Vector Competence and Viral Interactions of Zika Virus

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Abstract

Zika virus (ZIKV) (Flaviviridae: *Flavivirus*) is a mosquito-borne pathogen that has been linked to life-threatening health complications following its emergence in the Americas. As ZIKV continues its northern expansion, it becomes increasingly important to identify the risk for ZIKV transmission in North America through the determination of competent vector species as well as the potential for flavivirus co-infections.

The susceptibility to infection and the potential for ZIKV transmission was investigated in mosquito species local to Southern Ontario, Canada. Wild mosquitoes were exposed to a ZIKV-infected blood meal or sugar meal at a final titer of 10^5 plaque-forming units. Colony *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) mosquitoes were also fed a ZIKV-infected bloodmeal alongside the wild mosquitoes. ZIKV transmission was not detected among the blood-fed wild mosquitoes, however, low infection rates of 9.7% and 33.3% were observed in *Aedes vexans* (Meigan) and *Coquillettidia perturbans* (Walker), respectively. In the sugar-fed wild mosquitoes, a low infection rate (6.1%) and transmission efficiency (1.2%) was observed for *Culex pipiens* Linnaeus only. Among the colony mosquitoes, *Ae. albopictus* displayed a higher transmission efficiency. The results indicate that these mosquitoes are not likely to be competent ZIKV vectors.

The infection dynamics of ZIKV were further analyzed following simultaneous and sequential exposure to West Nile virus (WNV) (Flaviviridae: *Flavivirus*) in mammalian and insect cell lines. Cells were co-infected or superinfected with the viruses at a final multiplicity of infection of 0.01. Viral RNA was subsequently extracted and amplified from the supernatant samples. Viral interference was observed in the mammalian cell line but not in the insect cell line. Additionally, the infection order of the viruses had a significant impact on the estimated viral titer. These results may be applicable in areas where the viruses co-circulate and risk of co-infection exists.

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List of Abbreviations

ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
Ae.	*Aedes*
An.	*Anopheles*
C	Capsid
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CHIKV	Chikungunya Virus
CI	Confidence Intervals
CL3	Containment Level 3
CMC	Carboxymethyl Cellulose
CNS	Central Nervous System
Cq.	*Coquillettidia*
CT	Cycle Threshold
Cx.	*Culex*
CxFV	*Culex* Flavivirus
DENV	Dengue Virus
DIR	Disseminated Infection Rate
DMEM	Dulbecco's Modified Eagle's Medium
DPBS	Dulbecco's Phosphate-Buffered Saline
E	Envelope

ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
Gen	Generic
GBS	Guillain-Barré Syndrome
HI	Hemagglutination Inhibition
HIV	Human Immunodeficiency Virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IR	Infection Rate
ISM	Infected Sugar Meal
JEV	Japanese Encephalitis Virus
KUNV	Kunjin Virus
LSQ	Log Starting Quantity
M	Membrane
MEME	Minimum Essential Medium Eagle
MOI	Multiplicity of Infection
NCR	Non-Coding Region
NY99	New York 1999
ORF	Open Reading Frame
P/P	Primer/Probe
PAHO	Pan American Health Organization

PFU	Plaque-Forming Units
PHAC	Public Health Agency of Canada
PHEIC	Public Health Emergency of International Concern
prM	Pre-Membrane
PSG	Penicillin/Streptomycin/L-Glutamine
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RH	Relative Humidity
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SLEV	St. Louis Encephalitis Virus
TE	Transmission Efficiency
TR	Transmission Rate
USA	United States of America
WHO	World Health Organization
WNF	West Nile Fever
WNV	West Nile Virus
WNV-ZIKV CI	West Nile Virus and Zika Virus Co-infection
WNV/ZIKV SI	West Nile Virus First, Zika Virus Second Superinfection
YFV	Yellow Fever Virus
ZIKV	Zika Virus
ZIKV/WNV SI	Zika Virus First, West Nile Virus Second Superinfection

Chapter 1

Literature Review

1.1 Preface

This literature review will provide relevant information on two mosquito-borne flaviviruses: West Nile virus (WNV) and Zika virus (ZIKV); information that is essential for understanding the methodologies presented in this thesis. The review will begin with a general introduction to flavivirus biology and virology before moving on to specific information regarding the epidemiology, phylogeny, transmission, and health complications associated with WNV and ZIKV. Subsequently, the review will provide information on the mosquito vector including general biology and the complexities surrounding the phenomenon of vector competence. The review will conclude with a presentation of various viral detection and quantification strategies that are available to aid in laboratory investigations focused on flaviviruses.

1.2 Flaviviruses

Flaviviruses are viruses that belong to the genus *Flavivirus* within the family Flaviviridae. The genus *Flavivirus* contains a number of clinically relevant mosquito-borne viruses, including dengue virus (DENV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), WNV, yellow fever virus (YFV), and ZIKV. The symptoms associated with certain flavivirus infections may present as mild, flu-like symptoms while other viruses belonging to the genus have been shown to cause severe, life-threatening disorders (Mukhopadhyay et al., 2005). The mosquito-borne flaviviruses may be further divided into two major groups based on their clinical presentation in humans: the primary vertebrate reservoirs, and the associated mosquito vectors. The first group includes the encephalitic viruses, which cause neurological diseases in the host. These viruses utilize birds as their primary reservoirs and mosquitoes belonging to the genus *Culex* (*Melanoconion*) as their primary vectors. The second group includes the non-encephalitic viruses,

which cause hemorrhagic fever in the host. These viruses utilize primates as their primary reservoirs and mosquitoes belonging to the genus *Aedes* (*Stegomyia*) as their primary vectors (Guant et al., 2001). Given the potential for these viruses to case fatal human infection and disease epidemics in many parts of the world, flaviviruses are studied extensively.

1.2.1 Virology

Flaviviruses are small, spherical, enveloped viruses containing a positive-sense, single-strand of genomic ribonucleic acid (RNA). The genomic material of each virion is encased by capsid (C) proteins, forming the nucleocapsid at the core of the virion. The nucleocapsid is surrounded by a host cell-derived lipid bilayer containing 180 copies of the envelope (E) protein as well as 180 copies of either the pre-membrane (prM) protein or its mature cleavage product, the viral membrane (M) protein. Intracellular flavivirus virions are distinct from extracellular virions; the intracellular virions contain prM proteins in their lipid bilayers while virions that exit the cell predominantly contain M proteins (Chambers et al., 1990). Thus, immature virus particles have a diameter of 60 nm while mature virus particles have a diameter of 50 nm following furin-induced prM cleavage. The flavivirus virion surface proteins are icosahedral in symmetry (Perera et al., 2008).

1.2.2 Genome

The linear, single-stranded RNA genome is approximately 11 kilobases in length. The genomic RNA contains a 5’ methylated cap and lacks a 3’ terminal polyadenylated tail. The genomic RNA comprises a single open reading frame (ORF) that is flanked by 5’ and 3’ non-coding regions (NCR). The ORF is translated into one long continuous polyprotein chain, which

is subsequently cleaved by viral and cellular proteases into 10 individual viral proteins. Of these viral proteins, there are 3 structural proteins and 7 non-structural proteins. The structural proteins are located directly following the 5' NCR and include the C protein, the prM protein, and the E protein. The non-structural proteins are located following the structural proteins and include the large, highly conserved NS1, NS3, and NS5 proteins as well as the small, hydrophobic NS2A, NS2B, NS4A, and NS4B proteins (Chambers et al., 1990). A schematic diagram of the flavivirus genome structure is depicted in Figure 1.1A.

While the structural viral proteins are necessary for the formation of mature infectious flavivirus virions, the non-structural viral proteins have a variety of functions that are essential for the replication of the viral genomic RNA. Specifically, the NS1 protein has been found to play a role in early RNA replication (Lindenbach & Rice, 1997). Similarly, the NS3 protein plays a role in RNA replication as well as polyprotein cleavage. The NS3 protein possesses an N-terminal serine protease domain, which requires the NS2B protein as a cofactor. The NS2B-NS3 protease is responsible for the cleavages at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 junctions (Phoo et al., 2016). The NS5 protein plays a role in the capping, methylation and replication of the viral genomic RNA through an N-terminal methyltransferase domain as well as a C-terminal RNA-dependent RNA polymerase domain (Cox et al., 2015).

1.2.3 Replication Cycle

There are eight major steps in the flavivirus replication cycle: attachment, endocytosis, membrane fusion, translation, RNA replication, assembly, maturation, and release (Figure 1.1B). Initially, the virion must non-specifically attach to the host cell surface. The viral E proteins facilitate the attachment by binding to cellular receptors found on the host cell surface (Chen et

al., 1996; Chen et al., 1997). Following attachment, the virion must be internalized by clathrin-mediated endocytosis (Perera-Lecoin et al., 2014). Following endocytosis, low pH conditions within the endosome induce structural changes in the E protein, which permits fusion of the viral membrane with the membrane of the endosome. The fusion of membranes releases the viral genomic RNA from the nucleocapsid and into the cytoplasm of the host cell (Harrison, 2008). The uncoated viral genomic RNA is then transported to the rough endoplasmic reticulum (ER) where it is translated into a single polyprotein (Gillespie et al., 2010). Following translation, the polyprotein is cleaved by both host and viral proteases into the functional structural and non-structural proteins. The replication of viral genomic RNA takes place within viral replication complexes that are associated with ER-derived membranes and consist of viral proteins, viral RNA, and possible host cell factors (Fernandez-Garcia et al., 2009; Yun & Lee, 2014). Viral assembly of immature virions follows viral RNA replication. Viral genomic RNA along with C proteins bud into the ER lumen where E and prM proteins are subsequently incorporated into the budding particles, forming the immature virion. During the cellular secretory pathway, the immature virions are transported through the low pH conditions of the trans-Golgi network, which is responsible for inducing structural changes within the virions (Zhang et al., 2007). Most importantly, the cleavage site of the prM protein is made accessible to furin. This host-derived protease cleaves the immature prM protein to the mature M protein (Stadler et al., 1997). Following subsequent structural rearrangements of the M and E proteins, the mature virions are secreted from the infected host cell through exocytosis (Pierson & Diamond, 2012). Following release, the mature virions will attach to a new uninfected host cell in order to continue the viral replication cycle (Yun & Lee, 2014).

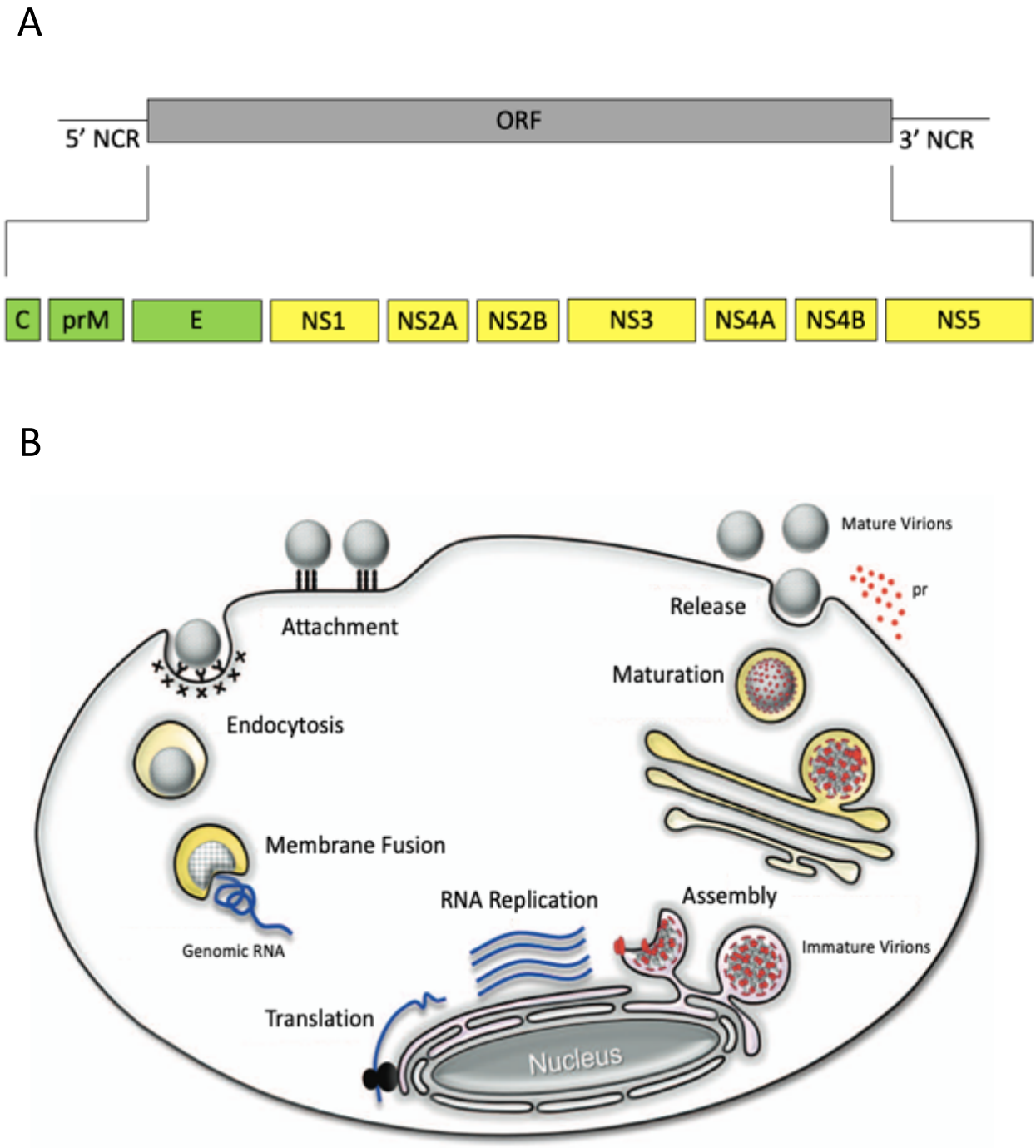

Figure 1.1: Flavivirus genome and replication cycle. (A) A schematic diagram of the flavivirus genome. The single open reading frame (ORF) is flanked by 5' and 3' non-coding regions (NCR). The ORF is translated to a polyprotein, which is subsequently cleaved into three structural (green) and seven non-structural (yellow) proteins. (B) A schematic diagram of the flavivirus replication cycle. The major steps of the replication cycle are: attachment, endocytosis, membrane fusion, translation, RNA replication, assembly, maturation, and release. Modified from Yun & Lee (2014).

1.3 West Nile Virus

WNV (Flaviviridae: *Flavivirus*) is a mosquito-borne positive-strand RNA virus that is recognized as one of the most widespread flaviviruses following its rapid geographic expansion from Africa and Eurasia to North America, where the virus is now well-established (Zdenek & Halouzka, 1999). The virus is primarily transmitted through the bite of an infected mosquito and utilizes avian hosts to maintain the transmission cycle, though humans and other vertebrates may also become infected. Although the majority of WNV infections are asymptomatic, approximately 20% of infected individuals may develop West Nile fever (WNF). Furthermore, approximately 1 in 150 WNV-infected individuals may develop serious, life-threatening illness targeting the central nervous system (CNS). Currently, there is no specific treatment or vaccination for WNF (Centers for Disease Control and Prevention [CDC], 2018A). The implementation of preventative measures and ongoing surveillance is imperative to reduce the public health concern of WNV in endemic areas.

1.3.1 Epidemiology

In 1937, WNV was isolated for the first time from a febrile woman in the West Nile district in the Northern Province of Uganda (Smithburn et al., 1940). In 1951, the virus was isolated for the second time in Israel following the first recognized WNV outbreak (Bernkopf et al., 1953). From 1951-1954, several WNV outbreaks were reported in Egypt and the virus was demonstrated to be endemic along the Nile Delta (Melnick et al., 1951; Taylor et al., 1956). In 1957, following another outbreak in Israel, neurologic illness was associated with WNV infection for the first time (Spigland et al., 1958). Neurologic events were also documented during subsequent outbreaks in France in 1962 (Panthier, 1968) and South Africa in 1974 (McIntosh et al., 1976). Large WNV

outbreaks were infrequent following 1974 as only smaller sporadic outbreaks characterized by mild febrile illnesses were reported. In 1996, however, a major WNV epidemic took place near Bucharest, Romania. The outbreak was notable as 90% of patients with serologically confirmed WNV infection had acute CNS infections (Tsai et al., 1998). Following the outbreak in Romania, an increase in the number of WNV outbreaks associated with high rates of severe CNS infection and fatality were observed throughout Africa, Asia, Europe, and the Middle East (Zdenek & Halouzka, 1999).

In the summer of 1999, WNV was detected in North America for the first time following reports of an unusual cluster of meningoencephalitis cases in Queens in New York City (Nash et al., 2001). Earlier in the year, high mortality rates among crows, as well as exotic bird species from the Bronx Zoo, had been reported in the New York City vicinity. Following genomic analyses, it was determined that WNV was the agent responsible for the avian deaths and the human outbreak (Lanciotti et al., 1999). An investigation of the bird species in New York City following the human epidemic demonstrated that 33% were positive for WNV neutralizing antibodies with half of the seropositive birds coming from Queens (Komar et al., 2001). Although the mechanism behind the introduction of WNV to North America remains unknown, the virus strain responsible for the New York epidemic is believed to have originated in the Middle East as it is closely related to a WNV strain isolated from the brain of a dead goose in Israel in 1998 (Lanciotti et al., 1999). Following the detection of WNV in New York City, the virus spread rapidly throughout the United States. In the summer of 2000, 21 cases of human WNV infection were reported in 10 counties spanning New York, New Jersey, and Connecticut (Marfin et al., 2001). In 2001, the number of human cases had increased to 66 in reports from 39 counties spanning 10 different states (CDC, 2002A). In the summer of 2002, the largest outbreak at the time was observed with 3,389 cases of human infection

reported from 619 counties and 37 states. Alarmingly, 69% of patients exhibited meningoencephalitis from this unprecedented outbreak (CDC, 2002B). The following year, 7,836 cases of human infection were reported from 45 states (CDC, 2003). By 2004, the virus had been detected in all contiguous states and was considered to be endemic for the foreseeable future (CDC, 2005). Between 1999-2017, a total of 48,183 disease cases were reported to the CDC, with 22,999 of these disease cases determined to be neuroinvasive. As of 2017, a total of 2,163 WNV-related deaths have been reported in the United States (CDC, 2018B).

WNV is also endemic in other parts of North America, specifically the provinces of Canada. Although the virus was first detected in 2001 in Ontario from dead birds and mosquito pools, the first cases of human infection were documented in 2002, with a total of 414 reported cases from Ontario and Quebec (Government of Canada, 2018A). In subsequent years, the virus spread west throughout the provinces until 2009 when it was detected in British Columbia for the first time (Roth et al., 2010). Between 2002-2016, a total of 5,593 disease cases were reported to the Government of Canada, with the largest outbreaks occurring in 2003 (1,481 cases) and 2007 (2,215 cases) (Government of Canada, 2018A).

1.3.2 Phylogeny

WNV is a member of the Japanese encephalitis serocomplex. Five distinct genetic lineages have been proposed for WNV. Lineage 1 is widely distributed around the world and has been associated with human encephalitis. This lineage may be further subdivided into two clades: 1a and 1b. Clade 1a includes isolates from Africa, Asia, Europe, and North America while clade 1b includes isolates of Kunjin virus (KUNV), which is a WNV subtype from Australia. WNV isolates from lineage 2 are responsible for sporadic zoonotic outbreaks originally in sub-Saharan Africa

and later in Europe (Petersen & Roehrig, 2001). Previously, lineage 2 isolates were not believed to cause human encephalitis however, WNV outbreaks in Greece (Danis et al., 2011) and Romania (Sirbu et al., 2011) have been associated with neuroinvasive disease. McMullen et al. (2013) found that lineage 2 has molecularly evolved in a way that mirrors lineage 1, which may explain the unexpected virulent phenotype. Lineage 3 includes the isolates collected in 1997 and 1999 from *Culex pipiens* Linnaeus mosquitoes in the Czech Republic (Bakonyi et al., 2005). Lineage 4 includes the isolates that have been circulating in Russia since 1988 (Platonov et al., 2011; Chancey et al., 2015). Lineage 5 was formerly known as clade 1c under lineage 1, however, it is now recognized as a separate lineage and includes the WNV isolates from India (May et al., 2011; Chancey et al., 2015). A phylogenetic tree for WNV is presented in Figure 1.2.

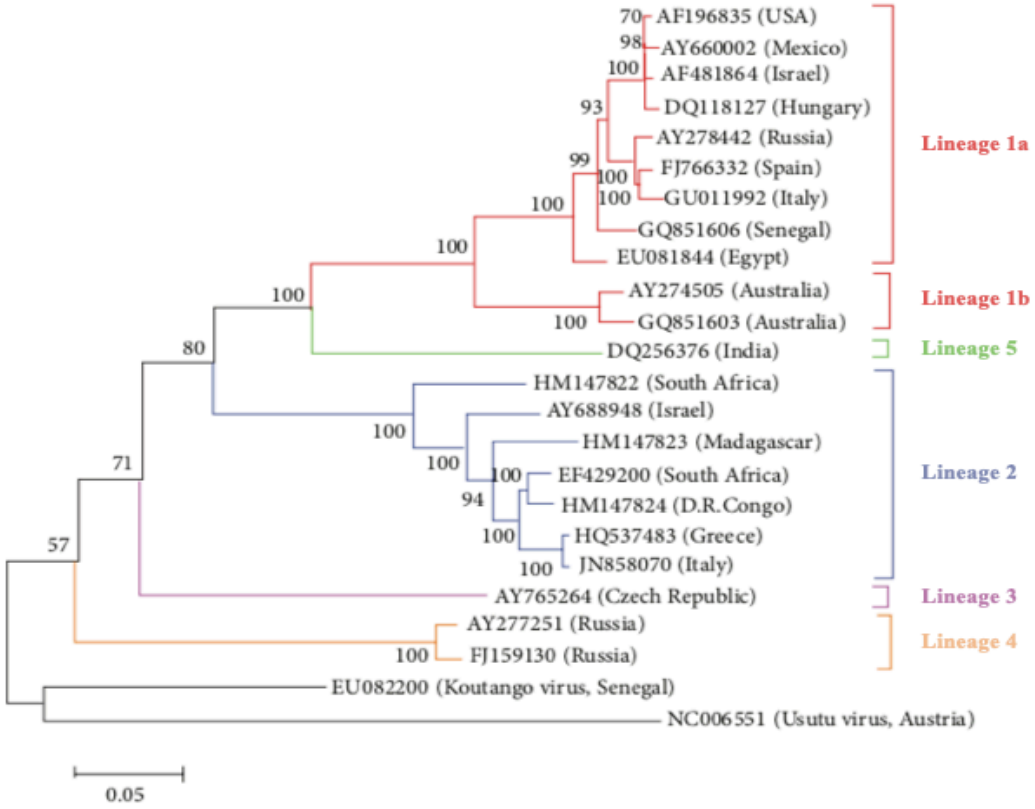

Figure 1.2: West Nile virus phylogenetic tree. The phylogenetic tree constructed from complete genome sequences depicting the five major West Nile virus lineages. Modified from Chancey et al. (2015).

