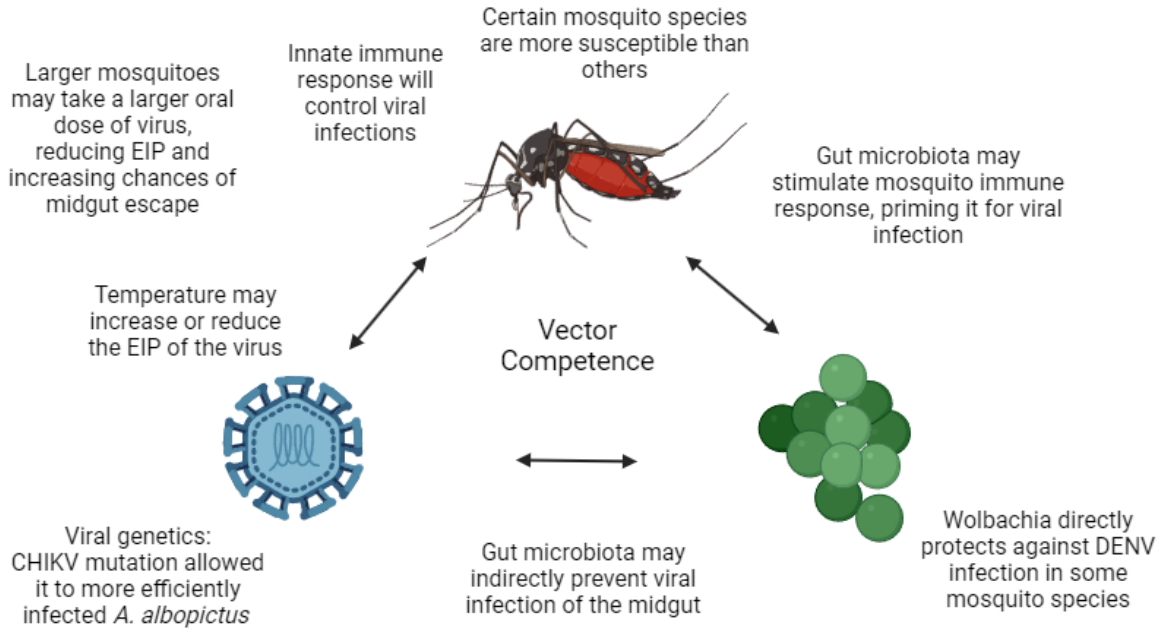


Figure 7.1 A schematic outlining the basic interactions between the virus, the mosquito and the mosquito microbiota in vector competence. Viral genetics: The E1-A226V mutation in CHIKV allowed for more efficient replication in *A. albopictus*. Mosquito: Certain mosquito species or strains are susceptible or refractory to certain arboviruses. Microbiota: Commensal gut bacteria such as Wolbachia protect against

DENV challenge in some mosquito species. Further interactions such as temperature altering the EIP, mosquito size influencing viral uptake during an infectious blood meal, mosquito immunity protecting against specific arboviruses and gut microbiota priming the immune system also influence vector competence.

Vector competence is the ability of a mosquito vector to become infected with and transmit a virus. It relies on the mosquito ingesting virus in an infectious blood feed, and the virus then efficiently replicating in and disseminating through the mosquito to the salivary glands. From here, it must efficiently replicate in the salivary glands and be transmitted to a new vertebrate host at a high enough titre to generate infection. Vector competence is influenced by a range of factors, including the genetics of the virus and the genetics, immune system and microbiota of the mosquito. This is an interlinked relationship which involves a large amount of complexity and often cannot be understood from individual experiments alone.