1.3.3 Transmission

WNV is an arthropod-borne virus that is transmitted through the bite of an infected mosquito. In nature, the virus is maintained in a sylvatic transmission cycle between mosquito vectors and bird hosts. Birds are considered the primary hosts for WNV transmission because they are able to develop sufficiently high viremia to infect mosquitoes. Bird species belonging to the order Passeriformes, such as the house finch (*Carpodacus mexicanus*), American crow (*Corvus brachyrhynchos*), blue jay (*Cyanocitta cristata*), house sparrow (*Passer domesticus*), and common grackle (*Quiscalus quiscula*), are most susceptible to infection and exhibit high levels of cloacal and oral shedding (Komar et al., 2003). Despite the fact that larger mammals such as humans and horses are also susceptible to WNV infection, these hosts are considered to be incidental as they do not contribute to the virus transmission cycle. This is because larger mammals are not able to develop sufficient levels of viremia that are necessary for infecting mosquitoes, which labels them as 'dead-end hosts' in WNV transmission (Bunning et al., 2002). Mosquito species that feed from both avian and mammalian hosts are considered to be bridge vectors because they form connections between the primary transmission cycle and the incidental hosts for WNV (Chancey et al., 2015).

Mosquitoes belonging to the genus *Culex* are considered to be the primary vectors for WNV. In North America, *Cx. pipiens* and *Culex restuans* Theobald are the dominant vectors for the virus as these mosquito species are relatively abundant and frequently use birds as hosts for blood meal acquisition (Ebel et al., 2005). Other implicated vectors for the virus include *Culex erraticus* (Dyar & Knab), *Culex nigripalpus* Theobald, *Culex quinquefasciatus* Say, and *Culex tarsalis* Coquillet (Turell et al., 2005). Mosquito species belonging to the genus *Aedes*, including *Aedes albopictus* (Skuse) and *Aedes vexans* (Meigan), have also been shown to act as competent

vectors for the virus (Turell et al., 2005; Tiawsirisup et al., 2008).

Non-vector-borne transmission has also been observed for WNV through blood transfusion (Pealer et al., 2003), organ transplant (Iwamoto et al., 2003), transplacental transmission (CDC, 2002C), and laboratory exposure (CDC, 2002D). Transmission of WNV through breastfeeding has also been proposed following the detection of the virus in breast milk. Although the infant showed no signs of WNF following breastfeeding, a serum sample taken three weeks following birth was found to be positive for WNV-specific immunoglobulin M (IgM) (CDC, 2002E). Despite the risk, transmission of WNV via breastfeeding is considered rare and it is not recommended for women infected with the virus to stop breastfeeding (CDC, 2018A).

1.3.4 West Nile Fever

Following transmission of the virus through a mosquito bite, the first cells believed to be infected are the keratinocytes and Langerhan cells. Initial replication occurs when these cells migrate to the regional lymph nodes. The virus then spreads to visceral organs, where replication is believed to take place in macrophages and epithelial cells (Lim et al., 2011). Approximately 80% of all WNV infections are asymptomatic. When symptoms do arise however, they may vary from mild flu-like symptoms to severe neuroinvasive disease. About 1 in 5 WNV infections results in WNF, which presents as a febrile illness. Symptoms associated with WNF include headache, body ache, joint pains, diarrhea, vomiting, and rash. The incubation period is estimated to range from 2-14 days. While most patients diagnosed with WNF are able to make a full recovery, fatigue and weakness may persist for months (CDC, 2018A). Despite the fact that the majority of WNV infections are non-neuroinvasive, a proportion of disease cases are associated with serious, life-threatening complications.

In approximately 1 in 150 WNV infections, the CNS is affected resulting in severe and possibly fatal consequences. The neuroinvasive complications typically associated with severe WNV illness include encephalitis and meningitis. In neuroinvasive disease cases, WNV virions cross the blood-brain barrier and accumulate in the neurons of CNS tissues such as the basal ganglia, brainstem, cerebellum, hippocampus, neocortex, spinal cord, and thalamus (Armah et al., 2007). The symptoms associated with these cases may include high fever, headache, disorientation, stupor, numbness, tremors, convulsions, paralysis, vision loss, and coma. Immunocompromised patients and individuals with medical conditions such as cancer, diabetes, hypertension, and kidney disease are at a higher risk of developing severe disease following WNV infection. Recovery may require several weeks or months though certain deleterious effects to the CNS have the potential to be permanent. About 1 in 10 patients with severe WNV illness may die (CDC, 2018A).

1.4 Zika Virus

ZIKV (Flaviviridae: *Flavivirus*) is a positive-strand RNA virus that is responsible for large-scale outbreaks in the Pacific Islands as well as the Americas. ZIKV is primarily transmitted through the bite of an infected mosquito, however, other modes of transmission, such as perinatal and sexual contact, make the development of control strategies more complicated. Although ZIKV disease cases are often mild in nature, there is evidence that infection with the virus may lead to more severe neurological conditions, such as Guillain-Barré syndrome (GBS) and microcephaly. Currently, there is no specific treatment or vaccination for ZIKV disease (CDC, 2019A). As the number of imported ZIKV cases increases in North America and the geographic expansion of the primary mosquito vectors moves further northwards, ZIKV has the potential to pose as a serious

public health concern in non-endemic areas of the world.

1.4.1 Epidemiology

In 1947, during the process of surveilling for YFV, ZIKV was isolated for the first time from the blood of a sentinel rhesus monkey in the Zika forest of Uganda. The virus was isolated for the second time in 1948 from pools of *Aedes africanus* (Theobald) mosquitoes, also captured within the Zika forest (Dick et al., 1952). The first human disease cases were recorded in Uganda and the United Republic of Tanzania in 1952, following the detection of neutralizing antibodies to ZIKV in sera (Smithburn, 1952). In 1954, ZIKV was isolated from a human for the first time in Nigeria (MacNamara, 1954). Following the first human isolation, sporadic ZIKV disease cases were reported in Africa, specifically Egypt (Smithburn et al., 1954), Kenya (Geser et al., 1970), Sierra Leone (Robin & Mouchet, 1975), Nigeria (Fagbami, 1979), Central African Republic (Saluzzo et al., 1981), Gabon (Saluzzo et al., 1982), and Senegal (Monlun et al., 1993). Sporadic human infection was also reported in Asia, specifically India (Smithburn et al., 1954), Malaysia (Smithburn, 1954; Pond, 1963), the Philippines (Hammon et al., 1958), Thailand (Pond, 1963), Vietnam (Pond, 1963), Indonesia (Olson et al., 1983), and Pakistan (Darwish et al., 1983).

In 2007, the first ZIKV outbreak was reported on Yap Island in the Federated States of Micronesia. It was estimated that 73% of Yap Island residents aged 3 years and older had been recently infected with ZIKV and *Aedes hensilli* Farner was proposed to be the main vector species during the outbreak (Duffy et al., 2009). Following the first ZIKV outbreak, sporadic ZIKV cases were continually reported in southeast Asian countries, including the first report from Cambodia (Heang et al., 2012). In 2013, the second ZIKV outbreak occurred in French Polynesia, an island in the South Pacific Ocean. This was the largest documented ZIKV outbreak at the time and the

first outbreak in French Polynesia that was not caused by DENV (Cao-Lormeau et al., 2014). The French Polynesia outbreak also provided the first reported case of GBS occurring immediately after ZIKV infection (Oehler et al., 2014). Following the second ZIKV outbreak, disease cases were reported on other islands in the South Pacific Ocean, specifically the Cook Islands (Roth et al., 2014), New Caledonia (Dupont-Rouzeyrol et al., 2015), and Easter Island (Tognarelli et al., 2016). Additionally, the virus was imported to countries outside of Africa, Asia, and the South Pacific Ocean, including Norway (Wæhre et al., 2014), Australia (Pyke et al., 2014), Japan (Kutsuna et al., 2014), and Italy (Zammarchi et al., 2015).

In early 2015 ZIKV infection was reported for the first time in Brazil (Campos et al., 2015). By the end of 2015 the Brazilian Ministry of Health estimated that the number of suspected ZIKV cases in Brazil ranged from 440,000-1,300,000 (European Centre for Disease Prevention and Control [ECDC], 2015). Alongside the increasing number of ZIKV disease cases reported in Brazil, an increase was also observed in the number of infants born with microcephaly in ZIKV-affected areas (Schuler-Faccini et al., 2016). Following the emergence of ZIKV in Brazil, the virus spread quickly throughout South America and the possible link between ZIKV and microcephaly was further strengthened. Thus, on February 1, 2016, the World Health Organization (WHO) declared that the observed increase in neurological disorders connected to reports of ZIKV infection constituted a Public Health Emergency of International Concern (PHEIC) (WHO, 2016A).

According to the most recent epidemiological update, confirmed vector-borne transmission of ZIKV has been reported in 48 countries and territories in the Americas. Sexually-transmitted ZIKV disease cases have also been confirmed in Argentina, Canada, Chile, Peru, and the United States of America (USA) (Pan American Health Organization [PAHO]/WHO, 2017). In addition,

there have been numerous reports of travel-related cases of ZIKV infection in non-endemic countries such as Canada (Fonseca et al., 2014), China (Yin et al., 2016), and various Western European countries (De Smet et al., 2016; Gyurech et al., 2016; Maria et al., 2016; Díaz-Menéndez et al., 2018). In fact, as of August 31, 2018, there have been 569 travel-related and 4 sexually-transmitted cases of ZIKV detected in Canada (Government of Canada, 2018B). However, following the declaration that ZIKV was no longer considered to be a PHEIC (WHO, 2016B), the number of cases reported in the Americas has decreased (PAHO/WHO, 2017). In 2018, 64 ZIKV disease cases were reported in the USA, all due to travellers returning from affected areas (CDC, 2019B) and as of May 1, 2019, one travel-related ZIKV case has been reported in California (CDC, 2019C).

1.4.2 Phylogeny

Since the initial discovery of ZIKV in 1947, a significant number of virus isolates have been obtained from Africa, Asia, the Americas, and the Pacific Islands. However, of these numerous ZIKV isolates only 29 have been fully or nearly fully sequenced. Phylogenetic analyses have revealed that the 29 available ZIKV genomes may be grouped into two distinct genetic lineages: African and Asian (Yun et al., 2016). The African lineage may be further divided into an East African lineage and a West African lineage (Lanciotti et al., 2008). The ZIKV strains that were isolated from the recent American epidemic were derived from a common ancestor in the Asian lineage (Yun et al., 2016; Song et al., 2017).

The complete genomes of three historically relevant and spatiotemporally distinct ZIKV strains have been sequenced and are often used in ZIKV research (Yun et al., 2016). The first is the MR-766 strain, which is the African prototype strain of ZIKV isolated in 1947 from the sentinel

rhesus monkey in Uganda (Dick et al., 1952). The second is the P6-740 strain, which is the first non-African strain of ZIKV isolated in 1966 from a pool of *Aedes aegypti* (Linnaeus) mosquitoes in Malaysia (Marchette et al., 1969). The third is the PRVABC59 strain, which is the strain associated with the American epidemic isolated in 2015 from a human patient in Puerto Rico (Lanciotti et al., 2016). Further research is required to determine the impact of viral genetic variation on the pathogenicity of the ZIKV isolates belonging to the two distinct lineages. A phylogenetic tree for ZIKV is presented in Figure 1.3.

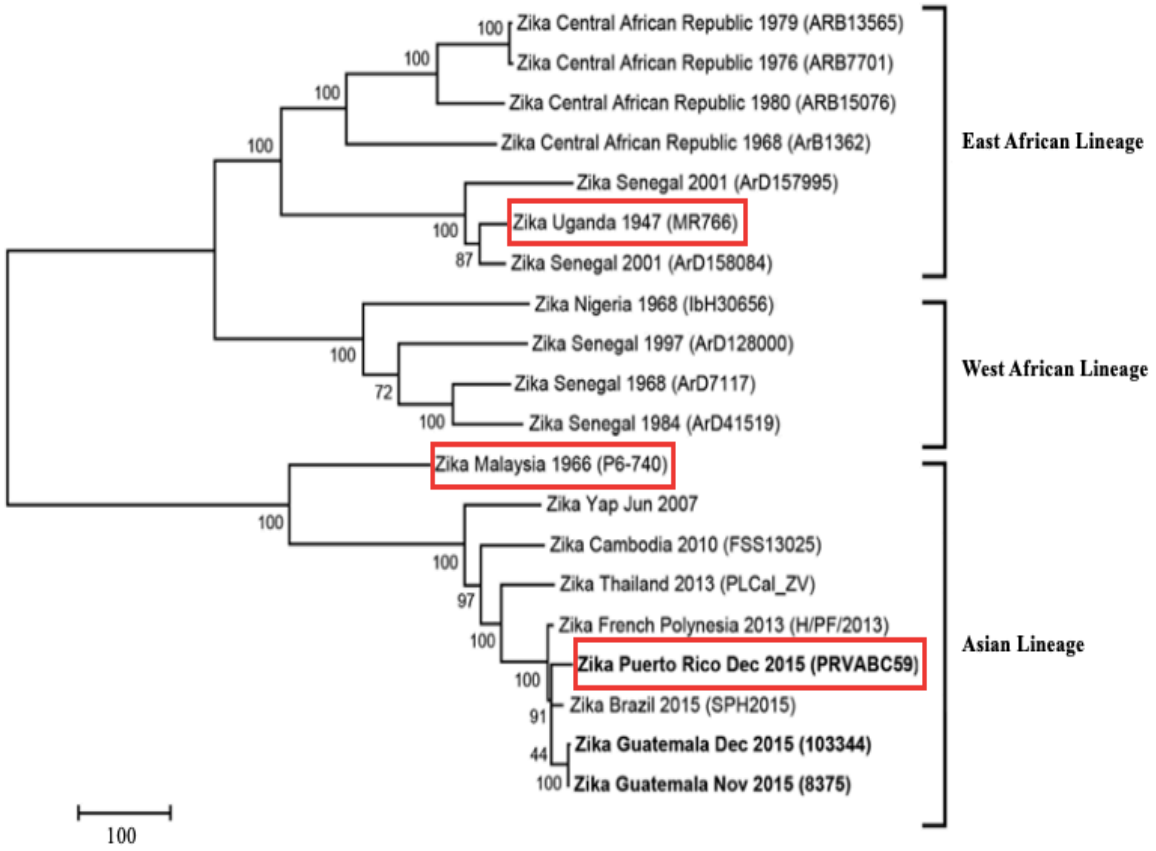

Figure 1.3: Zika virus phylogenetic tree. The phylogenetic tree constructed from complete genome sequences depicting the two major Zika virus lineages. The locations of the MR-766, P6-740, and PRVABC59 strains are indicated. Modified from Lanciotti et al. (2016).

1.4.3 Transmission

ZIKV is an arthropod-borne virus that is primarily transmitted through the bite of an infected mosquito. There are two distinct vector-borne transmission cycles associated with ZIKV, which include the sylvatic cycle and the urban cycle. The sylvatic cycle describes the circulation of the virus between arboreal mosquito vectors and non-human primate hosts while the urban cycle describes the transmission of the virus between peridomestic mosquito vectors and human hosts (Weaver et al., 2016). It is possible for the cycles to merge in cases where an arboreal mosquito acquires the virus from a non-human primate host in the sylvatic cycle and subsequently infects a human host in close proximity.

Mosquitoes belonging to the genus *Aedes* are predicted to be the primary vectors for ZIKV. In the urban cycle specifically, the primary vectors for ZIKV are hypothesized to be *Ae. aegypti* and *Ae. albopictus*. This is because the virus has been detected in wild-caught mosquito pools for each species (Diallo et al., 2014; Díaz-Quiñonez et al., 2016; Ferreira-de-Brito et al., 2016; Grard et al., 2014) and high infection and transmission rates have been reported following laboratory infections (Ciota et al., 2017; Di Luca et al., 2016; Li et al., 2012; Wong et al., 2013). Further experimentation is necessary in order to better define the vectoring capacities of these mosquito species for the virus as well as to identify additional mosquito species that may also be competent vectors.

Non-vector-borne transmission has also been observed for the virus through perinatal and sexual contact (CDC, 2019A). ZIKV may be transmitted during pregnancy from the infected mother to the fetus. The transmission has been confirmed following the detection of ZIKV RNA in the amniotic fluid and tissues of aborted fetuses diagnosed with microcephaly (Besnard et al., 2014; Jouannic et al., 2016; Mlakar et al., 2016; Sarno et al. 2016). Sexual transmission of the

virus has also been observed and confirmed following the detection of high virus titers in the semen of infected male patients (Atkinson et al., 2016; Deckard et al, 2016; Musso et al., 2015A). Transmission through methods such as blood transfusion and breastfeeding have been hypothesized but not yet observed (CDC, 2019A). However, the risk for transmission exists as ZIKV has been detected in breast milk, saliva, and urine (Barzon et al., 2016; Besnard et al., 2014; Musso et al., 2015B). Although vector-borne transmission is most common for the virus, further research is required to determine all non-vector-borne routes that exist for ZIKV transmission.

1.4.4 Zika Virus Disease

The majority of ZIKV infections are asymptomatic. In symptomatic cases, the symptoms are mild in nature and typically include fever, rash, headache, conjunctivitis, and pain in the joints and muscles. Symptoms associated with ZIKV infection typically only last for several days to a week. Due to the fact that symptoms, when present, resemble those of a flu-like illness, individuals may not realize they have been infected with the virus (CDC, 2019A). The incubation period is estimated to range from 3-14 days (Krow-Lucal et al., 2017). Despite these reports, evidence has been accumulated that suggests the virus is responsible for causing life-threatening autoimmune and developmental complications.

GBS is a rare autoimmune disorder where the peripheral nervous system is attacked by the immune system, resulting in muscle weakness and paralysis. GBS may result from a variety of causes, including viral infection (Yuki & Hartung, 2012). In fact, GBS has been associated with previous infection with chikungunya virus (CHIKV) (Wielanek et al., 2007) and DENV infection (Ralapanawa et al., 2015). The association between GBS and previous ZIKV infection was first observed during the 2013 French Polynesia ZIKV outbreak, where the incidence of GBS increased

20-fold (Oehler et al., 2014). In a case-control study conducted by Cao-Lormeau et al. (2016) during the French Polynesia outbreak, researchers detected anti-ZIKV immunoglobulin G (IgG) and IgM antibodies in 98% of patients diagnosed with GBS. Additionally, all GBS patients had neutralizing antibodies to ZIKV compared to the control group where 55.7% of patients had neutralizing antibodies (Cao-Lormeau et al., 2016). The link between GBS and ZIKV infection was also observed during the recent outbreak in the Americas (Thomas et al., 2016). The mechanism relating ZIKV infection to GBS is currently unknown.

Microcephaly is a neurological condition characterized by an abnormally small head size as a result of improper brain development (Cavalheiro et al., 2016). Microcephaly is divided into two distinct forms: congenital and postnatal. Congenital (primary) microcephaly occurs when the brain is unable to grow to the normal size during pregnancy, likely as a result of reduced neuron production. Postnatal (secondary) microcephaly occurs when the brain is a normal size at birth but subsequently fails to grow properly, likely as a result of decreased dendritic activity (Woods, 2004). Autosomal recessive primary microcephaly is an inherited genetic disorder (Woods, 2004), however, there are also non-genetic causes of primary microcephaly including heavy maternal alcohol consumption (Ouellette et al., 1977) and smoking (Van dan Eeden et al., 1990) during pregnancy, as well as viral infection (Baron et al., 1969). During the 2015 ZIKV outbreak in Brazil, the number of reported microcephaly cases dramatically increased, suggesting that a link might exist between the virus and the growth abnormality (Schuler-Faccini et al., 2016). Retrospective studies conducted in French Polynesia also indicated an increase in microcephaly cases following the 2013 outbreak (Cauchemez et al., 2016; Jouannic et al., 2016). Evidence to support this link was observed following the detection of ZIKV RNA in the amniotic fluid (Jouannic et al., 2016; Sarno et al., 2016) and tissues of fetuses diagnosed with microcephaly (Besnard et al., 2014;

Mlakar et al., 2016; Sarno et al. 2016). The first case-control study examining the link between ZIKV infection and microcephaly found that 41% of newborns diagnosed with microcephaly in the study had laboratory-confirmed ZIKV infection compared to no ZIKV infection in the control group (de Araújo et al., 2016). Tang et al. (2016) observed that ZIKV efficiently infects human cortical neural progenitor cells, which triggers cell cycle arrest and cell death. As these cells are important for human brain development, this observation may provide a possible mechanism for ZIKV-induced microcephaly (Tang et al., 2016).

Although GBS and microcephaly are currently the most common complications associated with ZIKV infection, the virus has also been linked to other neurological disorders. An 81-year-old man developed meningoencephalitis following CNS infection with ZIKV (Carteaux et al, 2016). Additionally, a 15-year-old girl developed acute myelitis following an infection with the virus (Mécharles et al., 2016). More research is required to strengthen the links between known neurological complications associated with ZIKV infection, as well as to determine other possible associated complications.