The mosquito microbiota has strong influences on vector competence, and so remains an important factor to consider. For example, a commensal gut bacterium, *Serratia marcescens*, secretes SmEnhancin, a mucin-degrading protein. This degrades the protective mucin layer on the midgut and promotes DENV infection (Wu *et al*., 2019). Two ISVs, Phasi Charoen-like virus and Humaitia Tubiacanga virus promote expression of the proviral gene H4, promoting DENV and ZIKV infection in *A. aegypi* (Olmo *et al*., 2023). Finally, a prime example of ustilising the mosquito microbiome for pathogen control is *Wolbachia*. When Aag2 or Aag2-wMel cells were infected with DENV, by day 5 there was 388-fold less intracellular copies of DENV detected in the Aag2-wMel cells and DENV titre was 4-fold lower than in the control cells (Thomas *et al*., 2018). This effect is dependent on both the virus, the mosquito and the *Wolbachia* strain. When *Wolbachia* wAlbB strain infects *Culex tarsalis,* an important vector of WNV, WNV infection rate was significantly higher compared to control mosquitoes (Dodson *et al*., 2014).

In chapter 5 we hypothesised that treatment with GLY and TRI may be impacting the microbiota of the mosquito. For both CHIKV and ZIKV this reduced the percentage of the tested *A. aegypti* population that became infected with either of these viruses. We expected the treatments to modulate the microbiota and therefore change the immune response, however we saw limited difference in immune gene

expression between treatments. This may be a result of our choice of genes. Given we looked at structural proteins for the JAK-STAT and Toll pathways we may have seen a larger difference had we chosen effector proteins and looked at different time points to see how this influenced early infection. To fully understand the full scope of this warrants another study. Furthermore, our work with the ISV NEGV showed that pre-exposure to NEGV increased the percentage of mosquitoes infected but significantly reduced dissemination rates, lowering the vector competence. Our work has highlighted how external factors influence vector competence against two alpha- and *flavivirus*es and outlines the need for in-depth studies to fully understand how these factors are influencing the mosquito or viral biology to change the competence. Further studies are needed to see the impact of the two treatments on the microbiota but suggest that they play a role in modulating vector competence. This opens new avenues of research to increase our knowledge of human contributions to changing vector competence, showing our actions with agricultural chemicals, which are used in farming practices globally, can change vector competence. This also highlights the need to further study the impact of external factors on the microbiome and its contribution to vector competence. Our work has shown that multiple factors can influence vector competence, and with the complex interplay between virus, mosquito and microbiota, it is likely that multiple external factors have a collective impact.

Our research primarily focuses on understanding the various factors that influence vector competence, a crucial component of vectorial capacity. Classic vectorial capacity is defined by the vector competence, the viral EIP, the size of the mosquito population, mosquito survival through one day and human biting rates. Moreover, this is subject to a multitude of external factors such as temperature, human population, enzootic mammal population, vaccination rates etc. with the multitude of factors involved, vector competence data cannot be a sole indicator of the probability of a mosquito in transmitting a viral disease. In Nigeria in 1969 an outbreak of WNV virus was facilitated by a population of *A. aegypti*. These mosquitoes were considered poorly competent for the virus, however due to high population density and high biting rates, became efficient vectors for the epidemic (Miller *et al*., 1989). Vectorial capacity tells us the importance of a mosquito as a vector for an arboviral disease and is key to

understand. Our work has added to our knowledge of vector competence and further work would be required to see the impact on vectorial capacity.

7.2.4 Future directions

To study vector competence and capacity well for a mosquito-virus pair as is needed, we would need multiple experiments and field data to map out the relationship and predict the risk. Chen (2023) surveyed 265 current studies and found that over 90% of the potential mosquito-virus combinations were untested in experimental settings and that entire regions and their corresponding vectors and viruses were under-sampled. This study stresses the importance of doing vector competence studies around the world with a range of vectors and viruses to avoid the inherent bias that is only testing pairings that are naturally found in the same geographical areas. Additionally, it shows that most of the research is done on a select few viruses, spurred on by their involvement in outbreak, which suggests that the research is largely driven by the economic and health impact of a current/previous outbreak, which doesn't account for new future viral outbreaks.