1.5 Mosquitoes

Mosquitoes are insects that belong to the family Culicidae within the order Diptera. These insects are found throughout the world, excluding areas that are permanently frozen. The majority of the approximately 3,500 different species of mosquito live in tropical and subtropical environments where the warm, humid climate is ideal for rapid development (Clements, 1992). However, there are many mosquito species that are able to thrive in temperate environments, such as those encountered in North America. In fact, there are currently 68 mosquito species that are native to Ontario, Canada (Giordano, 2018). Mosquitoes are notable for their modified mouthparts,

which may be used by females for piercing skin and ingesting blood in certain species of mosquitoes. The acquired blood meal acts as a source of protein for egg development. Mosquitoes are insects that exhibit complete metamorphosis as they pass through four distinct life stages, which include the egg, larval, pupal, and adult stages (Clements, 1992).

1.5.1 Egg Stage

Adult female mosquitoes deposit their eggs on water or on sites that will soon become flooded, as contact with water stimulates egg hatching. The number of eggs laid by a female mosquito at one time may range from 50-500 eggs. Egg laying behaviours vary among different mosquito genera. This variation is clearly evident among the *Aedes* and *Culex* genera as *Aedes* mosquitoes are known to lay their eggs individually, while *Culex* mosquitoes lay their eggs in rafts. Each egg is protected by an egg shell and embryonic development begins almost instantly after the egg has been laid. Depending on the temperature, full larval development within the egg may take anywhere from a few days to over a week. In most mosquito species, the larva will hatch from the egg stage once it is fully formed (Clements, 1992). *Aedes* mosquitoes are unique because their egg shells are capable of resisting desiccation in the absence of water, which allows for the fully-formed unhatched larva to survive for months until the egg encounters a source of water (Service, 1997).

1.5.2 Larval Stage

Once the mosquito larva has hatched from the egg stage, it is fully adapted for living in water. Despite their aquatic nature, mosquito larvae require atmospheric oxygen for respiration and utilize a siphon as their only functional respiratory opening. This requirement forces the larvae

to spend most of their time at the air/water interface or to make frequent visits to the surface of the water. Mosquito larvae feed on ‘particulate matter’ such as aquatic algae, bacteria, diatoms, and even detritus. As the organs found in mosquito larvae predominantly serve larval functions, their structures are very different from adult mosquito organs. However, groups of undifferentiated cells exist within the larval organs that ultimately form the organs of the adult mosquito (Clements, 1992). The larval stage is an important developmental stage as larval competition has been shown to have an impact on adult body size, which in turn may influence the vectoring capabilities of certain mosquito species (Alto et al., 2005). The mosquito larva undergoes four moults in the larval stage. Following the fourth moult, the mosquito enters the pupal stage (Clements, 1992).

1.5.3 Pupal Stage

Similar to the egg and larval stages, the pupal stage is also aquatic. In the pupal stage, the mosquito is not able to feed as the head and thorax of the insect are fused together to form the cephalothorax. The pupa now floats at the surface of the water and utilizes mesothoracic spiracles for respiration. In the pupal stage, certain larval organs are destroyed and replaced with adult organs. The final stages of metamorphosis are typically completed within a few days. Once the adult mosquito has formed fully within the pupal cuticle, it proceeds to swallow air at the water surface, which causes pressure to build within the pupal structure. The increased pressure splits the pupal cuticle, which allows for the adult mosquito to emerge (Clements, 1992).

1.5.4 Adult Stage

In contrast to the three earlier aquatic life stages, the adult stage in the mosquito life cycle is terrestrial. The primary behavioural activities carried out by all adult mosquitoes include

emergence, feeding, mating, and oviposition in the case of females (Clements, 1992). Adult male mosquitoes typically emerge before the females and are distinguishable based on their large plumose antennae (Thielman & Hunter, 2007). In tropical regions, the life span of the adult mosquito may range from a few days to several weeks while in temperate regions the life span is typically longer (Clements, 1992).

1.5.5 Blood Feeding

Similar to other insects within the order Diptera, mosquitoes are fluid feeders. However, mosquitoes are unique among the more primitive dipterans because their mouthparts have evolved into a long feeding structure known as the proboscis. Both male and female mosquitoes rely on these modified mouthparts for probing nectaries as adults utilize the sugars contained within plant juices as a source of energy (Joseph, 1970). Female mosquitoes of certain species are able to use their proboscis to pierce the skin of vertebrate hosts and ingest blood from peripheral blood vessels. These mosquito species utilize the blood meal as a source of protein for the development of large batches of eggs. Provided that the female mosquito is undisturbed during the acquisition of the blood meal, feeding will continue until the stretch receptors within the insect's abdomen signal satiation. Females have been shown to ingest blood meals with weights that are 2.5-4 times greater than their own body weights. The blood meal is diverted to the midgut of the mosquito where digestion of blood proteins yields amino acids used for vitellogenesis (Nyar & Sauerman, 1970).

1.6 Vector Competence

During the blood-feeding process, the female mosquito simultaneously delivers saliva to the vertebrate host through the salivary canal contained within its elongated mouthparts. The saliva

contains active molecules that act against the natural host defenses triggered following blood feeding. Anti-platelet proteins, which inhibit collagen- and adenosine diphosphate (ADP)-induced platelet aggregation (Yoshida et al., 2008; Ribeiro et al., 1984), thrombin inhibitors, which inhibit thrombin-mediated blood coagulation (Francischetti et al., 1999), and sialokinins, which have vasodilator properties (Champagne & Ribeiro, 1994), have been isolated from mosquito saliva. The saliva is also the means by which an infected female mosquito may be able to transmit a specific pathogen to a susceptible host. The blood feeding requirements of female mosquitoes provide a medium for acquiring pathogenic organisms from one vertebrate host and passing them on to another. Many different mosquito species are capable of acting as competent vectors to a variety of pathogens including viruses, bacteria, fungi, and nematodes. However, many specific ecological and physiological aspects must be appropriate for a mosquito to have the capacity to acquire and transmit a particular pathogen (Clements, 1992).

With respect to arthropod-borne viruses there is a specific pathway that must be followed within the mosquito in order to establish viral infection, dissemination, and ultimately transmission of the virus to a new, susceptible host. Following blood meal acquisition, the ingested virions must infect and replicate in the midgut epithelial cells before travelling to the salivary glands where, following replication, the virus particles pass with saliva into a new host (Clements, 1992). An illustration of the pathway taken by arthropod-borne viruses within the mosquito vector is depicted in Figure 1.2. However, barriers exist within the mosquito that may block the infection and transmission process, which prevents the mosquito from acting as a competent vector.

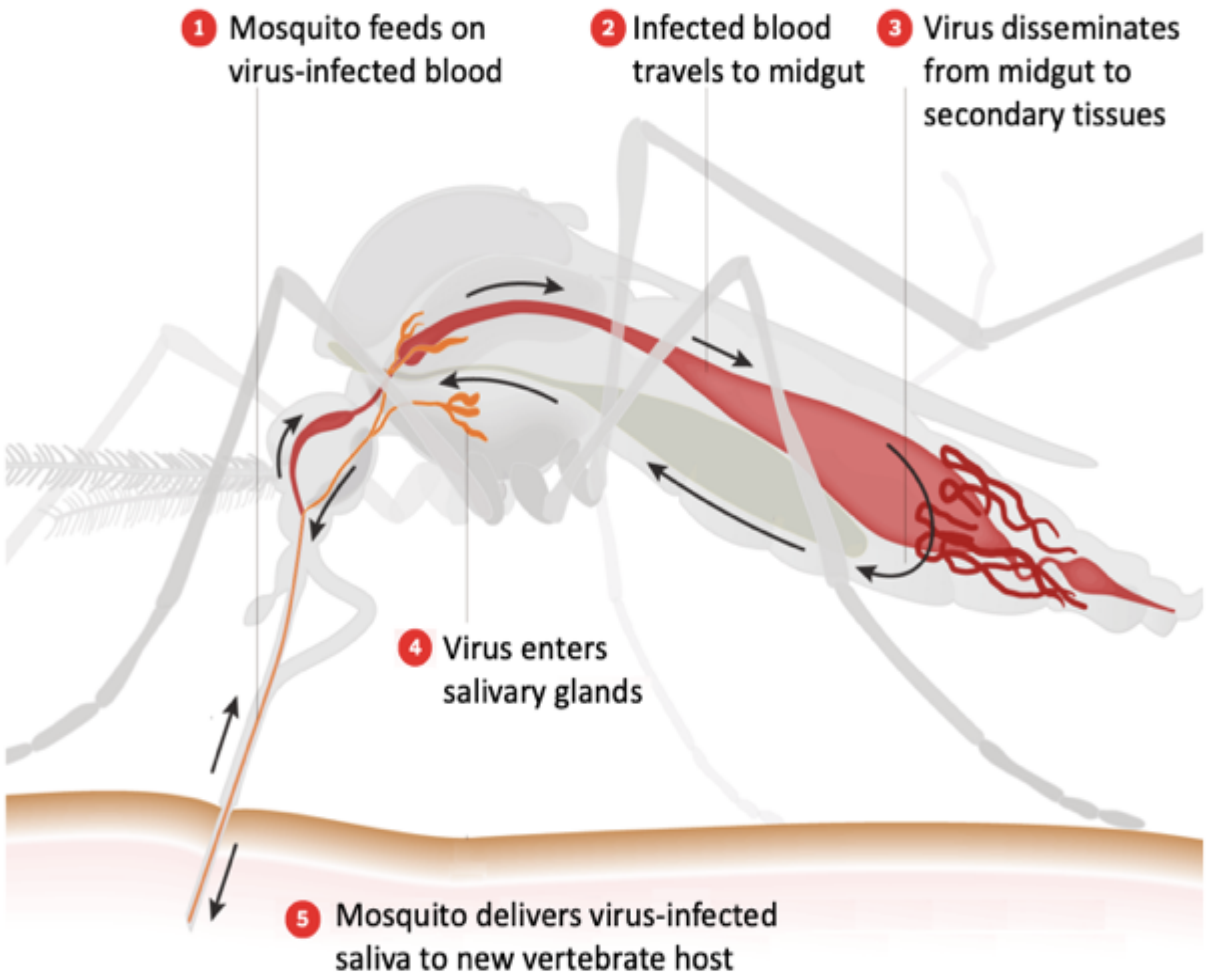

Figure 1.4: Viral dissemination pathway within the mosquito vector. An illustration depicting the progression of an arthropod-borne virus through a competent mosquito vector from blood meal acquisition to transmission through infected saliva. Modified from Kaushik et al. (2017).

1.6.1 Midgut Barriers

The first barrier to infection is encountered in the midgut of the mosquito vector. Following blood meal acquisition from an infected host, the viremic blood meal initially enters the midgut lumen. The virions must enter the midgut epithelial cells through the microvilli before the blood meal in the midgut lumen is surrounded by the peritrophic membrane, which is secreted during blood meal digestion (Okuda et al., 2002). Following the escape from the midgut lumen into the midgut epithelial cells, viral RNA replication occurs at the ER membrane followed by viral maturation. The virions must then disseminate from the midgut epithelial cells to the secondary tissues of the mosquito vector (Franz et al., 2015). It is suggested that the virions must pass through the basal lamina of the midgut epithelial cells to enter the haemocoel (Whitfield et al., 1973). Once the virus has entered the haemocoel, viral amplification within extramesenteronal cells and tissues is necessary so that the salivary glands may be efficiently infected (Hardy et al., 1983).

1.6.2 Salivary Gland Barriers

The final barrier to infection within the mosquito vector is encountered within the salivary glands. Infection of the salivary glands typically begins in the distal lateral lobes while viral replication appears to occur at the smooth membrane structures of the salivary glands. Following viral replication, the virus must move into the apical cavities of acinar cells in order to ensure transmission into a new host during a subsequent feeding (Forrester et al., 2014; Franz et al., 2015). While there are additional factors that influence vector competency, such as the genetic makeup of both the arthropod and the virus, the incubation temperature, and the concentration of virus within the acquired blood meal, an arthropod is typically regarded as a competent vector for a specific arthropod-borne virus when the virus is able to successfully pass through these barriers to

infection and infect a new vertebrate host (WHO, 1985).

1.7 Viral Detection and Quantification Strategies

There are a variety of laboratory protocols that are utilized to confirm the presence or absence of flaviviruses within samples of interest. Following viral detection, there are additional strategies available to quantify the virus through the determination of a viral titer. The following viral detection and quantification strategies are frequently utilized to study WNV and ZIKV.

1.7.1 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) relies on a highly specific antibody-antigen interaction. In an ELISA, an antigen is immobilized on a plate surface and complexed with an enzyme-linked antibody. Following incubation with the corresponding substrate, detection is accomplished from the observation of a visible colour change, indicating the presence of the antibody-antigen pair (Crowther, 2000). ELISA is utilized in the detection of antibodies with specificity for a variety of antigens, including viral antigens, and is often used for the detection of flaviviruses in samples of human sera (Broom et al., 1987).

1.7.2 Hemagglutination Inhibition Assay

The hemagglutination inhibition (HI) assay relies on the capacity of antibodies specific to the virus under investigation to inhibit the virus from agglutinating red blood cells. In the HI assay, serum dilutions are incubated with virus and red blood cells are subsequently added. Following an incubation period, the HI titer is determined by the highest serum dilution that inhibited hemagglutination (Clarke & Casals, 1958; Landolt et al., 2014).

1.7.3 Plaque Assay

The plaque assay is regarded as the gold standard for the detection and quantification of infectious virus particles through the determination of a viral titer. This assay involves the infection of confluent cell monolayers with serial dilutions of the virus under investigation. During an hour of incubation, the virions enter the cells and begin the viral replication cycle. As the infected cells lyse, a small, circular region may be observed on the cell monolayer following staining, which is referred to as a plaque. The viral titer may be subsequently estimated from the average number of plaque-forming units (PFU) per volume of viral suspension. However, this technique cannot be utilized for virus identification due to the fact that the plaques formed by different flaviviruses are not clearly distinguishable (Agbulos et al., 2016).

1.7.4 Reverse Transcriptase-Polymerase Chain Reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) is an efficient method for the detection of viral gene segments in a sample. As flaviviruses are single-stranded RNA viruses, a reverse transcriptase (RT) is used to transcribe the viral RNA to complementary DNA (cDNA). The cDNA target sequence is then amplified and monitored through the detection of fluorescence. The cycle threshold (CT) value indicates the cycle in the reaction where fluorescence increases above a fixed threshold value (Lanciotti et al., 2000). It is recommended to perform RT-PCR and plaque assay in succession to determine both viral identity and viral titer (Agbulos et al., 2016).

Objectives

Despite the fact that ZIKV has been circulating since 1947, a great deal of information remains unknown regarding the infection dynamics of the virus, particularly in North America. The overall objective presented in this dissertation is to investigate the risk of ZIKV infection and transmission within live vectors as well as host and vector-derived cell lines.

As the number of reported ZIKV disease cases increases in non-endemic countries, it becomes imperative to identify the mosquito species that may contribute to the transmission cycle by acquiring the virus and passing it to a new susceptible host. In Chapter 2, the vector competence of wild-caught mosquito species native to the Southern Ontario region was analyzed through the determination of infection, dissemination, and transmission rates following experimental infection with ZIKV. Based on previous investigations reporting low vector competence for North American mosquitoes (Dibernardo et al., 2017; O'Donnell et al., 2017), it is hypothesized that the wild mosquitoes tested in this study will produce low transmission efficiencies following experimental infection with ZIKV.

In addition to the increase in the number of reported ZIKV infections, cases of co-infection have also been identified involving ZIKV and additional mosquito-borne viruses, including DENV and CHIKV (Waggoner et al., 2016; Carrillo-Hernández et al., 2018; Mercado-Reyes et al., 2019). The health consequences for co-infections involving ZIKV are not well defined and the possibility for multiple infections involving other viruses is currently unknown. In Chapter 3, the infection dynamics of ZIKV and the possibility of viral interference were analyzed following concurrent and sequential exposure to the North America endemic flavivirus, WNV, in both mammalian and insect cell lines. As these viruses utilize identical replication pathways (Yun & Lee, 2014), it is hypothesized that viral interference will be observed in both cell lines following dual infections.

Chapter 2

Preliminary Vector Competence Results of Canadian Mosquitoes for Zika Virus

2.1 Abstract

As the number of reported Zika virus (ZIKV) cases increases in North America, it becomes increasingly important to identify the mosquito species that might be competent vectors for the virus. In this study, the vector competence of wild mosquito species collected in the Niagara region of Southern Ontario, Canada was investigated. Wild *Aedes punctor* (Kirby), *Aedes trivittatus* (Coquillet), *Aedes vexans* (Meigen), *Anopheles punctipennis* (Say), and *Coquillettidia perturbans* (Walker) mosquitoes, as well as colony *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) mosquitoes, were fed a ZIKV-infected blood meal. In addition, wild *Aedes japonicus* (Theobald) and *Culex pipiens* Linnaeus mosquitoes were orally exposed to an infected sugar meal. Infection, dissemination, and transmission rates for each mosquito were calculated. Among the wild bloodfed mosquitoes, infection was only observed for *Ae. vexans* and *Cq. perturbans*, with no evidence of transmission. Infection and transmission were observed in the colony mosquitoes, with *Ae. albopictus* showing 10% higher transmission efficiency than *Ae. aegypti*. Among the wild sugar-fed mosquitoes, low rates of infection and transmission were observed for *Cx. pipiens* only. The infection and transmission results indicated that mosquito species local to the Niagara region are not likely to be competent vectors for Zika virus. However, the recent establishment of *Ae. albopictus* in southern Ontario may facilitate Zika virus transmission in Canada given the higher transmission efficiency associated with this species.

2.2 Introduction

Zika virus (ZIKV) is an arthropod-borne virus (Flaviviridae: *Flavivirus*) that has caused considerable morbidity and economic loss throughout many tropical and sub-tropical areas around the world. ZIKV was first isolated from a rhesus monkey in 1947 within the Zika forest of Uganda (Dick et al., 1952). The virus has since spread causing sporadic disease in Africa and South East Asia (Monlun, 1993; Heang et al., 2012). ZIKV first reached the Americas in 2015 causing an unprecedented epidemic in Brazil, which affected more than 200,000 people (Zanluca et al., 2015). The virus continued to spread north into the Caribbean and throughout much of Central America (Petersen et al., 2016), reaching the Continental United States in July of 2016. Autochthonous (locally acquired) transmission has since been recorded in the Miami-Dade County of Florida, USA in 2015 and 2017 and Hidalgo County, Texas in 2017 (Centers for Disease Control and Prevention [CDC], 2019A).

ZIKV is primarily transmitted through the bite of an infected mosquito (ECDC, 2016). However, other modes of transmission such as sexual transmission (Foy et al., 2011; Musso et al., 2015A; Atkinson et al., 2016; Deckard et al., 2016) and perinatal transmission via infected breast milk (Besnard et al., 2014; Dupont-Rouzeyrol et al., 2016) have enabled the virus to persist in communities when conditions are not favourable for mosquito-borne transmission. The Caribbean is the major source of imported arbovirus infection to countries in the temperate zone (CDC, 2019A; Government of Canada, 2018C). The Continental United States has recorded 5,460 travel related cases, 251 locally acquired mosquito-borne transmission cases, 52 sexually transmission cases, 2 laboratory acquired infections, and 1 person-to-person contact since 2015 (CDC, 2019A). In Canada, a total of 569 travel related cases (of which 45 were pregnant women) and 4 sexual

transmission cases have been reported to date (Government of Canada, 2018C). Autochthonous ZIKV transmission has not yet been reported in Canada.

Prior to the World Health Organization (WHO) declaring ZIKV a Public Health Emergency of International Concern (PHEIC), few research laboratories in North America were studying this virus and surveillance programs were not yet established. As such there are gaps in knowledge limiting our ability to adequately assess whether or not mosquito species known from Canada are capable of transmitting ZIKV or if our local climate conditions will support autochthonous ZIKV transmission. The province of Ontario in Canada has a diverse and rich mosquito fauna comprised of North American native species and invasive species from Asia and Africa (Giordano et al., 2015; Giordano, 2018). Of the 68 mosquito species known to inhabit Ontario a dozen or so actively bite humans, including two recently introduced *Aedes* mosquitoes; *Aedes japonicus* (Theobald) and *Aedes albopictus* (Skuse) (Thielman & Hunter, 2006; Giordano, 2018). *Ae. japonicus* is an invasive species native to Japan that was first detected in Ontario in 2001 (Thielman & Hunter, 2006). This species is primarily mammophilic and is known to feed on humans (Apperson et al., 2004; Molaei et al., 2009). *Ae. albopictus,* a highly anthropophilic species and competent vector for ZIKV (Chouin-Carneiro et al., 2016), has been collected repeatedly in Windsor, Ontario since 2016 (Giordano, 2018).

Although *Aedes aegypti* (Linnaeus) and *Ae. albopictus* have been implicated as the primary mosquito vectors for ZIKV worldwide (Chouin-Carneiro et al., 2016; Musso et al., 2015A), numerous other species have tested positive for ZIKV in the wild. In the Americas, natural ZIKV infections have been detected in *Ae. albopictus, Ae. aegypti, Aedes vexans, Culex coronator* Dyar & Knab*, Culex quinquefasciatus* Say*,* and *Culex tarsalis* Coquillett (Díaz-Quiñonez et al., 2016; Ferreira-de-Brito et al., 2016; Guedes et al., 2017; Smartt et al., 2017; Elizondo-Quiroga et al.,

2018). Laboratory strains of *Aedes taeniorhynchus* (Wiedemann), *Anopheles freeborni* Aitken, *Anopheles quadrimaculatus* Say, *Cx. tarsalis*, and *Aedes triseriatus* (Say) have shown to be refractory to ZIKV transmission (Weger-Lucarelli et al., 2016; Dodson & Rasgon, 2017; Kenney et al., 2017). There are conflicting reports in the literature as to whether the northern and southern house mosquito, *Cx. pipiens* and *Cx. quinquefasciatus*, respectively, are competent vectors of ZIKV. Aliota et al. (2016), Amraoui et al. (2016), Huang et al. (2016), Weger-Lucarelli et al. (2016), Dodson & Rasgon (2017), Hart et al. (2017), and Kenney et al. (2017) found that *Cx. pipiens* and *Cx. quinquefasciatus* laboratory strains of North American origin were refractory to ZIKV infections. Guedes et al. (2017) and Elizondo-Quiroga et al. (2018) detected ZIKV in wild-caught *Cx. quinquefasciatus* in Brazil and Mexico, and Guo et al. (2016) observed that *Cx. quinquefasciatus* collected in China are competent vectors for ZIKV.

Few studies in North America evaluate mosquitoes captured from the wild. O'Donnell et al. (2017) tested *Ae. vexans* collected from Minnesota and North Dakota; Gendernalik et al. (2017) tested *Ae. vexans* from Colorado; and Dibernardo et al. (2017) investigated the vector competency for ZIKV of 8 field-collected mosquito species from Manitoba, Canada. In an effort to add to these works we set out to test the vector competence for ZIKV of field-collected mosquitoes from Ontario.

2.3 Materials and Methods

2.3.1 Mosquito Collection and Rearing

Wild adult mosquitoes were collected from 10 trapping sites in the Niagara region of Ontario, Canada during the summer months of 2016 using CO_2-baited CDC miniature light traps (John W. Hock Co., Gainesville, FL). *Culex* egg rafts were collected from a backyard pond in the

city of Thorold in August and September of 2016. Mosquito eggs (non-*Culex*) were also collected from oviposition traps set in Thorold during the summer months of 2016. All captured mosquitoes were transported to the Containment Level 3 (CL3) insectary located in Brock University's Cairns Family Health and Bioscience Research Complex. Eggs (individual and rafts) and larvae were reared to adulthood and maintained in an incubator at approximately 24˚C and 75-80% relative humidity (RH) under a 16-hour light, 8-hour dark cycle. Adult mosquitoes were housed in mosquito cages (BioQuip) and fed daily using cotton pads soaked with a 10% sucrose solution.

Laboratory colony mosquitoes were prepared for testing alongside the wild-caught mosquitoes. *Ae. aegypti* (Rockefeller strain, Rutgers University, New Brunswick, NJ) and *Ae. albopictus* (Monmouth and Mercer strain, Rutgers University, New Brunswick, NJ) eggs were reared to adults and maintained under the same conditions as the wild caught mosquitoes. Adult colony mosquitoes were maintained on 10% sucrose daily and offered a bovine blood meal (Cedarlane, Burlington, ON) once a week to facilitate egg laying. The blood meal was fed to the mosquitoes using the Hemotek membrane feeding apparatus (Hemotek Ltd., Blackburn, UK). The colony mosquitoes selected for the ZIKV experimental infection were 5 days old and had never been offered a blood meal prior to the infected blood meal. Twenty-four hours prior to blood-feeding, the sucrose meal was removed from the colony and wild-caught mosquito cages in order to promote feeding from the infected blood meal.

2.3.2 Zika Virus Strain

The ZIKV Thai 2013 strain used in this study was supplied by the National Microbiology Laboratory (Winnipeg, MB) on behalf of the Public Health Agency of Canada (PHAC). The virus was isolated from the urine of a Canadian traveller exhibiting symptoms of fever and rash

following her return from Thailand (Fonseca et al. 2014; GenBank Accession No. KF993678). The virus stock (passage 5) was propagated in an African green monkey kidney cell line (VERO E6; ATCC CRL-1586) and the viral titer was subsequently determined using a plaque assay. Protocols utilized for viral propagation and the plaque assay are described in Agbulos et al. (2016). Following the plaque assay, the final titer of the virus stock was determined to be 10^6 plaque-forming units (PFU) per mL. Aliquots of this stock solution were stored at -80°C.

2.3.3 Mosquito Infection

Infections of wild-caught and colony mosquitoes were carried out in the CL3 laboratory. Mosquito samples were kept in sealed collection vessels with fine netting over the exposed end in order to facilitate feeding. The ZIKV infected blood meal was prepared by diluting 200 µL of ZIKV stock in 1.8 mL of citrated sheep blood (Cedarlane, Burlington, ON) in order to achieve a blood meal with a final titer of 10^5 PFU/mL ZIKV. Adenosine triphosphate (ATP, Sigma-Aldrich) was added to the blood meal at a final concentration of 5 mM (Chouin-Carneiro et al., 2016). The infected blood meal was fed to the mosquitoes using the Hemotek membrane feeding apparatus.

We performed an additional infection using the infected sugar meal (ISM) protocol as described by Causarano (2017). Female mosquitoes will sometimes divert a portion of a sugar meal to the crop as a way to store energy for use during host-seeking or mating activities (Clements, 1992). Therefore, if an ISM was ingested it is possible that virions could by-pass the midgut barrier and disseminate from the crop into the hemolymph. We only included specimens that had an engorged abdomen, indicating that the majority of the ISM was in the midgut. A 10^5 PFU/mL ZIKV-ISM was prepared by mixing 200 µL of ZIKV stock in 1,549 µL of Dulbecco's Modified Eagle's Medium (DMEM), 250 µL of a 40% sucrose solution, and 1 µL of green food

colouring. The ISM was warmed to approximately 30˚C and the mosquitoes were allowed to feed for 30 minutes. Following 30 minutes of feeding, all mosquito samples were cold-anaesthetized by placing them in the fridge at 4 ˚C for approximately 4 to 5 minutes. Mosquitoes whose abdomens were visibly red (infected blood meal) or green (ISM) and distended were separated for further testing. Blood-fed and sugar-fed mosquitoes were incubated within sealed paper cups with a mesh top at 28˚C and 75-80% RH under a 16-hour light, 8-hour dark cycle for 10 days. Mosquitoes were allowed to feed on 10% sucrose solution *ad libitum*.

2.3.4 Mosquito Identification and Dissection

Following the 10-day incubation period, the surviving wild mosquitoes were cold-anesthetized for identification purposes. Wild mosquito samples were identified to species using the photographic key by Thielman & Hunter (2007). Legs and wings were removed from each mosquito and placed in 500 µL of a 2% fetal bovine serum (FBS) in DMEM solution. Following the removal of the legs and wings, the proboscis of each mosquito was inserted into a microcapillary tube containing a feeding solution of 1:1 FBS to 50% sucrose. In order to induce salivation, 1 µL of 1% pilocarpine hydrochloride solution (Sigma-Aldrich) was applied to the thorax of each mosquito. Following 30 minutes of feeding the contents of each capillary tube were emptied into 100 µL of 2% FBS in DMEM. The abdomen of each mosquito was collected in 500 µL of 2% FBS in DMEM solution. All samples were stored at -80˚C until further processing.

2.3.5 RNA Extraction and Amplification

A sterile copper-coated BB (Crosman Corporation, Bloomfield, NY) was added to each tube and samples were homogenized using a Mixer Mill at 30 shakes/second for 2 minutes and

subsequently centrifuged at 6,000 rpm for 4 minutes. RNA was extracted from the samples using the Total RNA Purification 96-Well Kit (Norgen Biotek, Thorold, ON) according to the manufacturer's instructions. Real time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was used to detect the presence of ZIKV in the samples. We followed the protocols reported by Agbulos et al. (2016) for the amplification of viral RNA using the primer/probe (P/P) sets described by Lanciotti et al. (2008).

Samples initially producing a cycle threshold (CT) value lower than 40 were re-tested using both of the real-time P/P sets specific for the ZIKV 2007 strain (Lanciotti et al., 2008). Samples producing a CT value lower than 37 by both P/P sets were regarded as positive for the presence of ZIKV. The qRT-PCR results for legs/wings and saliva samples were only considered if the virus had previously been detected in the body of the same mosquito sample. Additionally, 100 μL aliquots of body and legs/wings samples and 30 μL aliquots of saliva samples from mosquitoes testing positive following qRT-PCR were used to infect VERO E6 cells. Following a 4-day incubation period, the supernatant was collected and tested to confirm the presence of infectious ZIKV.

2.3.6 Vector Competence Calculations

In order to examine the vector competence of the mosquito samples, four rates were determined. The infection rate (IR) is the proportion of mosquitoes whose bodies tested positive for the presence of ZIKV compared to the total number of mosquitoes tested for that species. The disseminated infection rate (DIR) is the proportion of mosquitoes whose legs/wings tested positive for the presence of ZIKV compared to the number of mosquitoes whose bodies also tested positive from that species. The transmission rate (TR) is the proportion of mosquitoes whose saliva tested

positive for ZIKV compared to the number of mosquitoes whose legs/wings also tested positive from that species. The transmission efficiency (TE) is the proportion of mosquitoes whose saliva tested positive for ZIKV compared to the total number of mosquitoes tested from that species (Chouin-Carneiro et al. 2016). We calculated the standard error and 95% confidence intervals (CI) of each IR, DIR, TR, and TE.

2.4 Results

2.4.1 Blood Infections

A total of 37 wild-caught specimens from the blood meal infection trials survived the incubation period (Table 1). ZIKV was detected in the bodies of 9.7% (n = 31) *Ae. vexans* and 33.3% (n = 3) of the *Cq. perturbans* specimens. ZIKV was not detected among the single *Ae. punctor*, *Ae. trivittatus*, and *An. punctipennis* specimens tested (Table 2.1). Although select body samples from *Ae. vexans* and *Cq. perturbans* mosquitoes tested positive for ZIKV, there were no legs/wings or saliva samples that tested positive for the wild-caught mosquitoes.

In regard to the colony mosquitoes, relatively low IR and DIR values were obtained. The TR for both *Ae. aegypti* and *Ae. albopictus* mosquitoes was 100%, indicating that ZIKV was consistently detected in the saliva following detection in the legs/wings for these mosquito samples (Table 2.2).

2.4.2 Sugar Infections

Fourteen *Ae. japonicus* and 82 *Cx. pipiens* specimens survived the incubation period in the ISM trials (Table 2.3). *Ae. japonicus* was refractory to ZIKV infection. ZIKV was detected in 6.1% of the *Cx. pipiens* bodies tested. *Cx. pipiens* had a DIR of 20% (Table 3). We recovered ZIKV in each of the body, appendages, and saliva secretions from a single *Cx. pipiens* specimen.

Table 2.1: Blood infection results for wild-caught mosquitoes. Vector competence results for wild-caught mosquito species following oral exposure to a ZIKV-infected blood meal.

Species	IR (N, 95% CI)	DIR (N, 95% CI)	TR (N, 95% CI)	TE (N, 95% CI)
Aedes punctor	0% (1, 0)	0%	0%	0%
Aedes trivittatus	0% (1, 0)	0%	0%	0%
Aedes vexans	9.7% (31, 0-20)	0% (3, 0)	0%	0%
Anopheles punctipennis	0% (1, 0)	0%	0%	0%
Coquillettidia perturbans	33.3% (3, 0-87)	0% (1, 0)	0%	0%

IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency; N, number tested; 95% CI, 95% confidence intervals.

Table 2.2: Blood infection results for colony mosquitoes. Vector competence results for colony mosquito species following oral exposure to a ZIKV-infected blood meal. N, number tested; 95% CI, 95% confidence intervals.

Species	IR (N, 95% CI)	DIR (N, 95% CI)	TR (N, 95% CI)	TE (N, 95% CI)
Aedes aegypti	12.7% (55, 4-22)	14.3% (7, 0-40)	100% (1, 100)	1.8% (55, 0-5)
Aedes albopictus	36.4% (11, 8-65)	50% (4, 1-99)	100% (2, 100)	18.2% (11, 0-41)

IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency; N, number tested; 95% CI, 95% confidence intervals.

Table 2.3: Sugar infection results for wild-caught mosquitoes. Vector competence results for wild-caught mosquito species following oral exposure to a ZIKV-infected sugar meal. N, number tested; 95% CI, 95% confidence intervals.

Species	IR (N, 95% CI)	DIR (N, 95% CI)	TR (N, 95% CI)	TE (N, 95% CI)
Aedes japonicus	0% (14, 0)	0%	0%	0%
Culex pipiens	6.1% (82, 1-11)	20.0% (5, 0-55)	100% (1, 100)	1.2% (82, 0-4)

IR, infection rate; DIR, disseminated infection rate; TR, transmission rate; TE, transmission efficiency; N, number tested; 95% CI, 95% confidence intervals.

2.5 Discussion

A major challenge in assessing the vector competency of wild-caught mosquitoes is their reluctance to feed from an infectious meal. We had low success rates in bloodfeeding wild-caught host-seeking adults and reared mosquitoes. Bloodfeeding is a complex process that is dependent on a number of host and environmental cues, and thus mimicking these conditions in the laboratory can be difficult. Our low blood feeding success rates supported the decision to pursue the ISM protocol. However, by this time the field season was coming to an end and *Ae. japonicus* and *Cx. pipiens* were the most abundant species in our search area.

The results from this investigation indicated that the wild-caught mosquito species collected from the Niagara region are not likely to be competent ZIKV vectors. Of the 7 species we collected and tested, 3 have not previously been evaluated as potential vectors for ZIKV. This is the first report of *Ae. punctor, Ae. trivittatus*, and *An. punctipennis* vector competency for ZIKV. It should be noted that the samples sizes were small (n = 1) and therefore, we cannot rule out these species as potential vectors of ZIKV until they are re-evaluated with a larger sample size.

Newly emerged *Ae. japonicus* and *Cx. pipiens* would not feed on the infectious blood meal that was offered, which is why we decided to implement the ISM protocol. We found that *Ae. japonicus* was refractory to ZIKV infection. A recent study conducted in Germany reported a high IR (66.7%) for *Ae. japonicus* following consumption of a ZIKV-infected blood meal but the TE was low at 9.5% (Jansen et al., 2018). The researchers concluded that the risk of ZIKV transmission by *Ae. japonicus* in central Europe was likely low. We found that *Cx. pipiens* was a poor vector of ZIKV with an IR of 6.1% and TE of 1.2%. The results presented here conflict with previous reports, which observed *Cx. pipiens* to be refractory to ZIKV infection (Aliota et al., 2016; Huang et al., 2016) and dissemination (Kenney et al., 2017).

Although ZIKV was detected in the body samples of some *Ae. vexans* and *Cq. perturbans* specimens (IR = 9.7% and 33.3%, respectively), there was no evidence of dissemination or transmission of the virus for either species. These results are consistent with previous studies investigating the vector competence of North American mosquitoes for ZIKV. An investigation using specimens from southern Manitoba, Canada observed an IR of 13% for wild-caught *Ae. vexans* specimens following oral exposure to ZIKV while the IR for *Cq. perturbans* specimens was 0% (Dibernardo et al., 2017). Interestingly, 2 *Ae. vexans* samples from the 131 tested by Dibernardo et al. (2017) were able to transmit ZIKV following oral exposure. Likewise, a study investigating *Ae. vexans* vector competence in Montana, USA and North Dakota, USA found that wild-caught samples had an overall transmission potential of only 1% (O'Donnell et al., 2017). In contrast, a study conducted in Colorado, USA observed a very high IR of 80% for wild-caught *Ae. vexans* samples (Gendernalik et al., 2017). Despite a low DIR of 16% and TE of 5%, Gendernalik et al. (2017) concluded that *Ae. vexans* is a competent vector for ZIKV.

The results for colony *Ae. aegypti* indicated a low vector competence for these species (TE = 1.82%) despite the fact that previous studies have shown *Ae. aegypti* to be a competent vector for ZIKV (Li et al., 2012; Di Luca et al., 2016; Duchemin et al., 2017; Guedes et al., 2017; Pompon et al., 2017). However, there have been conflicting studies that reported low ZIKV transmission for *Ae. aegypti* samples despite high IR values (Diagne et al., 2015; Chouin-Carneiro et al., 2016; Jupille et al., 2016). Similarly, Richard et al. (2016) observed high rates of ZIKV infection in French Polynesian *Ae. aegypti* samples with low transmission efficiencies until 14 days post infection, suggesting that some populations of these mosquitoes may possess a delayed capacity to transmit ZIKV. Weger-Lucarelli et al. (2016) and Roundy et al. (2017) found that vector competency in *Ae. aegypti* is strain specific, which may explain the large variation in published

literature. Interestingly, Ciota et al. (2017) observed that freeze/thawing stock solutions significantly impairs infectivity to mosquitoes, which also has the potential to affect vector competence results in the laboratory.

Although *Ae. aegypti* has been viewed as the primary vector of ZIKV during the recent South American outbreak, it should be noted that in our laboratory colony *Ae. albopictus* mosquitoes were more competent (TE = 18.2%). Climate change models predicted that the environmental conditions for *Ae. albopictus* would soon be met in southern Ontario (Campbell et al., 2015); our research team discovered *Ae. albopictus* in Windsor, Ontario in 2016. This species was also found to be a competent vector of ZIKV in Australia (Duchemin et al., 2017), Singapore (Wong et al., 2013) and the United States (Azar et al., 2017; Ciota et al., 2017) and has been implicated in the transmission of Chikungunya virus (CHIV) in Italy in 2007 (Rezza et al., 2007) as well as France in 2014 (Roiz et al., 2015). *Ae. albopictus* is now well established in New Jersey, New York, Ohio, and Pennsylvania, as well as in Windsor, Ontario (Farajollahi et al., 2012; Giordano, 2018).

In conclusion, the wild-caught mosquitoes captured from the Niagara Region in Southern Ontario, Canada do not appear to be competent ZIKV vectors. However, in addition to the presence of competent vector species, there are further conditions that must be met in order for the virus to persist in Southern Ontario, such as suitable climate conditions as well as the presence of susceptible hosts to maintain the transmission cycle. While non-human primate hosts are the main reservoirs in the sylvatic ZIKV transmission cycle, it is important to identify additional animal hosts that may also have the potential to uphold virus transmission in North America. Additionally, the recent establishment of *Ae. albopictus* in Southern Ontario is very concerning as this species is a competent vector for ZIKV. It is important to continue this investigation by targeting larger

sample sizes of North American mosquito species that are known to preferentially feed on humans. An understanding of the environmental temperatures that would contribute to ZIKV transmission is a crucial next step to aid public health officials in communicating potential peaks in transmission risk to the public.

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Chapter 3

Dual Flavivirus Infection Dynamics in Host and Vector Cells

3.1 Abstract

As the range of vector mosquitoes for Zika virus (ZIKV) expands northwards, the likelihood for co-circulation and co-infection with other North American endemic viruses, such as West Nile virus (WNV), is increased. In this study, the infection dynamics of WNV and ZIKV following co-infections and superinfections were investigated in order to determine the possibility for viral interference. VERO E6 cells and C6/36 cells, derived from African green monkey kidney epithelial cells and *Aedes albopictus* (Skuse) larva, respectively, were co-infected or superinfected with WNV and ZIKV at a final multiplicity of infection (MOI) of 0.01. Supernatant samples were collected each day following the infections for a total of five days, at which time viral RNA was extracted and subjected to quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in order to verify the presence of WNV and ZIKV in the samples. Cycle threshold (CT) values were converted to log starting quantity (LSQ) values using a standard curve generated by the qRT-PCR. In regard to the co-infections, WNV had consistently higher LSQ values in VERO E6 cells while there was no significant difference observed in C6/36 cells. When WNV was the first virus to infect cells in the superinfections, the LSQ values were significantly higher than those for ZIKV in both cell lines. When ZIKV was the first virus in the superinfections, ZIKV had consistently higher LSQ values in C6/36 cells while there was no significant difference in LSQ on the fifth day of infection in VERO E6 cells. The results indicated a level of viral interference associated with two flavivirus infections in VERO E6 cells but not in the C6/36 cell line.

3.2 Introduction

Following its arrival in South America in 2015 and the subsequent spread to Central and North America, Zika virus (ZIKV) (Flaviviridae: *Flavivirus*) has gained international attention as a threat to public health. Prior to that point, the virus had only been responsible for sporadic disease cases in Africa and Asia as well as small outbreaks in the Pacific Islands (Song et al., 2017). The ZIKV outbreaks reported in South America were unprecedented due to the fact that the virus, which had previously been characterized by mild, self-limiting illness, was being linked to severe, life-threatening autoimmune and neurological disorders. Along with the increase in reported ZIKV diseases cases, there was an accompanying increase in the number of microcephaly and Guillain-Barré syndrome (GBS) cases (Schuler-Faccini et al., 2016; Thomas et al., 2016). The observed increase in these serious health disorders following ZIKV infection saw the World Health Organization (WHO) declare the virus as a Public Health Emergency of International Concern (PHEIC) (WHO, 2016A).

In addition to the increased reports of GBS and microcephaly developing following ZIKV infection, there have also been reports of patients suffering from ZIKV co-infections, which have the potential to enhance disease severity. The arrival of ZIKV in South America allowed for the co-circulation of the virus with other endemic viruses such as chikungunya virus (CHIKV) (Togaviridae: *Alphavirus*) and dengue virus (DENV) (Flaviviridae: *Flavivirus*). Confirmed co-infection involving ZIKV and one or more other viruses have been reported. Cases of CHIKV-ZIKV co-infection have been observed in Brazil (Sardi et al., 2016) and Ecuador (Zambrano et al., 2016). In a study conducted in Columbia, 28 patients tested positive for a CHIKV-ZIKV co-infection while 3 patients tested positive for a DENV-ZIKV co-infection. Two of the CHIKV-ZIKV co-infections were fatal (Mercado-Reyes et al., 2019). In a study conducted in Nicaragua,

16 patients tested positive for a CHIKV-ZIKV co-infection, 6 patients tested positive for a DENV-ZIKV co-infection, and 6 patients tested positive for a CHIKV-DENV-ZIKV triple co-infection (Waggoner et al., 2016). Finally, at the Colombian-Venezuelan border, the prevalence of CHIKV-ZIKV and DENV-ZIKV co-infections were approximately 5% and 6%, respectively, with a prevalence of 2% for co-infection with all three viruses (Carrillo-Hernández et al., 2018).

The increase in reports of co-infections involving ZIKV, CHIKV, and DENV in South America is facilitated by the fact that the three viruses are circulating in the same areas and utilize similar mosquito species as vectors for their primary mode of transmission. However, as the range of ZIKV expands further northwards, the risk of co-infection with CHIKV and DENV decreases as these viruses are not as well-established in North America (Centers for Disease Control and Prevention [CDC], 2018C; CDC, 2019D). Despite this, the northward expansion of ZIKV may allow for co-infections to occur with certain viruses that are endemic to North America. West Nile virus (WNV) (Flaviviridae: *Flavivirus*) is endemic to both Canada and the United States and is similar to ZIKV in the sense that both are mosquito-borne flaviviruses. While WNV disease cases are typically mild in nature, about 1% of WNV infections target the central nervous system and may have fatal consequences (CDC, 2018A). As presumed mosquito-borne ZIKV transmission has been reported in the southern states of the United States, it is possible that areas exist in North America where ZIKV and WNV may co-circulate (CDC, 2019A).