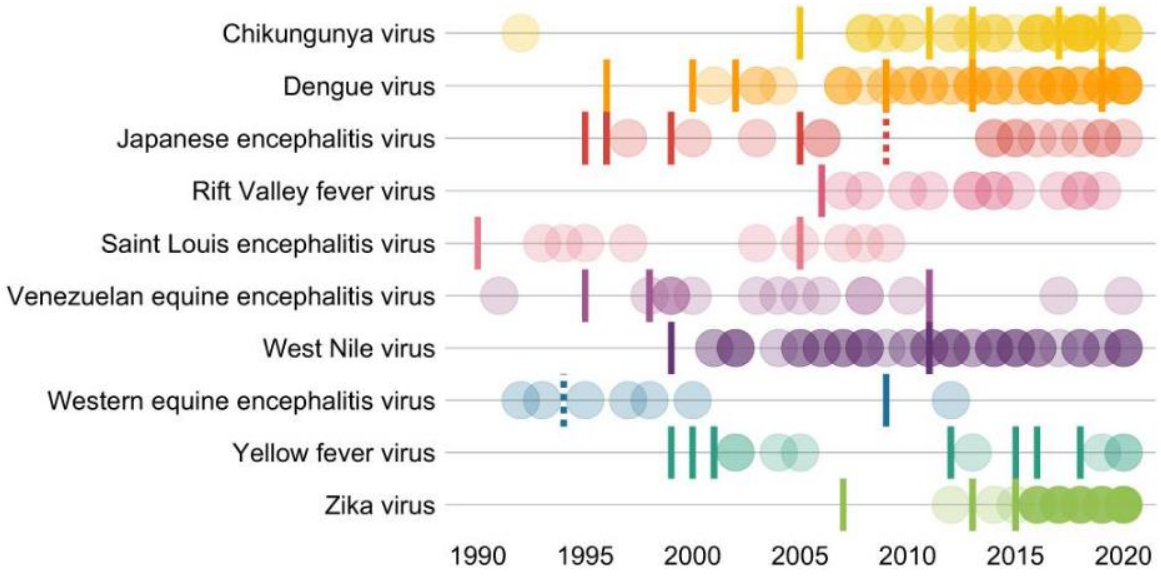


Figure 7.2 Vector competence studies over time broken down by the 10 viruses most studied. Solid lines represent outbreaks that motivated further enquiry. Dashed lines are other notable developments in viral history such as in 1994 the last known human case of WEEV in North America and 2009 creation of WHO WPRO and SEARO Japanese Encephalitis Virus Reference Laboratories (Chen *et al*., 2023).

Studies such as these lend us the clarity that we need to move away from individual labs testing individual factors. This approach has limited cohesion and results in a large amount of variables remaining unaccounted for. One approach to resolve this would be the generation of a global consortium for vector competence and vectorial capacity research that aims to identify the current and future risks of mosquitoes as arboviral vectors. This will require global surveillance to identify which mosquito species are found in which countries, that can also be correlated with habitat preference data which can influence control strategies. This approach can also identify ISV populations linked to mosquitoes which can further studies into the impact of ISVs on vector competence. Further labs can be conducting competence studies of viruses that are both currently in that region or predicteds to be in future. Mathematical modelling can be used for these predictions. Several labs can be collaborating on testing current and predicted temperatures for each region to understand how this will impact viral replication in future. This requires an open access global repository where the data of individual experiments can be stored. This allows us to systematically work through what needs to be done for each individual mosquito-virus pairing and what has already been done. It requires an institution to oversee the data and allocate tasks to specific laboratories, and ensures that we systematically cover the different conditions required to truly understand vector competence and capacity for each virus-vector pairing. Across 68 vector competence studies on Australian mosquitoes that covered 111 mosquito-virus pairings across 389 individual expeirments, variation in vector competence between species within the same genus were identified. Furthermore the study identified which types of mosquitoes were more/less susceptible (Kain *et al*., 2022). This allowed them to determine which species of mosquito were more important for control measures. This highlights the usefullness of a global repository and collaboration, and how quickly we would begin to see patterns emerging.

This knowledge would allow us to understand the competence and capacity of vectors within each region for current and predictive scenarios. This will provide us information to influence public health guidelines and mosquito control measures. It will give us greater understanding to develop gene control

systems and vaccines for areas where appropriate and allow us to generate a guided response, as gene therapy has done for modern medicine.

7.3 How do we assess viral evolution and model bottlenecks in the laboratory

Viral diversity and evolution are often studied through DNA and RNA sequencing. It allows scientists to see how a viral genome has changed over time and generations. This can be done using field or patient samples during an outbreak, *in vivo* mosquito infections or in *in vitro* cell culture models. Analysis from the field allows us to map viral evolution in real-time and understand how viruses adapt in a completely natural environment, with all external factors accounted for and from this, patterns can be drawn. In 2005, a CHIKV strain evolved a mutation in its E1 glycoprotein (E1-A226V) that allowed for more efficient replication in *A. albopictus*, leading to an outbreak in the Indian Ocean Islands (Tsetsarkin *et al*., 2007). Before the Caribbean outbreak*, A. albopictus* had not been determined a prime vector for CHIKV, with the lineages mainly transmitted by *A. aegypti*. However, there were several clear instances of *A. albopictus* being the primary vector in the outbreak. During oral feeding experiments, infectious E1-A226V CHIKV was detectable in saliva at 2dpi and reached higher titres of 10^3.3 pfu/ml in *A. albopictus* compared to 10^2.5 pfu/ml in *A. aegypti* (Dubrulle *et al*., 2009). This is a clear example of how sequencing has been used to understand vector competence and has been used in conjunction with *in vivo* experiments.

In vivo mosquito systems are useful because they replicate natural viral infection and account for the mosquito microbiome and immune system. However, mosquitoes can be difficult to work with and do not always replicate natural infection as well as we would think. Mosquito biology demands specialised facilities and expertise for maintenance, rearing and handling. Providing the correct conditions for certain mosquitoes can be labour intensive and time-consuming and often leads to little reward. There is variability in mosquito feeding behaviour which can impact the consistency of infection rates and transmission studies. *In vivo* work with mosquitoes allows for alterations such as differences in rearing larvae and adults may alter the adult mosquito microbiome, which in turn may impact its vector competence, as will the variety of different feeding methods used to entice wild-caught mosquitoes. Furthermore, to maintain efficient *in vivo* studies requires vertebrate hosts such as rodents in the case

for VEE, which raises ethical concerns, time-constraints and adds another layer of complexity to the experimental design.

Establishing an efficient cell culture model allows us to investigate viral evolution and behaviour under controlled conditions without the need for the higher insect containment systems needed for *in vivo* mosquito infections. Cell culture reduces the ethical considerations within the experimental design and is generally more cost-effective than using *in vivo* systems. With cell culture models it is easier to control the environment which leads to more reproducible conditions. This control is crucial when studying bottlenecks, as it allows for the manipulation of specific factors such as cell type, viral titre and temperature. Additionally, cell culture allows for rapid turnover which can be advantageous when working to tighter time frames.

Our cell culture model considers the time it takes VEE to generate diversity following a bottleneck and uses a low MOI to replicate the size of the viral population that normally traverses a bottleneck. From this, we can alternate from insect to mammalian cells (with efficient replication in mammalian cells at 2 days) and understand how the viral population is evolving and adapting to the dual host system without the requirement of mosquito and rodent *in vivo* work. Our cell model has allowed us to see how viruses with different mutational spectrums struggle to overcome multiple bottlenecks and has increased our understanding of how important mammalian cells are in the arboviral life cycle. Within the space of five to six weeks we were able to monitor viral evolution and adaptation to alternating passage whilst the same work would have taken five to six months in *in vivo* systems, giving us the same results. Sequencing is beneficial and would have greatly improved this study, but this provides brilliant preliminary results for a study with further replicates that allows for sequencing of isolates over the duration of the passages. This new model is essential as it allows us to watch viral evolution in real time without the constraints of *in vivo* systems and is proven accurate due to replication of the attenuation of the mutants seen in previous *in vivo* work. Here, our experiment has answered questions the *in vivo* work could not, showing that the altered diversity did not allow the viruses to survive repeated bottlenecks. However, this model requires refining which the time period of this work did not allow. For instance, for more accurate MOI between each bottleneck plaque assays would need to be conducted on samples

to account for titre changes between passages. We are also looking at this from a simplistic view and not accounting for instance for the fact mosquitoes may take multiple blood meals and how this would impact survival of the bottlenecks nor account for the effect of the microbiota in mosquito infection. Whilst there are limitations, the model can be adapted to include these and still requires less time and equipment/containment than the same *in vivo* mosquito work, allowing us to mimic viral evolution in a mosquito.