There are key differences in the transmission cycles of ZIKV and WNV that have the potential to impede co-infection. The main difference between the two viruses is that humans and other large mammals are considered to be incidental hosts for WNV, due to the fact that they are not able to develop levels of viremia that are sufficient for infecting mosquitoes, while this is not the case for ZIKV (Bunning et al., 2002). In the urban cycle of ZIKV transmission, humans act as

the main hosts from which peridomestic mosquitoes are able to acquire the virus (Weaver et al., 2016). In WNV transmission, however, birds, specifically passerines, are the primary hosts from which mosquito vectors may become infected (Komar et al., 2003). There is also variability in the mosquito species that act as competent vectors for the different viruses. Mosquitoes belonging to the genus *Aedes* (*Stegomyia*), particularly *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse), are predicted to be the primary vectors for ZIKV (Ferreira-de-Brito et al., 2016; Grard et al., 2014) while *Culex* (*Melanoconion*) mosquitoes, particularly *Culex pipiens* Linnaeus and *Culex restuans* Theobald, are the primary vectors for WNV (Ebel et al., 2005). Despite the fact that WNV is maintained in a sylvatic transmission cycle, human infection may be established through bridge vectors, which are mosquito species that are known to feed from both avian and mammalian hosts (Chancey et al., 2015). *Ae. aegypti* and *Ae. albopictus* have both been shown to have the potential to act as competent bridge vectors for WNV (Turell et al., 2005) and the virus has been detected in both species in the United States (CDC, 2016).

It is important to note that while *Ae. aegypti* and *Ae. albopictus* may have the capacities to transmit both WNV and ZIKV to a human host, it is unlikely that these vectors will be able to acquire both viruses from the same host. While it is clear that WNV cannot be acquired from a human host, the possibility of acquiring ZIKV from a bird host is not well-studied. Okia et al. (1971) tested 221 birds in Uganda and found that 43% possessed antibodies to WNV while 15% possessed antibodies to ZIKV. Further studies are required to determine whether ZIKV is able to be maintained in a sylvatic transmission cycle using birds as hosts. However, human infection by both viruses may be possible in areas where co-circulation occurs by superinfection, which takes place when a host is infected with a second virus after already being infected with a different virus from a previous mosquito bite. Thus, while serologic surveys might indicate that a patient is co-

infected with both viruses, the viruses were actually acquired through a superinfection. Superinfections are well-characterized for human immunodeficiency virus (HIV) cases (Smith et al., 2005), but it is difficult to determine whether co-infections of arthropod-borne viruses are the result of simultaneous or sequential viral infections through serological surveys.

Co-infections involving ZIKV and WNV have not been reported to date but the risk exists in areas where both viruses co-circulate. It is important to examine the effects of simultaneous and sequential infections involving these viruses in order to identify possible variation in infectivity or severity that may be applicable to human disease cases. In an effort to investigate this relationship, the infection dynamics of ZIKV and WNV were analyzed following both co-infections and superinfections in mammalian and insect cell lines.

3.3 Materials and Methods

3.3.1 West Nile Virus Strain

The WNV strain utilized in this investigation was the New York 1999 (NY99) strain (Accession No. AF196835). The NY99 strain was supplied by the National Microbiology Laboratory (Winnipeg, MB) on behalf of the Public Health Agency of Canada (PHAC). The virus was isolated from the brain of a dead Chilean flamingo (*Phoenicopterus chilensis*) from the Bronx Zoo in New York following an outbreak of human encephalitis (Lanciotti *et al*., 1999).

The virus stock was propagated in an African green monkey kidney epithelial cell line (VERO E6; ATCC CRL-1586). Working in a Containment Level 3 (CL3) laboratory, VERO E6 cell monolayers were infected with a multiplicity of infection (MOI) of 0.02. Following the observation of notable cytopathic effect, the cells were centrifuged at 5,000 g and 4˚C for 30 minutes. The viral supernatant was subsequently collected and frozen at -80˚C in 1 mL aliquots.

In order to determine the viral titer of the working stocks, a plaque assay was performed. VERO E6 cell monolayers seeded onto 6-well culture plates were infected with serial 10-fold dilutions of the working stock virus. The viral dilutions were prepared using a 2% fetal bovine serum (FBS) solution. Following a 60-minute infection period, the viral dilutions were removed from the cell monolayers and replaced with an overlay consisting of a 1:1 preparation of a carboxymethyl cellulose (CMC) solution and a 2% FBS solution. Following a 3-day incubation period at 37˚C and 5% CO_2 conditions, cells were stained using a crystal violet solution. The protocols used for preparing all reagents and solutions used in this investigation are included in Appendix I. Following the determination of the number of plaques per well, the viral titer of the NY99 working stock used in this experiment was 4.48×10^7 plaque-forming units (PFU)/mL. The calculations used for determining viral titer are included in Appendix II.

3.3.2 Zika Virus Strain

The ZIKV strain utilized in this investigation was the PRVABC59 strain (Accession No. KU501215). The PRVABC59 strain was supplied by the National Microbiology Laboratory (Winnipeg, MB) on behalf of the PHAC. In 2015, the virus was isolated by the CDC Arbovirus Diagnostic Laboratory from human serum in Puerto Rico (Lanciotti *et al*., 2016). The virus stock was propagated in VERO E6 cells to create a working stock and viral titer was determined by plaque assay following the same protocols detailed in Section 3.3.1. The viral titer of the PRVABC59 working stock used in this experiment was 2.24×10^5 PFU/mL (Appendix II).

3.3.3 Cellular Infections

Two different cell lines were examined in this study. The first was a VERO E6 cell line

and the second was an *Ae. albopictus* larva (whole) cell line (C6/36; ATCC CRL-1660). The VERO E6 cell line was incubated at 37˚C while the C6/36 cell line was incubated at 28˚C. Both cell lines were kept under 5% CO_2. During the experimental infections, the VERO E6 cell line was supplemented with 2% FBS medium while the C6/36 cell line was supplemented with C6/36 culture medium.

Under CL3 conditions, three different methods of infection were used. The first method of infection was a WNV and ZIKV co-infection (WNV-ZIKV CI). For this co-infection, designated cells were infected simultaneously with a 1:1 preparation of both viruses at a final MOI of 0.01. The second method of infection carried out was a WNV first, ZIKV second superinfection (WNV/ZIKV SI). For this superinfection, designated cells were initially infected with WNV (0.01 MOI) and subsequently infected with ZIKV (0.01 MOI) 24 hours following the initial infection. The third method of infection carried out was a ZIKV first, WNV second superinfection (ZIKV/WNV SI), which followed the same infection protocol as the WNV/ZIKV SI, the only difference being the reversal of the order of the viruses used to infect the designated cells. The calculations used for determining the volumes of inoculum necessary to achieve a 0.01 MOI are included in Appendix III.

VERO E6 and C6/36 cells were seeded onto five 12-well culture plates each and incubated until the cell monolayers had reached 80-90% confluency. On each 12-well culture plate, three wells were designated to each method of infection (WNV-ZIKV CI, WNV/ZIKV SI, and ZIKV/WNV SI) (Figure 3.1). The final three wells on the culture plate were designated as negative controls. Once cells had reached the appropriate confluency, the prepared culture media was removed to allow for cells to be washed with Dulbecco's phosphate-buffered saline (DPBS). The DPBS was then removed and the pre-determined volumes of inoculum were added to the

appropriate wells. In order to ensure that the entire cell monolayer was covered during the infection period, the appropriate type of media, depending on the cell line, was added alongside the viral inoculum until a final volume of 400 µL was met per well. Following the addition of virus, the cells were incubated for 1 hour. Once this incubation period had lapsed, the viral inocula were removed from the wells and another DPBS wash was performed. Each well was then supplemented with 1 mL of the appropriate media, depending on the cell line, and all plates were returned to the incubator for 2-5 days. On each subsequent day following the initial infection, all supernatant samples were collected from one full plate. In addition, single WNV and ZIKV cellular infections were carried out following the same infection protocols that were utilized for the co-infections and superinfections in both cell lines.

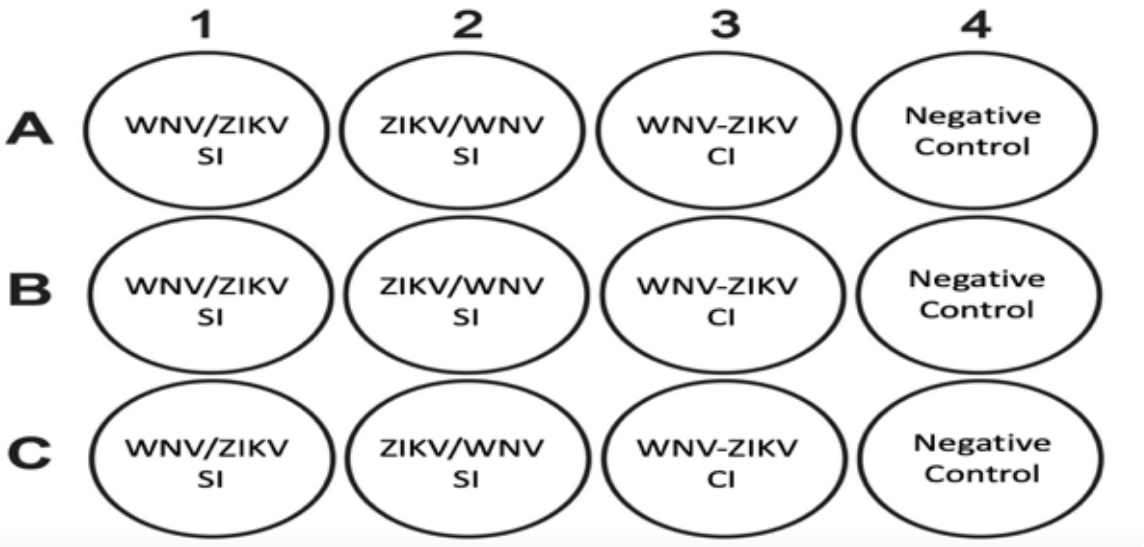

Figure 3.1: Cellular infection well map. Well map depicting the locations for the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in VERO E6 and C6/36 cells.

3.3.4 Viral RNA Extraction

An aliquot of 100 µL was taken from each supernatant sample and added to 200 µL of lysis buffer (Norgen Biotek, Thorold, ON). RNA was extracted from the supernatant samples using the

Total RNA Purification 96-Well Kit (Norgen Biotek) according to the manufacturer's instructions. All supernatant samples were subsequently stored at -80˚C.

3.3.5 Viral RNA Amplification

Viral RNA was detected in the supernatant samples using a one-step real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). All reactions were performed using the iTaq universal probe one-step master mix (Bio-Rad). All supernatant samples were tested for the presence of both WNV and ZIKV RNA. For WNV RNA detection, samples were initially tested with the WNV generic (Gen) primer/probe (P/P) set, which targets the WNV 3' non-coding region (NCR). Samples that tested positive for the presence of WNV RNA using the Gen P/P set were subsequently tested with the P/P set responsible for targeting the WNV envelope (E) (Lanciotti *et al*., 2000). For ZIKV RNA detection, samples were initially tested with the ZIKV 1 P/P set, which targets the ZIKV M glycoprotein. Samples that tested positive for the presence of ZIKV RNA using the Zika 1 P/P set were subsequently tested with the ZIKV 2 P/P set, which is responsible for targeting the ZIKV E (Lanciotti *et al*., 2008). Full sequences for the P/P sets used in this experiment, as well as the master mix formulas and thermocycler conditions, are included in Appendix IV.

3.3.6 Standard Curve Generation

Serial 10-fold dilutions were prepared using a 2% FBS solution for both virus stocks, ranging from 10^{-1} and 10^{-7}. Viral RNA was subsequently extracted from the prepared viral dilutions following the same protocol used in Section 3.3.4. The extracted RNA from the serial dilutions was amplified with the supernatant samples using qRT-PCR to generate a standard curve.

3.3.7 Statistical Analyses

In order to be able to compare the qRT-PCR results between WNV and ZIKV in the supernatant samples, the trendline equation generated from each standard curve was used to calculate log starting quantity (LSQ) values using cycle threshold (CT) values. The CT value determined through the qRT-PCR reaction was substituted into the standard curve trendline equation as the known x-value in order to solve for the unknown y-value, which corresponded to the LSQ of virus for the supernatant sample under investigation.

Once all LSQ values had been calculated, single factor analys of variance (ANOVA) tests ($\alpha = 0.05$) were run using Microsoft Excel in order to determine whether a significant difference in LSQ existed between the viruses based on the different methods of infection. In cases where a significant difference was detected among groups a Tukey's test was used to determine the source of the difference within the group.

3.4 Results

3.4.1 Dual Flavivirus Infections

The cell lines yielded different results depending on the type of infection. In regard to the WNV-ZIKV CI, the LSQ values were significantly higher for WNV when compared to ZIKV throughout the duration of the experiment conducted in VERO E6 cells (Figure 3.2). In the C6/36 cells, however, there was no significant difference observed in LSQ values (Figure 3.2). Both cell lines showed similar results for WNV/ZIKV SI with the LSQ values associated with WNV significantly higher than those for ZIKV on each day of the experiment (Figure 3.3). Finally, while the LSQ values for ZIKV were significantly higher in the C6/36 cells for the ZIKV/WNV SI, there was no significant difference in LSQ values between the two viruses on the fifth and final day of

experimentation in the VERO E6 cells (Figure 3.4). In addition, LSQ values were typically higher in the VERO E6 cells when compared to the C6/36 cells. The peak LSQ values attained in the VERO E6 cells were 6.86 for WNV (WNV-ZIKV CI, Day 5) and 4.85 for ZIKV (ZIKV/WNV SI, Day 5) while in the C6/63 cells the peak LSQ values attained were 4.38 for WNV (WNV/ZIKV SI, Day 5) and 4.58 for ZIKV (ZIKV/WNV SI, Day 5).

3.4.2 Single Flavivirus Infections

The growth curves comparing single and dual virus infections indicated viral interference of WNV in dual infections. In the VERO E6 cells, the LSQ values for the WNV single infection were consistently higher than those for the dual infections, with a peak LSQ value of 7.46 for the single WNV infection compared to peak LSQ values of 6.86 (WNV-ZIKV CI), 6.73 (WNV/ZIKV SI), and 5.27 (ZIKV/WNV SI) (Figure 3.5A). In the C6/36 cells, however, the single WNV infection yielded similar LSQ values when compared to the dual infections until the fifth day of experimentation when the single infection had the highest LSQ value of 4.89 compared to 4.38 (WNV/ZIKV SI), 3.91 (WNV-ZIKV CI), and 3.50 (ZIKV/WNV SI) (Figure 3.5B). With regard to ZIKV, the LSQ values for the single infection were also highest in the VERO E6 cells, with a peak LSQ value of 5.87 for the single ZIKV infection compared to peak LSQ values of 4.85 (ZIKV/WNV SI), 4.47 (WNV-ZIKV CI), and 2.10 (WNV/ZIKV SI) (Figure 3.6A). However, in the C6/36 cells, the ZIKV/WNV SI consistently had the highest LSQ values, even when compared to the single ZIKV infection. On the fifth day of experimentation, the LSQ value for the ZIKV/WNV SI was 4.58 while the single ZIKV infection had an LSQ value of 3.78 (Figure 3.6B).

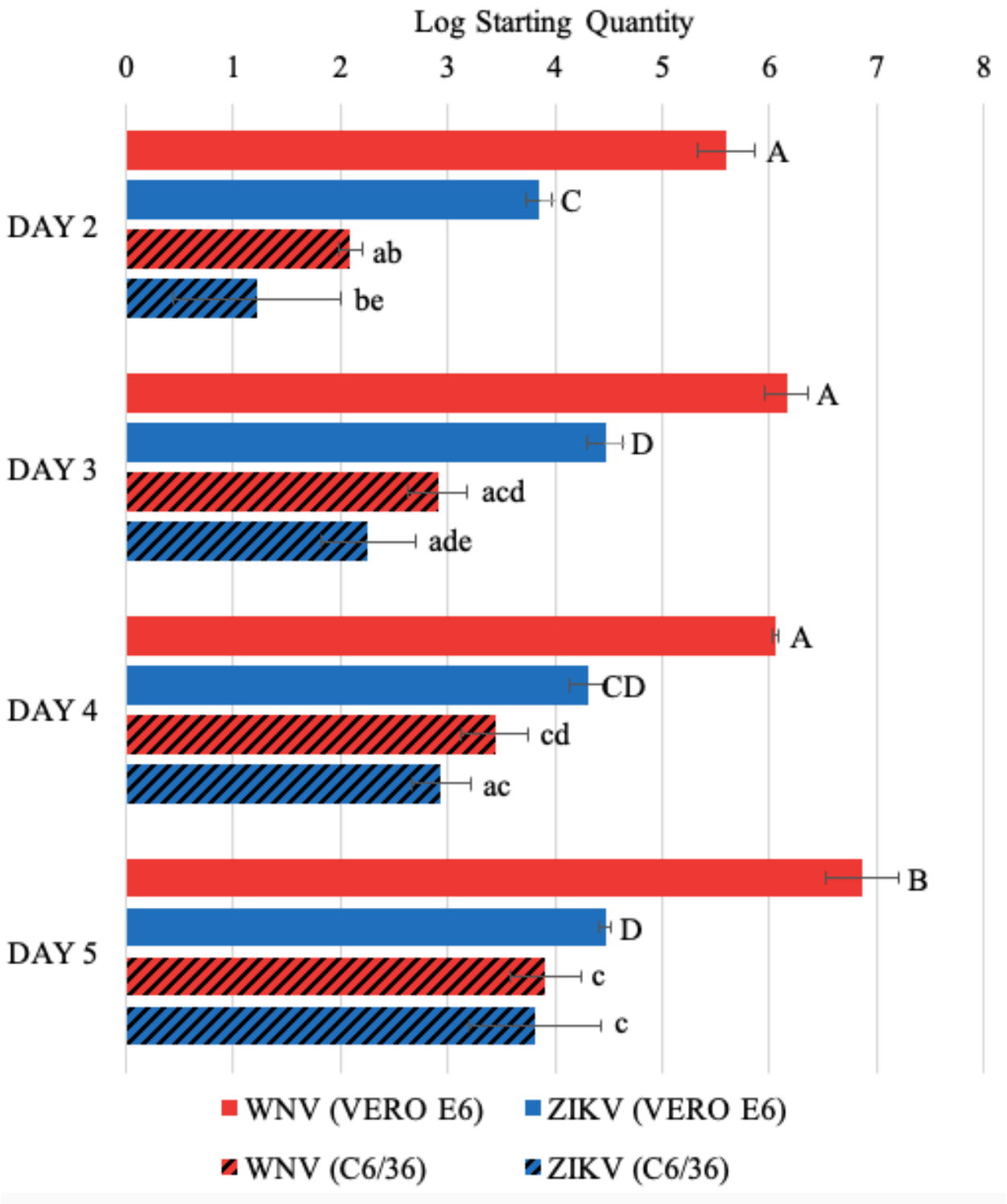

Figure 3.2: WNV and ZIKV co-infection results in VERO E6 and C6/36 cells. The mean log starting quantity (LSQ) values ± SD from triplicate samples for the simultaneous infection of VERO E6 cells (solid bars) and C6/36 cells (striped bars) with WNV and ZIKV (WNV-ZIKV CI) at a final MOI of 0.01. Bars with different capital or lower-case letters are significantly different.

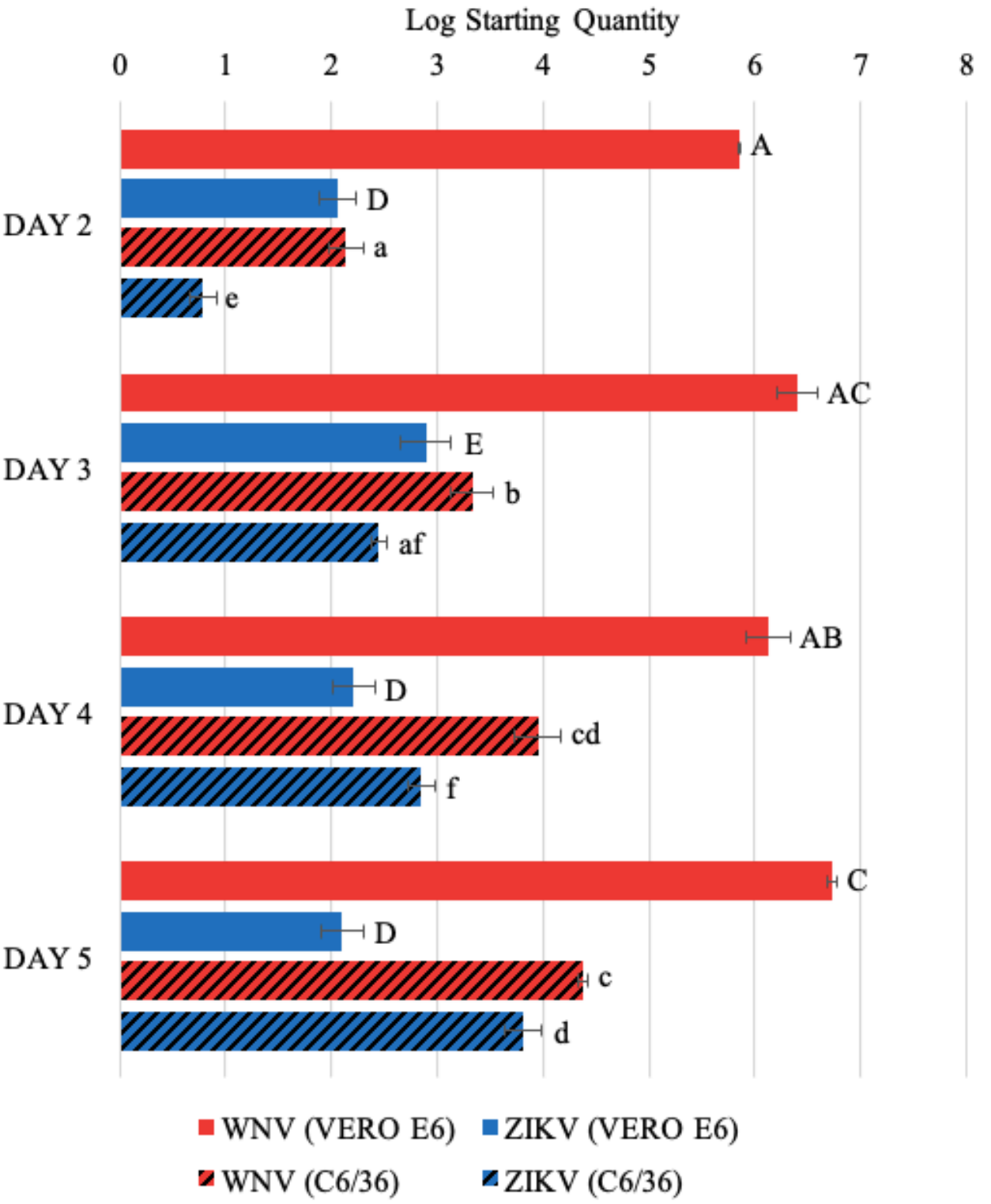

Figure 3.3: WNV first superinfection results in VERO E6 and C6/36 cells. The mean log starting quantity (LSQ) values ± SD from triplicate samples for the sequential infection of VERO E6 cells (solid bars) and C6/36 cells (striped bars) with WNV followed by ZIKV (WNV/ZIKV SI) at an MOI of 0.01. Bars with different capital or lower-case letters are significantly different.