7.2.6 How has this changed our perception of bottlenecks?

The importance of viral diversity in traversing a bottleneck has been clear. Our work shows that within a mosquito, increasing the mutation rate of a viral population impacts the ability of it to traverse a bottleneck. Similar attenuation is seen over bottlenecks with high fidelity viruses (G64S polio) (Pfeiffer and Kirkegaard, 2005). This suggests that the viral population has evolved to produce a certain level of diversity directly adapted to the bottlenecks it faces. This improves our understanding of factors that control viral diversity by suggesting that the co-evolution of the virus and mosquito has fine-tuned this as a method of overcoming the specific bottlenecks. In mosquitoes that are refractory to viral infection, could it be that due to lack of co-evolution, the virus has not evolved the correct mutation rate to survive the bottlenecks in that specific vector? Would altering the mutation rate of a virus and then infecting those mosquitoes make them susceptible to viral infection? This can alter the way we look at the situation. A virus evolves due to environmental pressures, and this puts more importance on the mosquito itself as a strong factor in developing viral diversity. Kain (2022) identified across multiple studies that vector competence was influenced by infection barriers within the mosquito. It is important that we understand the role of the mosquito and bottlenecks in generating viral diversity, rather than assuming they are independent factors. This will give us a better understanding of vector competence and which viruses may be better adapted to which mosquitoes. We still need to determine a way to figure out how to correlate mutation rate with bottleneck survival, but once we do, we can screen viruses against mosquito bottlenecks during future vector competence studies.

8 Conclusion

In conclusion, our work has provided a multifaceted view of the complex interactions between viruses, mosquitoes and their environment in the context of vector competence. We have identified several key findings including the inability of low-fidelity VEEV variants to traverse bottlenecks in their mosquito host. Through this we developed a cell culture model adapted to VEEV that allows us to accurately model VEEV mosquito infection and highlights the need to adapt cell culture models to individual virus-mosquito pairs for viral evolution and bottleneck research. A key finding is that the internal and external factors tested did not impact the RNAi response, demonstrating its remarkable resilience as a key antiviral response. This suggests a pivotal role in the co-evolution of mosquitoes and viruses, enabling the persistence of viral infection that is key for arboviral transmission. Furthermore, this highlights the importance of harnessing the RNAi response as a control measure for arboviral infection and transmission.

Our research into agricultural chemicals, specifically GLY and TRI on *A. aegypti* has shown that these may alter the vector competence for both CHIKV and ZIKV in an experimental setting. Further work is required to determine the effects these chemicals have on the mosquito microbiota and the reason for the reduction in competence. This sheds a light on the need to test commonly used agricultural chemicals against mosquito/virus pairings in within regions.

Finally, the ISV NEGV increases replication of CHIKV and ZIKV in the bodies of their mosquito vector however reduces the dissemination rates, lowering the overall vector competence. This sheds light on the virome of mosquitoes and provides avenues for research both into understanding the role of the microbiome and virome of the mosquito and its potential for manipulating vector competence.

As we navigate the complexities of vectorial capacity, it becomes increasingly clear that a multitude of factors influence the transmission of mosquito-borne diseases, making it a challenge to address these accurately within a single study. To tackle this, we propose the establishment of a global consortium tasked with cataloguing mosquito distribution and factors that influence vector competence and capacity within each individual region. This will allow for an accurate assessment of the risk posed by vectors

within each country and influence targeted control strategies. There are currently several global research collaborations looking at different aspects of the arbovirus threat, such as the WHO Global Arbovirus Initiative and the Arbovirus Research Consortium based in Colorado. To fully understand the threat of arbovirus spread, the data from these needs to be consolidated into one platform.

Ultimately, this work has contributed significantly to our understanding of bottlenecks as an evolutionary pressure on viruses. We have proposed the illusion that viruses adapt to the specific bottlenecks imposed by their vector hosts and that survival of bottlenecks is a result of continued co-evolution of mosquito and virus. These insights deepen our understanding of viral evolution and offer potential targets for future interventions aimed at disrupting the transmission of mosquito-borne diseases. At a time when the threat of viral outbreaks is seemingly on the rise, our research is a vital steppingstone toward more effective disease control strategies.

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