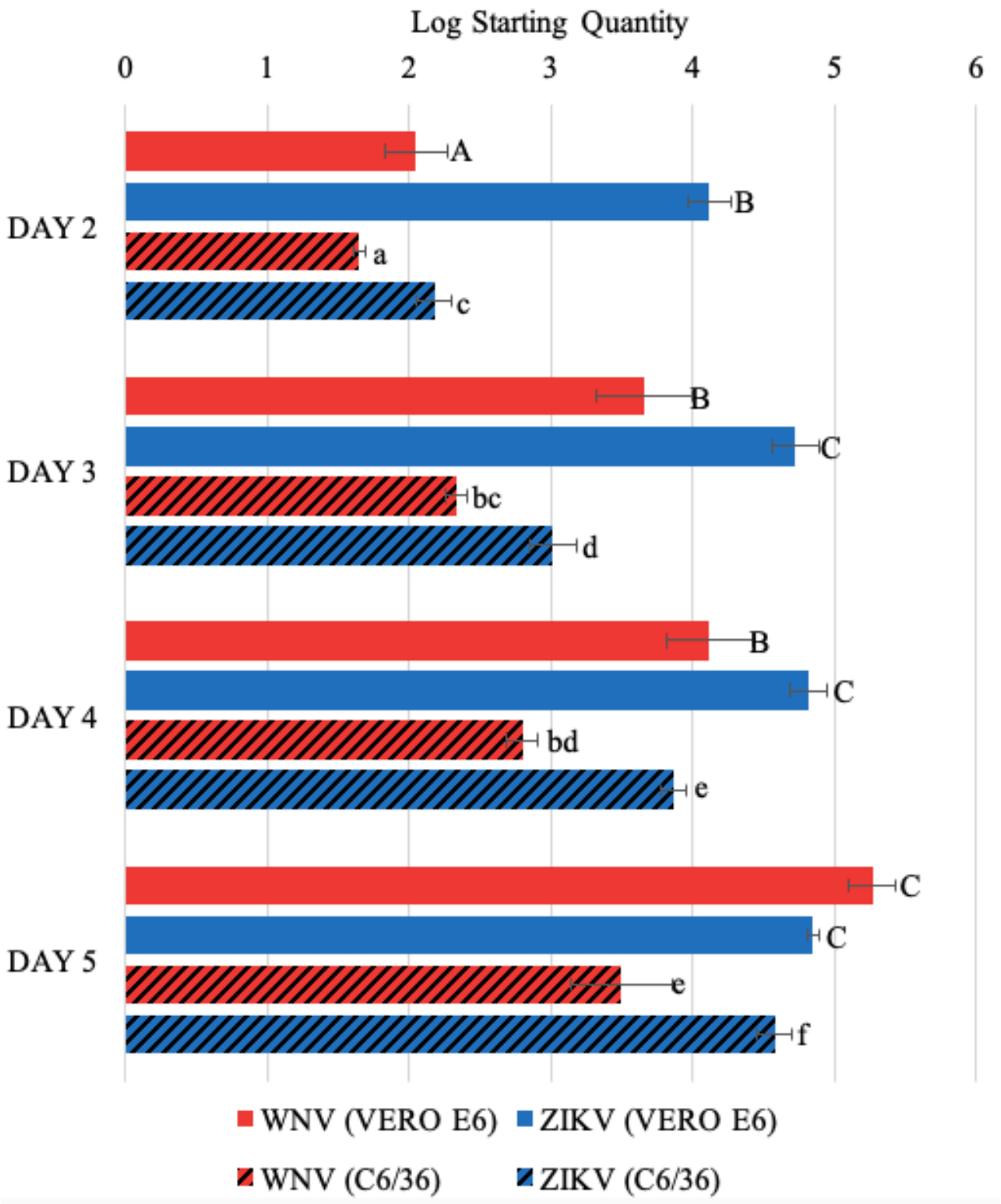

Figure 3.4: ZIKV first superinfection results in VERO E6 and C6/36 cells. The mean log starting quantity (LSQ) values ± SD from triplicate samples for the sequential infection of VERO E6 cells (solid bars) and C6/36 cells (striped bars) with ZIKV followed by WNV (ZIKV/WNV SI) at an MOI of 0.01. Bars with different capital or lower-case letters are significantly different.

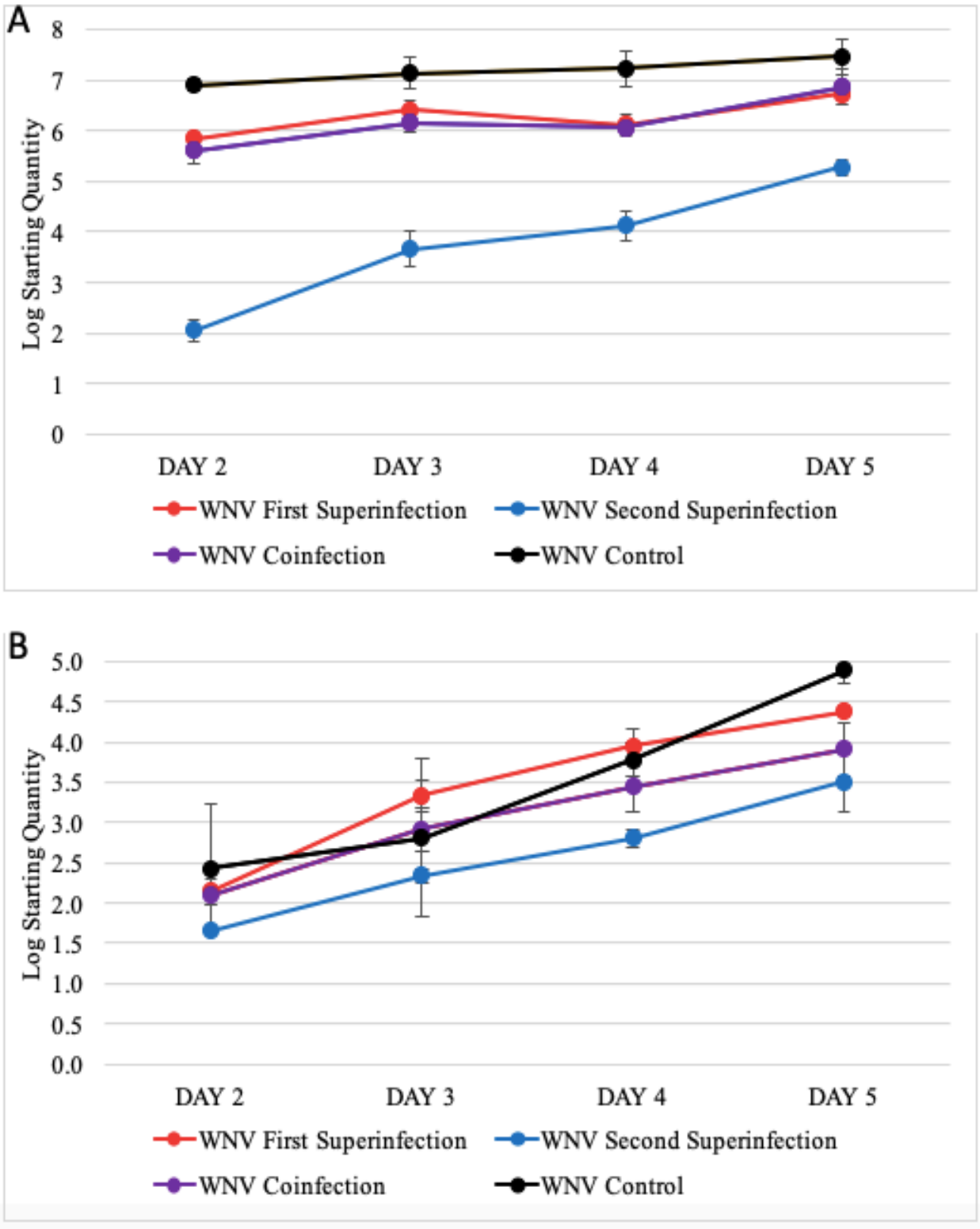

Figure 3.5: WNV growth curves following single and dual infections. The mean log starting quantity (LSQ) values ± SD from triplicate samples for the WNV single infection (WNV Control) compared to the co-infection and superinfections carried out with ZIKV in (A) VERO E6 cells and (B) C6/36 cells at an MOI of 0.01.

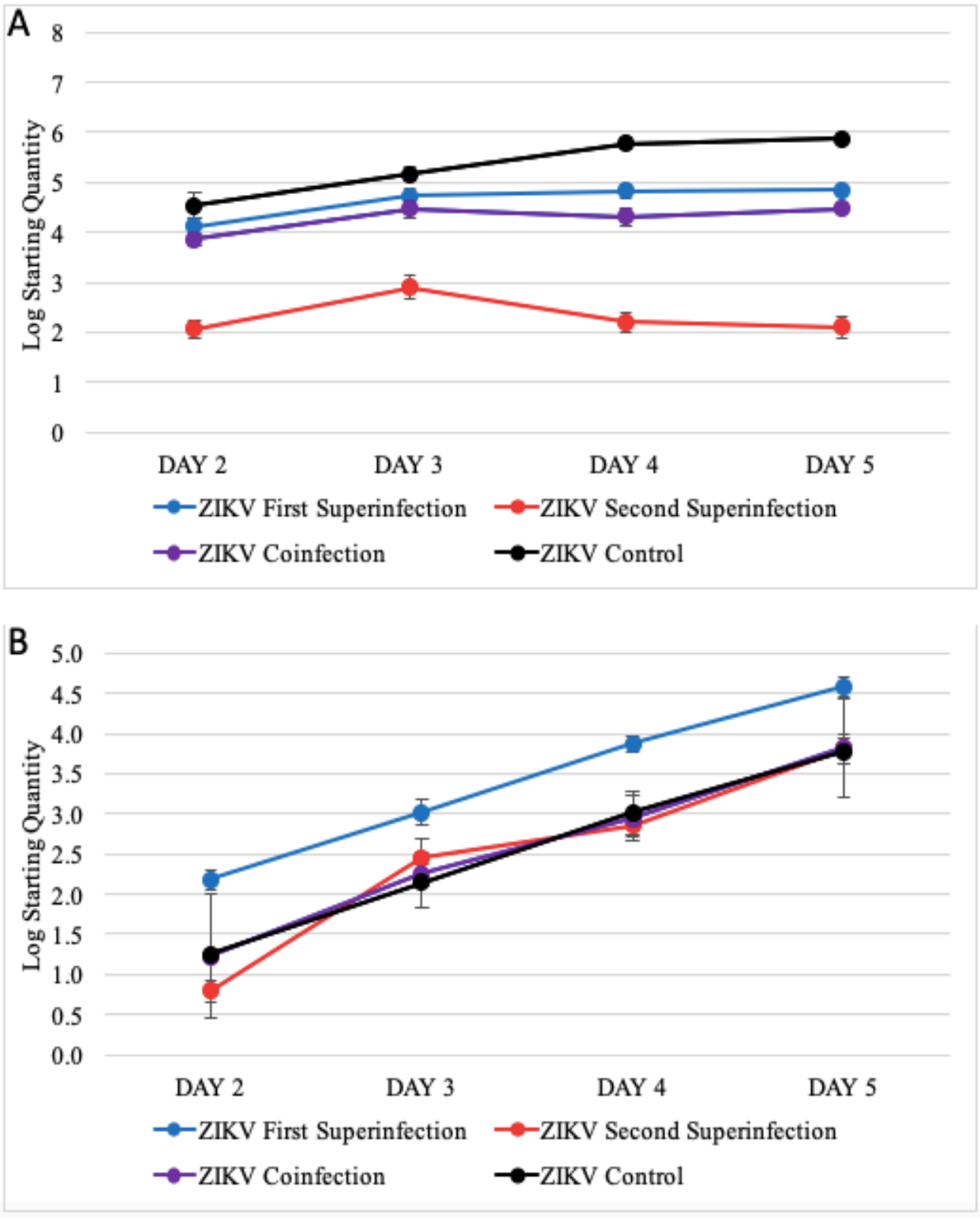

Figure 3.6: ZIKV growth curves following single and dual infections. The mean log starting quantity (LSQ) values ± SD from triplicate samples for the ZIKV single infection (ZIKV Control) compared to the co-infection and superinfections carried out with WNV in (A) VERO E6 cells and (B) C6/36 cells at an MOI of 0.01.

3.5 Discussion

Following the introduction of ZIKV to the Americas, the virus has the potential to co-circulate with other endemic arthropod-borne viruses. Co-infections have been observed for ZIKV, CHIKV, and DENV in South America where active transmission has been reported (Waggoner et al., 2016; Carrillo-Hernández et al., 2018; Mercado-Reyes et al., 2019). However, it remains unclear whether the viruses involved in the co-infections were delivered to the hosts through a single mosquito bite or through separate biting events. The latter infection method, referred to as a superinfection, is more likely to occur in cases where the viruses are maintained in transmission cycles utilizing different vertebrate hosts, which is the case for ZIKV and WNV. As co-infections involving WNV and ZIKV have not been reported to date, the aim of this study was to analyze the effects of simultaneous and sequential exposure of these viruses to host and vector cells.

The results showed that WNV and ZIKV were able to replicate in both VERO E6 cells and C6/36 cells following dual infection. However, both viruses attained higher LSQ values in the VERO E6 cells when compared to the C6/36 cells. This observation was consistent with other studies using both cell lines in viral growth kinetics experiments. Prow et al. (2016) consistently observed higher virus titers in VERO cells using the NY99 strain of WNV for the first three days following the initial infection. Culture supernatants were harvested for two additional days in the C6/36 cells, at which time the viral titer seemed to increase to levels comparable to the VERO trials, indicating that the NY99 strain of WNV might require a longer propagation period in C6/36 cells (Prow et al., 2016). Similarly, Moser et al. (2018) observed the PRVABC59 strain of ZIKV to replicate more quickly in VERO cells and attained higher titers on each of the three days following the initial inoculation. Samples were also taken for four additional days in the C6/36 cells, at which time the viral titers increased to match those attained in the VERO cells and

subsequently plateaued (Moser et al., 2018). Göertz et al. (2017) observed higher titers for ZIKV (Suriname strain) following co-infection with CHIKV in the VERO E6 cells, however, CHIKV had substantially higher titers in the C6/36 cells when compared to the VERO E6 cells following the co-infection. Thus, it appears that there are many factors, including the cell line, virus strain, and infection period, that may influence viral growth kinetics experiments.

Viral interference describes the phenomenon where the infection with one virus inhibits the infection of other viruses (Salas-Benito & De Nova-Ocampo, 2015). In this study, viral interference was observed for both viruses in VERO E6 cells following the co-infection and superinfections. Göertz et al. (2017) also observed viral interference for ZIKV in VERO E6 cells following co-infection with CHIKV. The viral titers from the co-infection were approximately 3 logs lower than the single ZIKV infection on the second- and third-days post infection (Göertz et al., 2017). Despite the viral interference observed for both viruses following the VERO E6 co-infection in the present study, WNV had consistently higher LSQ values when compared to ZIKV. This is to be expected as the NY99 strain of WNV has been shown to reach higher titers in VERO cells when compared with the PRVABC59 strain of ZIKV (Prow et al., 2016; Moser et al., 2018).

In cases where viral interference is a direct result of a superinfection, the infection inhibition is termed superinfection exclusion (Salas-Benito & De Nova-Ocampo, 2015). In this study, primary WNV infection was shown to interfere with subsequent ZIKV infection in the WNV/ZIKV SI. In contrast, ZIKV had the higher LSQ values in the ZIKV/WNV SI for the first four days following the initial infection after which time, there was no significant difference in LSQ value between the viruses. Unfortunately, studies investigating superinfections in VERO E6 cells, particularly flavivirus superinfections, are scarce as most studies focus on C6/36 cells or other mosquito-derived cell lines. However, it is reasonable to assume that the capacity of the

WNV strain used in this experiment to attain high titers in the VERO E6 cell line allowed for the virus to attain the same LSQ level as ZIKV in the ZIKV/WNV SI. Future superinfection studies should lengthen the number of days following the initial infection in order to investigate whether the LSQ values for WNV will plateau or continue to increase following the fifth day of the ZIKV/WNV SI in VERO E6 cells.

The viral interference observed in VERO E6 cells is likely due to increased competition between the viruses in infecting cells. As the number of cells infected by each virus increases, there will be a direct decrease in the number of cells available for further virus production. Thus, the titers observed in the co-infection and superinfections are lower than those for the single infections because the viruses are in competition for resources. Furthermore, as WNV and ZIKV are both flaviviruses, they utilize the same cellular and viral factors in their replication cycles, which may result in a greater level of interference when compared to simultaneous infections with viruses that are not as closely related (Salas-Benito & De Nova-Ocampo, 2015).

In contrast to viral interference, viral accommodation describes the phenomenon where the infection by two viruses does not result in interference (Salas-Benito & De Nova-Ocampo, 2015). Viral accommodation was observed in this study following the C6/36 dual flavivirus infections. Göertz et al. (2017) observed viral accommodation for ZIKV and CHIKV following co-infection in C6/36 cells as well. The authors attributed this result to the fact that ZIKV and CHIKV belong to different families and consequently utilize different viral replication pathways in cells, which results in lower levels of viral competition and interference (Göertz et al., 2017). However, viral accommodation was also observed by Kathong et al. (2010) following co-infections with three different viruses, including the flaviviruses DENV and Japanese encephalitis virus (JEV). These discrepancies might indicate that viral interference is variable among different flaviviruses and

may also be dependent on the type of cell line used in the infections. Additionally, the time interval between the first and second infection in superinfection experiments may also influence the observed level of viral interference (Salas-Benito & De Nova-Ocampo, 2015). Pepin et al. (2008) found that replication was more greatly suppressed in superinfections between the DENV2 and DENV4 serotypes when the interval between the first and second viral infection was increased. In the present study, the virus infected first in the C6/36 superinfections consistently had significantly higher LSQ values while there was no significant difference in LSQ between WNV and ZIKV following the co-infection in the same cell line. Perhaps increasing the superinfection time interval in C6/36 cells would have resulted in a greater level of viral interference between the viruses.

Surprisingly, in the C6/36 cells ZIKV was found to attain the highest LSQ values through the ZIKV/WNV SI for the entirety of the experiment compared to the other dual infections as well as the single ZIKV infection (Figure 3.6B). This result has not been previously observed in cell culture, however, vector competence studies have shown that ZIKV may possess an advantage in dual viral infections in the mosquito vector. Following co-infection with ZIKV and DENV, Chaves et al. (2018) found that *Ae. aegypti* mosquitoes were more efficient vectors for ZIKV and that the virus was preferentially transmitted following the co-infection. Similarly, Magalhaes et al. (2018) found that superinfection with CHIKV slightly enhanced ZIKV transmission one week following the second blood meal. The authors suggested that dual viral infections within the mosquito vector may be overwhelming to the mosquito immune system, thereby resulting in less efficient defences (Magalhaes et al., 2018). These studies provide evidence that ZIKV is able to perform well in co-infections and superinfections with other viruses. Interestingly, Vázquez-Calvo et al. (2017) found that mice infected with WNV fourteen days following a previous infection with ZIKV had significantly higher survival rates when compared to mice infected with WNV only. Further

experimentation is required in order to better understand the high levels of viral accommodation observed in the C6/36 cells used in this experiment following the ZIKV and WNV superinfection.

The viral interference results observed in the cellular model may be applicable in the mosquito vector model. Bolling et al. (2012) superinfected C6/36 cells with WNV and the insect-specific flavivirus, *Culex* flavivirus (CxFV), and observed viral interference of WNV but not of CxFV. Following superinfection in live *Cx. pipiens* mosquitoes, samples persistently infected with CxFV had significantly lower WNV dissemination rates at 7 days post-infection, indicating that previous infection with CxFV suppresses early WNV replication in the mosquito vector (Bolling et al., 2012). Similarly, Colmant et al. (2018) observed viral interference of WNV by Bamaga virus in C6/36 cells and live *Culex annulirostris* (Skuse) mosquitoes. Göertz et al. (2017) did not observe viral interference among ZIKV and CHIKV following co-infection in C6/36 or *Ae*. *aegypti* Aag2 cells and subsequently found that vector competence of live *Ae. aegypti* mosquitoes did not change following co-infection with the viruses. Thus, co-infection studies carried out *in vitro* provide a good indication of what may be observed *in vivo*.

This is the first study to investigate co-infection and superinfection for WNV and ZIKV in different cell lines. The results provide the first evidence of superinfection exclusion for these viruses as well as the occurrence of viral interference in the VERO E6 cell line but not in C6/36 cells following dual infections. It is important to continue this investigation in the mosquito model as the potential for co-circulation of ZIKV and WNV increases in North America.

Chapter 4

Discussion

4.1 Discussion

The objectives presented in this dissertation were to investigate the risk for Zika virus (ZIKV) transmission in Southern Ontario as well as the factors that may influence viral infection. These objectives were addressed through a vector competence investigation of local mosquito species following experimental infection with the virus as well as an analysis of ZIKV infection dynamics in host and vector-derived cell lines following simultaneous and sequential exposure to an additional flavivirus.

Despite the fact that autochthonous ZIKV transmission has yet to be reported in Canada, a total of 569 travel-rated cases and 4 sexually-transmitted cases have been reported in the country (Government of Canada, 2018B). Thus, it is important to determine the vectoring capacities of wild mosquitoes in Canada in order to identify the risk for mosquito-borne ZIKV transmission. Vector competence studies investigating ZIKV are scarce in Canada, particularly involving wild-caught mosquito samples. In an effort to provide an indication of the risk for ZIKV transmission by mosquitoes found in Southern Ontario, a vector competence investigation was performed (Chapter 2). Following exposure to a ZIKV-infected blood meal, there was no evidence of viral transmission for wild-caught *Aedes punctor*, *Aedes trivittatus*, *Aedes vexans*, *Anopheles punctipennis*, or *Coquillettidia perturbans* mosquito samples. Despite this observation, viral infection was detected in the bodies of approximately 10% of *Ae. vexans* samples and 33% of *Cq. perturbans* samples. However, the fact that there was no evidence of viral dissemination into the legs or wings indicates that the virus may not be able to overcome the midgut barrier of these mosquito species. Additionaly, following the delivery of a ZIKV-infected sugar meal, *Aedes japonicus* samples were found to be refractory to infection while a low infection rate (IR) (6%) and transmission efficiency (TE) (1%) were exhibited by *Culex pipiens* samples. These are

preliminary results that provide the first indication of the vectoring capabilities of these mosquito species collected from Southern Ontario.

In addition to the wild mosquitoes, the vector competency of colony *Aedes aegypti* and *Aedes albopictus* mosquitoes was also examined through the use of an infected blood meal. While viral infection, dissemination, and transmission were observed for *Ae. aegypti* and *Ae. albopictus*, the associated TE values were low at approximately 2% and 18%, respectively. While these results conflict with previous studies reporting high infection and transmission rates following experimental infection (Ciota et al., 2017; Di Luca et al., 2016; Li et al., 2012; Wong et al., 2013), low levels of transmission have also been observed for these mosquito species (Diagne et al., 2015; Chouin-Carneiro et al., 2016; Jupille et al., 2016). This variation in the literature may be attributable to a number of different factors, such as mosquito population, viral strain, infection period, and incubation conditions.

In addition to vector competence investigations, it is also important to identify the factors that may complicate ZIKV infection and transmission. Reports of simultaneous infection between ZIKV and additional arthropod-borne viruses have been reported in South America in areas where these viruses co-circulate (Waggoner et al., 2016; Carrillo-Hernández et al., 2018; Mercado-Reyes et al., 2019). As cases of ZIKV have also been reported in North America, the potential for co-circulation and subsequently co-infection with North America endemic viruses, such as West Nile virus (WNV), exists. *Ae. aegypti* and *Ae. albopictus* have both been shown to have the potential to act as competent vectors for WNV (Turell et al., 2005) and ZIKV (Ferreira-de-Brito et al., 2016; Grard et al., 2014). The potential range and ability to live and reproduce in the Northeastern USA has been predicted to be “very likely” for *Ae. albopictus* mosqutioes (CDC, 2018D). As this mosquito species has also been established in Windsor, Ontario (Giordano, 2018), the risk of co-

circulation of WNV and ZIKV in Southern Ontario by a competent vector species is feasible. Despite the fact that human co-infections between WNV and ZIKV have not been reported to date, it is important to consider the consequences resulting from such an interaction. In an effort to investigate this relationship, the infection dynamics of simultaneous and sequential exposure to WNV and ZIKV were analyzed in mammalian and insect cells (Chapter 3).

Following the experimental infections, viral interference was detected for both viruses in mammalian cells. The observed interference might be attributable to the increased competition among the viruses for viral replication architectures within available cells (Salas-Benito & De Nova-Ocampo, 2015). In the insect cells, however, viral accommodation was observed as the dual infections either did not result in lower viral titers, in the case of WNV, or achieved titers higher than those attained through single infections, in the case of ZIKV. There is a great deal of variation among published literature investigating multiple flavivirus infections in insect cell lines, indicating that there are many factors, such as viral strain and timing between infections, that may influence the outcome of viral co-infection studies. Additionally, it remains unclear whether co-infections with multiple flaviviruses have the potential to enhance disease severity in human cases thus, it is important to further this research as ZIKV continues its northern expansion.

In conclusion, the results presented in this thesis add to the growing body of information concerning ZIKV and the risk for viral transmission in North America. The vector competence results indicate that the wild mosquitoes collected in Southern Ontario are not likely to act as competent vectors for the virus. These results provide relevant information in regards to the possibility for autochthonous ZIKV transmission in Canada and are in agreement with previous literature reporting poor vector competence for North American mosquito species. Additionally, viral interference was observed in host-derived cells following the dual flavivirus infection assays

but not in vector-derived cells. The differences in viral growth kinetics between the cell lines may provide an indication of what may be observed in the host and vector model following dual flavivirus infections. The implementation of ongoing mosquito surveillance and preventative measures is imperative to prevent local ZIKV transmission in North America.

4.2 Limitations

As a result of the hot and dry conditions encountered in the summer of 2016 during the wild mosquito collection period, the sample sizes for certain wild-caught mosquito species were very low. The reluctance of the wild mosquitoes to feed from the infected blood meal further reduced the sample numbers as did the high mortality rates observed during the viral dissemination period. Bloodfeeding is a very complex process where certain mosquito species might exhibit high levels of specificity for the host from which the blood meal is acquired (Clements, 1992). Low sample sizes were likely observed due to the difficulties associated with mimicking the appropriate host and environmental cues in a Containment Level 3 (CL3) laboratory setting. Only one sample from *Ae. punctor*, *Ae. trivittatus*, and *An. punctipennis* survived the virus dissemination period while *Cq. perturbans* had a sample size of three. Thus, these species should not be disregarded as potential vectors for ZIKV until they are re-evaluated with a larger sample size. While the ISM was implemented as a possible solution to the low success rate observed with bloodfeeding, this method of infection does not adequately portray the process by which the mosquito vector may be infected with the virus in nature. Furthermore, female mosquitoes may divert a portion of their sugar meal from the midgut to the crop as a means of energy storage (Clements, 1992). Thus, virions ingested through a sugar meal may have potentially been diverted to the crop and disseminated into the haemolymph, effectively avoiding the midgut barrier to infection. In order

to avoid this, only those mosquitoes whose abdomens were visibly distended and green, due to the food colouring included in the ISM, were selected for further testing. However, the vectoring potential for *Cx. pipiens* should be re-evaluated through the use of an infected blood meal in order to help validate the results observed using the ISM.

While it was clear that WNV and ZIKV were able to replicate in both mammalian and insect cell lines following dual infections, the infectivity of the viruses was not determined. The gold standard for the detection and quantification of infectious virus particles is the plaque assay method (Agbulos et al., 2016). However, as WNV and ZIKV are both flaviviruses, the plaques formed by each virus would not be distinguishable from one another. Thus, a standard curve was generated as a method to compare the viruses and infectivity was detected through the observation of notable cytopathic effect in the infected cell cultures.

4.3 Future Prospects

This vector competence investigation involved only 7 of the 68 known mosquito species that are native to the province of Ontario (Giordano, 2018). Future studies investigating the vector competence of Canadian mosquitoes should utilize an expanded range when trapping wild mosquitoes in an effort to acquire a more diverse sample of mosquito species. Additionally, while the ZIKV Thai 2013 strain was utilized in the present study, it would also be beneficial to repeat this investigation using the PRVABC59 strain of the virus that was isolated in Puerto Rico in 2015. This is because the PRVABC59 strain has been associated with the recent ZIKV epidemic reported in South America and is therefore likely to be the strain that is currently circulating in the Americas (Lanciotti et al., 2016). As previously mentioned, there are many factors that may impact vector competence, the viral strain being one such factor. It may be that the mosquito species local to

Southern Ontario show a higher susceptibility to a different strain of virus. In addition, it would be beneficial to examine the infection and transmission capabilities of wild mosquitoes using live animal models in subsequent investigations in order to analyze the vectoring capabilities of the mosquito species under more natural and relevant conditions.

In this study, the viral dynamics following dual WNV and ZIKV infections were analyzed for the first time in host and vector-derived cell lines. In an effort to add to the results acquired through this investigation, future studies should examine the effects of different viral strains, additional cell lines, and variable time periods between sequential infections. Additionally, it is important to determine whether the results observed in the vector-derived cell line will also be applicable within the vector model through additional vector competence investigations analyzing the simultaneous transmission of both viruses. Finally, as the clinical manifestations following co-infections are not well-characterized, simultaneous infections in live animal models may provide an indication as to how disease severity may be potentially impacted.

Literature Cited

Abrao, E. P., & B. A. da Fonseca. 2016. Infection of mosquito cells (C6/36) by dengue-2 virus interferes with subsequent infection by yellow fever virus. Vector Borne Zoonotic Dis. 16: 124-130.

Agbulos, D. S., L. Barelli, B. V. Giordano, & F. F. Hunter. 2016. Zika virus: quantification, propagation, detection, and storage. Curr Protoc Microbiol. 43: 15D.4.1-15D.4.16.

Aliota, M. T., S. A. Peinado, J. E. Osorio, & L. C. Bartholomay. 2016. *Culex pipiens* and *Aedes triseriatus* mosquito susceptibility to Zika virus. Emerging Infect. Dis. 22: 1857-1859.

Alto, B. W., L. P. Lounibos, S. Higgs, & S. A. Juliano. 2005. Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes. Ecology. 86: 3279-3288.

Amraoui, F., C. Atyame-Nten, A. Vega-Rúa, R. Lourenço-de-Oliveira, M. Vazeille, & A. B. Failloux. 2016. *Culex* mosquitoes are experimentally unable to transmit Zika virus. Euro. Surveill. 21: 30333.

Apperson, C. S., H. K. Hassan, B. A. Harrison, H. M. Savage, S. E. Aspen, A. Farajollahi, W. Crans, T. J. Daniels, R. C. Falco, M. Benedict, M. Anderson, L. McMillen, & T. R. Unnasch. 2004. Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. Vector Borne Zoonotic Dis. 4: 71-82.

Armah, H. B., G. Wang, B. I. Omalu, R. B. Tesh, K. A. Gyure, D. J. Chute, R. D. Smith, P. Dulai, H. V. Vinters, B. K. Kleinschmidt-DeMasters, & C. A. Wiley. 2007. Systemic distribution of West Nile virus infection: post-mortem immunohistochemical study of six cases. Brain Pathol. 17: 354-362.

Atkinson, B., P. Hearn, B. Afrough, S. Lumely, D. Carter, E. J. Aarons, A. J. Simpson, T. J.

Brooks, & R. Hewson. 2016. Detection of Zika virus in semen. Emerging Infect. Dis. 22: 940.

Azar, S. R., C. M. Roundy, S. L. Rossi, J. H. Huang, G. Leal, R. Yun, I. Fernandez-Salas, C. J. Vitek, I. A. D. Paploski, P. M. Stark, J. Vela, M. Debboun, M. Reyna, U. Kitron, G. S. Ribeiro, K. A. Hanley, N. Vasilakis, & S. C. Weaver. 2017. Differential vector competency of *Aedes albopictus* populations from the Americas for Zika virus. Am. J. Trop. Med. Hyg. 97: 330-339.

Bakonyi, T., Z. Hubálek, I. Rudolf, & N. Nowotny. 2005. Novel flavivirus or new lineage of West Nile virus, Central Europe. Emerging Infect. Dis. 11: 225-231.

Baron, J., L. Youngblood, M. F. Siewers, D. N. Medearis. 1969. The incidence of cytomegalovirus, herpes simplex, rubella, and toxoplasma antibodies in microcephalic, mentally retarded, and normocephalic children. Pediatrics. 44: 932-939.

Barzon, L., M. Pacenti, A. Berto, A. Sinigaglia, E. Franchin, E. Lavezzo, P. Brugnaro, & G. Palù. 2016. Isolation of infectious Zika virus from saliva and prolonged viral RNA shedding in a traveller returning from the Dominican Republic to Italy, January 2016. Euro Surveill. 21: 30159.

Bernkopf, H., S. Levine, & R. Nerson. 1953. Isolation of West Nile virus in Israel. J. Infect. Dis. 93: 207-218.

Berthet, N., E. Nakouné, B. Kamgang, B. Selekon, S. Descorps-Declière, A. Gessain, J. C. Manuguerra, & M. Kazanji. 2014. Molecular characterization of three Zika flaviviruses obtained from sylvatic mosquitoes in the Central African Republic. Vector Borne Zoonotic Dis. 14: 862-865.

Besnard, M., S. Lastere, A. Teissier, V. Cao-Lormeau, & D. Musso. 2014. Evidence of perinatal

transmission of Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill. 19: 20751.

Bolling, B. G., F. J. Olea-Popelka, L. Eisen, C. G. Moore, & C. D. Blair. 2012. Transmission dynamics of an insect-specific flavivirus in a naturally infected *Culex pipiens* laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology. 427: 90-97.

Broom, A. K., J. Charlick, S. J. Richards, & J. S. Mackenzie. 1987. An enzyme-linked immunosorbent assay for detection of flavivirus antibodies in chicken sera. J. Virol. Methods. 15: 1-9.

Bunning, M. L., R. A. Bowen, B. C. Cropp, K. G. Sullivan, B. S. Davis, N. Komar, M. Godsey, D. Baker, D. L. Hettler, D. A. Holmes, B. J. Biggerstaff, & C. J. Mitchell. 2002. Experimental infection of horses with Wet Nile virus. Emerging Infect. Dis. 8: 380-386.

Campbell, L. P., C. Luther, D. Moo-Llanes, R. Danis-Lozano, & A.T. Peterson. 2015. Climate change influences on global distributions of dengue and chikungunya virus vectors. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 370: 20140135.

Campos, G. S., A. C. Bandeira, & S. I. Sardi. 2015. Zika virus outbreak, Bahia, Brazil. Emerging Infect. Dis. 21: 1885-1886.

Cao-Lormeau, V. M., C. Roche, A. Teissier, E. Robin, A. L. Berry, H. P. Mallet, A. A. Sall, & D. Musso. 2014. Zika virus, French Polynesia, South Pacific, 2013. Emerging Infect. Dis. 20: 1085-1086.

Cao-Lormeau, V. M., A. Blake, S. Mons, S. Lastere, C. Roche, J. Vanhomwegen, T. Dub, L. Baudouin, A. Teissier, P. Larre, A. L. Vial, C. Decam, V. Choumet, S. K. Halstead, H. J. Willison, L. Musset, J. C. Manuguerra, P. Despres, E. Fournier, H. P. Mallet, D. Musso, A.

Fontanet, J. Neil, & F. Ghawché. 2016. Guillain-Barré syndrome outbreak associated with Zika virus infection in French Polynesia: a case-control study. Lancet. 387: 1531-1539.

Carrillo-Hernández, M. Y., J. Ruiz-Saenz, L. J. Villamizar, S. Y. Gómez-Rangel, & M. Martínez-Gutierrez. 2018. Co-circulation and simultaneous co-infection of dengue, chikungunya, and Zika viruses in patients with febrile syndrome at the Colombian-Venezuelan border. BMC Infect. Dis. 18: 61.

Carteaux, G., M. Maquart, A. Bedet, D. Contou, P. Brugières, S. Fourati, L. C. de Langavant, T. de Broucker, C. Brun-Buisson, I. Leparc-Goffart, & A. M. Dessap. 2016. Zika virus associated with meningoencephalitis. N. Engl. J. Med. 374: 1595-1596.

Cauchemez, S., M. Besnard, P. Bompard, T. Dub, P. Guillemette-Artur, D. Eyrolle-Guignot, H. Salje, M. D. Van Kerkjove, V. Abadie, C. Garel, A. Fontanet, & H. P. Mallet. 2016. Association between Zika virus and microcephaly in French Polynesia, 2013-15: a retrospective study. Lancet. 387: 2125-2132.

Causarano, J. 2017. Secondary transmission dynamics of the West Nile virus in mosquitoes. M.S. dissertation, Brock University, Ontario.

Cavalheiro, S., A. Lopez, S. Serra, A. Da Cunha, M. D. da Costa, A. Moron, & H. M. Lederman. 2016. Microcephaly and Zika virus: neonatal neuroradiological aspects. Childs Nerv Syst. 32: 1057-1060.

Centers for Disease Control and Prevention. 2002A. West Nile virus activity – United States, 2001. MMWR Morb. Mortal. Wkly. Rep. 51: 497-501.

Centers for Disease Control and Prevention. 2002B. Provincial surveillance summary of the West Nile virus epidemic – United States, January – November 2002. MMWR Morb. Mortal. Wkly. Rep. 51: 1135-1136.

Centers for Disease Control and Prevention. 2002C. Intrauterine West Nile virus infection – New York, 2002. MMWR Morb. Mortal. Wkly. Rep. 51: 1129-1133.

Centers for Disease Control and Prevention. 2002D. Laboratory-acquired West Nile virus infections – United States, 2002. MMWR Morb. Mortal. Wkly. Rep. 51: 1133-1135.

Centers for Disease Control and Prevention. 2002E. Possible West Nile virus transmission to an infant through breast-feeding – Michigan, 2002. MMWR Morb. Mortal. Wkly. Rep. 51: 877-878.

Centers for Disease Control and Prevention. 2003. West Nile virus activity – Untied States, October 16-22, 2003. MMWR Morb. Mortal. Wkly. Rep. 52: 1253-1256.

Centers for Disease Control and Prevention. 2005. West Nile virus activity – Untied States, January 1-December 1, 2005. MMWR Morb. Mortal. Wkly. Rep. 54: 1022-1023.

Centers for Disease Control and Prevention. 2016. Mosquito species in which West Nile virus has been detected, United States, 1999-2016. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 28, 2019 from: https://www.cdc.gov/westnile/resources/pdfs/MosquitoSpecies1999-2016.pdf.

Centers for Disease Control and Prevention. 2018A. West Nile virus. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 4, 2019 from: https://www.cdc.gov/westnile/index.html.

Centers for Disease Control and Prevention. 2018B. Final cumulative maps & data for 1999-2017. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 5, 2019 from: https://www.cdc.gov/westnile/statsmaps/cumMapsData.html#one.

Centers for Disease Control and Prevention. 2018C. Chikungunya virus. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 27, 2019 from:

https://www.cdc.gov/chikungunya/index.html.

Centers for Disease Control and Prevention. 2018D. Potential range in US. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved August 18, 2019 from: https://www.cdc.gov/zika/vector/range.html.

Centers for Disease Control and Prevention. 2019A. Zika virus. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved February 25, 2019 from: https://www.cdc.gov/zika/index.html.

Centers for Disease Control and Prevention. 2019B. 2018 case counts in the US. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved February 24, 2019 from: https://www.cdc.gov/zika/reporting/2018-case-counts.html.

Centers for Disease Control and Prevention. 2019C. 2019 case counts in the US. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 25, 2019 from: https://www.cdc.gov/zika/reporting/2019-case-counts.html.

Centers for Disease Control and Prevention. 2019D. Dengue. Atlanta, GA: Centers for Disease Control and Prevention. Retrieved May 27, 2019 from: https://www.cdc.gov/dengue/.

Chambers, T. J., C. S. Hahn, R. Galler, & C. M. Rice. 1990. Flavivirus genome, organization, expression, and replication. Annu. Rev. Microbiol. 44: 649-688.

Champagne, D. E., & J. M. Ribeiro. 1994. Sialokinin I and II: vasodilatory tachykinins from the yellow fever mosquito *Aedes aegypti*. Proc. Natl. Acad. Sci. U.S.A. 91: 138-142.

Chancey, C., A. Grinev, E. Volkova, & M. Rios. 2015. The global ecology and epidemiology of West Nile virus. Biomed Res Int. 2015: 1-20.

Chaves, B. A., A. S. Orfano, P. M. Nogueira, N. B. Rodrigues, T. B. Campolina, R. Nacif-Pimenta, A. C. A. M. Pires, A. B. V. Júnior, A. D. C. Paz, E. B. D. C. Vaz, M. D. G. V. B. Guerra, B.

M. Silva, F. F. de Melo, D. E. Norris, M. V. G. de Lacerda, P. F. P. Pimenta, & N. F. C. Secundino. 2018. Coinfeciton with Zika virus (ZIKV) and dengue virus results in preferential ZIKV transmission by vector bite to vertebrate host. J. Infect. Dis. 218: 563-571.

Chen, Y., T. Maguire, R. M. Marks. 1996. Demonstration of binding of dengue virus envelope protein to target cells. J. Virol. 70: 8765-8772.

Chen, Y., T. Maguire, R. E. Hileman, J. R. Fromm, J. D. Esko, R. J. Linhardt, & R. M. Marks. 1997. Dengue virus infectivity depends on envelope protein binding to target cell heparin sulfate. Nat. Med. 3: 866-871.

Chouin-Carneiro, T. A. Vega-Rua, M. Vazeille, A. Yebakima, R. Girod, D. Goindin, M. Dupont-Rouzeyrol, R. Lourenço-de-Oliveira, & A. B. Failloux. 2016. Differential susceptibilities of *Aedes aegypti* and *Aedes albopictus* from the Americas to Zika virus. PLoS Negl. Trop. Dis. 10: 1-11.

Ciota, A. T., S. M. Bialosuknia, S. D. Zink, M. Brecher, D. J. Ehrbar, M. N. Morrissette, & L. D. Kramer. 2017. Effects of Zika virus strain and *Aedes* mosquito species on vector competence. Emerging Infect. Dis. 23: 1110-1117.

Clarke, D. H., & J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med Hyg. 7: 561-573.

Clements, A. N. 1992. The biology of mosquitoes. Volume 1: Development, nutrition and reproduction. Chapman & Hall, London, UK.

Colmant, A. M. G., S. Hall-Mendelin, S. A. Ritchie, H. Bienlefeldt-Ohmann, J. J. Harrison, N. D. Newton, C. A. O'Brien, C. Cazier, C. A. Johansen, J. Hobsen-Peters, R. A. Hall, & A. F. van den Hurk. 2018. The recently identified flavivirus Bamaga virus is transmitted horizontally by *Culex* mosquitoes and interferes with West Nile virus replication *in vitro* and

transmission *in vivo*. PLoS Negl Trop Dis. 21: e0006886.

Cox, B. D., R. A. Stanton, & R. F. Schinazi. 2015. Predicting Zika virus structural biology: challenges and opportunities for intervention. Antivir. Chem. Chemother. 24: 118-126.

Crowther, J. R. 2000. The ELISA guidebook. Methods Mol. Biol. 149: 1-413.

Danis, K., A. Papa, G. Theocharopoulos, G. Dougas, M. Athanasiou, M. Detsis, A. Baka, T. Lystras, K. Mellou, S. Bonovas, & T. Panagiotopoulos. 2011. Outbreak of West Nile virus in Greece, 2010. Emerging Infect. Dis. 17: 1868-1872.

Darwish, M. A., H. Hoogstraal, T. J. Roberts, I. P. Ahmed, & F. Omar. 1983. A sero-epidemiological survey for certain arboviruses (Togaviridae) in Pakistan. Trans. R. Soc. Trop. Med. Hyg. 77: 442-445.

de Araújo, T. V. B., L. C. Rodrigues, R. A. A. Ximenes, D. B. Miranda-Filho, U. R. Montarroyos, A. P. L. de Melo, S. Valongueiro, M. F. P. M. de Albuquerque, W. V. Souza, C. Braga, S. P. B. Filho, M. T. Cordeiro, E. Vazquez, D. C. S. Cruz, C. M. P. Henriques, L. C. A Bezerra, P. M. S. Castanha, R. Dhalia, E. T. A. Marques-Júnior, & C. M. T. Martelli. 2016. Association between Zika virus infection and microcephaly in Brazil, January to May, 2016: preliminary report of a case-control study. Lancet Infect Dis. 16: 1356-1363.

De Smet, B., D. Van den Bossche, C. van de Werve, J. Mairesse, J. Schmidt-Chanasit, J. Michiels, K. K. Ariën, M. Van Esbroeck, & L. Cnops. 2016. Confirmed Zika virus infection in a Belgian traveler returning from Guatemala, and the diagnostic challenges of imported cases into Europe. J. Clin. Virol. 80: 8-11.

Deckard, D. T., W. M. Chung, J. T. Brooks, J. C. Smith, S. Woldai, M. Hennessey, N. Kwit, & P. Mead. 2016. Male-to-male sexual transmission of Zika virus – Texas, January 2016. MMWR Morb. Mortal. Weekly. Rep. 65: 372-374.

Di Luca, M. D., F. Severini, L. Toma, D. Boccolini, R. Romi, M. E. Remoli, M. Sabbatucci, C. Rizzo, G. Venturi, G. Rezza, & C. Fortuna. 2016. Experimental studies of susceptibility of Italian *Aedes albopictus* to Zika virus. Euro Surveill. 21: 30233.

Diagne, C. T., D. Diallo, O. Faye, Y. Ba, O. Faye, A. Gaye, I. Dia, O. Faye, S. C. Weaver, A. A. Sall, & M. Diallo. 2015. Potential of selected Senegalese *Aedes spp.* mosquitoes (*Diptera*: *Culicidae*) to transmit Zika virus. BMC Infect. Dis. 15: 492.

Diallo, D., A. A. Sall, C. T. Diagne, O. Faye, O. Faye, Y. Ba, K. A. Hanley, M. Beunemann, S. C. Weaver, & M. Diallo. 2014. Zika virus emergence in mosquitoes in southeastern Senegal, 2011. PLoS One. 9: e109442.

Díaz-Menéndez, M., F. de la Calle-Prieto, D. Montero, E. Antolín, A. Vazquez, M. Arsuaga, E. Trigo, S. García-Bujalance, M. de la Calle, P. S. Seco, F. de Ory, & J. R. Arribas. 2018. Initial experience with imported Zika virus infected in Spain. Enferm. Infecc. Microbiol. Clin. 36: 4-8.

Díaz-Quiñonez, J. A., I. López-Martínez, B. Torres-Longoria, M. Vázquez-Pichardo, E. Cruz-Ramírez, J. E. Ramírez-González, C. Ruiz-Matus, & P. Kuri-Morales. 2016. Evidence of the presence of the Zika virus in Mexico since early 2015. Virus Genes. 52: 855-857.

Dibernardo, A., M. J. Turell, R. Lindsay, C. Loomer, & M. Iranpour. 2017. Vector competence of some mosquito species from Canada for Zika virus. J. Am. Mosq. Control. Assoc. 33: 276-281.

Dick, G. W. A., S. F. Kitchen, & A. J. Haddow. 1952. Zika virus (I). Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46: 509-520.

Dodson, B. L. & J. L. Rasgon. 2017. Vector competence of *Anopheles* and *Culex* mosquitoes for Zika virus. PeerJ. 14: e3096.

Duchemin, J., P. T. Mee, S. E. Lynch, R. Vedururu, L. Trinidad, & P. Paradkar. 2017. Zika vector transmission risk in temperate Australia: a vector competence study. Virol. J. 14: 108.

Duffy, M. R., T. H. Chen, W. T. Hancock, A. M. Powers, J. L. Jool, R. S. Lanciotti, M. Pretrick, M. Marfel, S. Holzbauer, C. Dubray, L. Guillaumot, A. Griggs, M. Bel, A. J. Lambert, J. Laven, O. Kosoy, A. Panella, B. J. Biggerstaff, M. Fischer, & E. B. Hayes. 2009. Zika virus outbreak on Yap Island, Federated States of Micronesia. N. Engl. J. Med. 360: 2536-2543.

Dupont-Rouzeyrol, M., O. O'Connor, E. Calvez, M. Duarès, M. John, J. P. Grangeon, & A. C. Gourinat. 2015. Co-infection with Zika and dengue viruses in 2 patients, New Caledonia, 2014. Emerging Infect. Dis. 21: 381-382.

Dupont-Rouzeyrol, M., A. Biron, O. O'Connor, E. Huguon, & E. Descloux. 2016. Infectious Zika viral particles in breastmilk. Lancet. 387: 1051.

Ebel, G. D., I. Rochlin, J. Longacker, & L. D. Kramer. 2005. *Culex restuans* (Diptera: Culicidae) relative abundance and vector competence for West Nile virus. J. Med. Entomol. 42: 838-843.

Elizondo-Quiroga, D., A. Medina-Sánchez, J. M. Sánchez-González, K. A. Eckert, E. Villalobos-Sánchez, A. R. Navarro-Zúñiga, G. Sánchez-Tejeda, F. Correa-Morales, C. González-Acosta, C. F. Arias, S. López, R. M. Del Ángel, V. Pando-Robles, & A. E. Elizondo-Quiroga. 2018. Zika virus in salivary glands of five different species of wild-caught mosquitoes from Mexico. Sci. Rep. 8: 809.

European Centre for Disease Prevention and Control. 2015. Zika virus epidemic in the Americas: potential association with microcephaly and Guillain-Barré syndrome. Stockholm, Sweden: European Centre for Disease Prevention and Control. Retrieved February 24, 2019 from https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/zika-virus-

americas-association-with-microcephaly-rapid-risk-assessment.pdf.

European Centre for Disease Prevention and Control. 2016. Factsheet about Zika virus disease. Stockholm, Sweden: European Centre for Disease Prevention and Control. Retrieved October 10, 2018 from https://ecdc.europa.eu/en/zika-virus-infection/facts/factsheet.

Fagbami, A. H. 1979. Zika virus infections in Nigeria: virological and seroepidemiological investigations in Oyo State. J Hyg (Lond). 83: 213-219.

Farajollahi, A., S. P. Healy, I. Unlu, R. Gaugler, & D. M. Fonseca. 2012. Effectiveness of ultra-low volume nighttime applications of an adulticide against diurnal *Aedes albopictus*, a critical vector of dengue and chikungunya viruses. PLoS ONE. 7: e49181.

Fernandez-Garcia, M. D., M. Mazzon, M. Jacobs, & A. Amara. 2009. Pathogenesis of flavivirus infections: using and abusing the host cell. Cell Host Microbe. 5: 318-328.

Ferreira-de-Brito, A., I. P. Ribeiro, R. M. Miranda, R. S. Fernandes, S. S. Campos, K. A. Silva, M. G. Castro, M. C. Bonaldo, P. Brasil, & R. Lourenço-de-Oliveira. 2016. First detection of natural infection of *Aedes aegypti* with Zika virus in Brazil and throughout South America. Mem. Inst. Oswaldo Cruz. 111: 655-658.

Fonseca, K., B. Meatherall, D. Zarra, M. Drebot, J. MacDonald, K. Pabbaraju, S. Wong, P. Webster, R. Lindsay, & R. Tellier. 2014. First case of Zika virus infection in a returning Canadian traveler. Am. J. Trop. Med. Hyg. 91: 1035-1038.

Forrester, N. L., L. L. Coffey, & S. C. Weaver. 2014. Arboviral bottlenecks and challenges to maintaining diversity and fitness during mosquito transmission. Viruses. 6: 3991-4004.

Foy, B. D., K. C. Kobylinski, J. L. C. Foy, B. J. Blitvich, A. T. da Rosa, A. D. Haddow, R. S. Lanciotti, & R. B. Tesh. 2011. Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg. Infect. Dis. 17: 880-882.

Francischetti, I. M., J. G. Valenzuela, & J. M. Ribeiro. 1999. Anophelin: kinetics and mechanisms of thrombin inhibition. Biochemistry. 38: 16678-16685.

Franz, A. W. E., A. M. Kantor, A. L. Passarelli, & R. J. Clem. 2015. Tissue barriers to arbovirus infection in mosquitoes. Viruses. 7: 3741-3767.

Gendernalik, A., J. Weger-Lucarelli, S. M. G. Luna, J. R. Fauver, C. Rückert, R. A. Murrieta, N. Bergren, D. Samaras, C. Nguyen, R. C. Kading, & G. D. Ebel. 2017. American *Aedes vexans* mosquitoes are competent vectors of Zika virus. Am. J. Trop. Med. Hyg. 96: 1338-1340.

Geser, A., B. E. Henderson, & S. Christensen. 1970. A multipurpose serological survey in Kenya. 2. Results of arbovirus serological tests. Bull. World Health Organ. 43: 539-552.

Gillespie, L. K., A. Hoenen, G. Morgan, & J. M. Mackenzie. 2010. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. J. Virol. 84: 10438-10447.

Giordano, B. V. 2018. Transmission dynamics and epidemiology of West Nile virus in Ontario, Canada. Ph.D. dissertation, Brock University, Ontario.

Giordano, B. V., A. Gasparotto, & F. F. Hunter. 2015. A checklist of the 67 mosquito species of Ontario, Canada. J. Am. Mosq. Control Assoc. 31: 101-103.

Göertz, G. P., C. B. F., Vogels, C. Geertsema, C. J. M. Koenraadt, & G. P. Pijlman. 2017. Mosquito co-infection with Zika and chikungunya virus allows simultaneous transmission without affecting vector competence of *Aedes aegypti*. PLoS Negl Trop Dis. 11: e0005654.

Government of Canada. 2018A. Surveillance of West Nile virus. Government of Canada. Retrieved May 5, 2019 from: https://www.canada.ca/en/public-health/services/diseases/west-nile-virus/surveillance-

west-nile-virus.html.

Government of Canada. 2018B. Surveillance of Zika. Government of Canada. Retrieved February 24, 2019 from: https://www.canada.ca/en/public-health/services/diseases/zika-virus/surveillance-zika-virus.html?id=zikacases%20-%20s1.

Government of Canada. 2018C. Zika virus: symptoms and treatment. Government of Canada. Retrieved October 10, 2018 from: https://www.canada.ca/en/public-health/services/diseases/zika-virus.html.

Grard, G., M. Caron, I. M. Mombo, D. Nkoghe, S. M. Ondo, D. Jiolle, D. Fontenille, C. Paupy, & E. M. Leroy. 2014. Zika virus in Gabon (Central Africa) – 2007: a new threat from *Aedes albopictus*? PLoS Negl Trop Dis. 8: e2681.

Guant, M. W., A. A. Sall, X. de Lamballerie, A. K. Falconar, T. I. Dzhivanian, & E. A. Gould. 2001. Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. J. Gen. Virol. 82: 1867-1876.

Guedes, D. R., M. H. Paiva, M. M. Donato, P. P. Barbosa, L. Krokovsky, S. W. D. S. Rocha, K. Saraiva, M. M. Crespo, T. M. Rezende, G. L. Wallau, R. M Barbosa, C. M. Oliveira, M. A. Melo-Santos, L. Pena, M. T. Cordeiro, R. F. O. Franca, A. L. Oliveira, C. A. Peixoto, W. S. Leal, & C. F. Ayres. 2017. Zika virus replication in the mosquito *Culex quinquefasciatus* in Brazil. Emerg Microbes Infect. 9: 69.

Guo, X. X., C. X. Li, Y. Q. Deng, D. Xing, Q. M. Liu, Q. Wu, A. J. Sun, Y. D. Dong, W. C. Cao, C. F. Qin, & T. Y. Zhao. 2016. *Culex pipiens quinquefasciatus*: a potential vector to transmit Zika virus. Emerg. Microbes Infect. 7: 102.

Gyurech, D., J. Schilling, J. Schmidt-Chanasit, P. Cassinotti, F. Kaeppeli, & M. Dobec. 2016.

False positive dengue NS1 antigen test in a traveller with an acute Zika virus infection imported into Switzerland. Swiss Med Wkly. 146: w14296.

Hammon, W. M., W. D. Schrack, & G. E. Sather. 1958. Serological survey for a arthropod-borne virus infections in the Philippines. Am. J. Trop. Med. Hyg. 7: 323-328.

Hardy, J. L., E. J. Houk, L. D. Kramer, & W. C. Reeves. 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu. Rev. Entomol. 28: 229-262.

Harrison, S. C. 2008. Viral membrane fusion. Nat. Struct. Mol. Biol. 15: 690-698.

Hart, C. E., C. M. Roundy, S. R. Azar, J. H. Huang, R. Yun, E. Reynolds, G. Leal, M. R. Nava, J. Vela, P. M. Stark, M. Debboun, S. Rossi, N. Vasilakis, S. Thangamani, & S. C. Weaver. 2017. Zika virus vector competency of mosquitoes, Gulf Coast, United States. Emerging Infect. Dis. 23: 559-560.

Heang, V., C. Y. Yasuda, L. Sovann, A. D. Haddow, A. P. T. da Rosa, R. B. Tesh, & M. R. Kasper. 2012. Zika virus infection, Cambodia, 2010. Emerging Infect. Dis. 18: 349-351.

Huang, Y. J., V. B. Ayers, A. C. Lyons, I. Unlu, B. W. Alto, L. W. Cohnstaedt, S. Higgs, & D. L. Vanlandingham. 2016. *Culex* species mosquitoes and Zika virus. Vector Borne Zoonotic Dis. 16: 673-676.

Iwamoto, M., D. B. Jernigan, A. Guasch, M. J. Trepka, C. G. Blackmore, W. C. Hellinger, S. M. Pham, S. Zaki, R. S. Lanciotti, S. E. Lance-Parker, C. A. DiazGranados, A. G. Winquist, C. A. Perlino, S. Wiersma, K. L. Hillyer, J. L. Goodman, A. A. Marfin, M. E. Chamberland, & L. R. Petersen. 2003. Transmission of West Nile virus from an organ donor to four transplant recipients. N. Engl. J. Med. 348: 2196-2203.

Jansen, S., A. Heitmann, R. Lühken, H. Jöst, M. Helms, O. Vapalahti, J. Schmidt-Chanasit, & E. Tannich. 2018. Experimental transmission of Zika virus by *Aedes japonicus japonicus*

from southwestern Germany. Emerg. Microbes Infect. 7:192.

Joseph, S. R. (1970). Fruit feeding of mosquitoes in nature. Proc. N. J. Mosq. Exterm. Ass. 57: 125-131.

Jouannic, J. M., S. Friszer, I. Leparc-Goffart, C. Garel, & D. Eyrolle-Guignot. 2016. Zika virus infection in French Polynesia. Lancet. 387: 1051-1052.

Jupille, H., G Seixas, L. Mousson, C. A. Sousa, & A. B. Failloux. 2016. Zika virus, a new threat for Europe?. PLoS Negl. Trop. Dis. 10: e0004901.

Kaushik, A., S. Tiwari, R. D. Jayant, A. Vashist, R. Nikkhah-Moshaie, N. El-Hage, & M. Nair. 2017. Electrochemical biosensors for early stage Zika diagnostics. Trends Biotechnol. 35: 308-317.

Kathong, N., N. Khemnu, S. N. Pattanakitsakul, P. Malasit, & T. W. Flegel. 2010. Persistent, triple-virus co-infections in mosquito cells. BMC Microbiol. 10: 14.

Kenney, J. L. H. Romo, N. K. Duggal, W. Tzeng, K. L. Burkhalter, A. C. Brault, & H. M. Savage. 2017. Transmission incompetence of *Culex quinquefasciatus* and *Culex pipiens pipiens* from North America for Zika virus. Am. J. Trop. Med. Hyg. 96: 1235-1240.

Komar, N., N. A. Panella, J. E. Burns, S. W. Dusza, T. M. Mascarenhas, & T. O. Talbot. 2001. Serologic evidence for West Nile virus infection in birds in the New York City vicinity during an outbreak in 1999. Emerging Infect. Dis. 7: 621-625.

Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, & M. Bunning. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerging Infect. Dis. 9: 311-322.

Krow-Lucal, E. R., B. J. Biggerstaff, & J. E. Staples. 2017. Estimated incubation period for Zika virus disease. Emerging Infect. Dis. 23: 841-844.

Kutsuna, S., Y. Kato, T. Takasaki, M. Moi, A. Kotaki, H. Uemura, T. Matono, Y. Fujiya, M. Mawatari, N. Takeshita, K. Hayakawa, S. Kanagawa, & N. Ohmagari. 2014. Two cases of Zika fever imported from French Polynesia to Japan, December 2014 to January 2014. Euro Surveill. 19: 20683.

Lanciotti, R.S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J.H. Scherret, R.A. Hall, J.S. MacKenzie, C.B. Cropp, B. Panigrahy, E. Ostlund, B. Scmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H.M. Savage, W. Stone, T. McNamara, & D.J. Gubler. 1999. Origin of the West Nile Virus responsible for an outbreak of encephalitis in the northeastern United States. Science, 286: 2333-2337.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, & J. T. Roehrig. 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J. Clin. Microbiol. 38: 4066-4071.

Lanciotti, R. S., O. L. Kosoy, J. J. Laven, J. O. Velez, A. J. Lambert, A. J. Johnson, S. M. Stanfield, & M. R. Duffy. 2008. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerging Infect. Dis. 14: 1232-1239.

Lanciotti, R. S., A. J. Lambert, M. Holodniy, S. Saavedra, & C. S. Ldel. 2016. Phylogeny of Zika virus in Western Hemisphere, 2015. Emerging Infect. Dis. 22: 933-935.

Landolt, G. A., H. G. G. Townsend, & D. P. Lunn. 2014. Equine influenza infection. Equine Infectious Diseases. 7: 141-151.

Li, M. I., P. S. Wong, L. C. Ng, & C. H. Tan. 2012. Oral susceptibility of Singapore *Aedes* (*Stegomyia*) *aegypti* (Linnaeus) to Zika virus. PLoS Negl. Trop. Dis. 6: e1792.

Lim, S. M., P. Koraka, A. D. Osterhaus, & B. E. Martina. 2011. West Nile virus: immunity and

pathogenesis. Viruses. 3: 811-828.

Lindenbach, B. D., & C. M. Rice. 1997. *trans*-Complementation of yellow fever virus NS1 reveals a role in early RNA replication. J. Virol. 71: 9608-9617.

MacNamara, F. N. 1954. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans. R. Soc. Trop. Med. Hyg. 48: 139-145.

Magalhaes, T., A. Robison, M. C. Young, W. C. Black, B. D. Foy, G. D. Ebel, & C. Rückert. 2018. Sequential infection of *Aedes aegypti* mosquitoes with chikungunya virus and Zika virus enhances early Zika virus transmission. Insects. 9: E177.

Marchette, N. J., R. Garcia, & A. Rudnick. 1969. Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. Am. J. Trop. Med. Hyg. 18: 411-415.

Marfin, A. A., L. R. Petersen, M. Eidson, J. Miller, J. Hadler, C. Farello, B. Werner, G. L. Campbell, M. Layton, P. Smith, E. Bresnitz, M. Caartter, J. Scaletta, G. Obiri, M. Bunning, R. C. Craven, J. T. Roehrig, K. G. Julian, S. R. Hinten, & D. J. Gubler. 2001. Widespread West Nile virus activity, eastern United States, 2000. Emerging Infect. Dis. 7: 730-735.

Maria, A. T., M. Maquart, A. Makinson, O. Flusin, M. Segondy, I. Leparc-Goffart, V. Le Moing, & V. Foulongne. 2016. Zika virus infections in three travellers returning from South America and the Caribbean respectively, to Montpellier, France, December 2015 to January 2016. Euro Surveill. 21: 30131.

May, F. J., C. T. Davis, R. B. Tesh, & A. D. T. Barrett. 2011. Phylogeography of West Nile virus: from the cradle of evolution in Africa to Eurasia, Australia, and the Americas. J. Virol. 85: 2964-2974.

McIntosh, B. M., P. G. Jupp, I. Dossantos, & G. M. Meenehan. 1976. Epidemics of West Nile and Sindbis viruses in South Africa with *Culex* (*Culex*) *univittatus* Theobald as vector. S. Afr. J.

Sci. 72: 295-300.

McMullen, A. R., H. Albayrak, F. J. May, C. T. Davis, D. W. Beasley, & A. D. Barrett. 2013. Molecular evolution of lineage 2 West Nile virus. J. Gen Virol. 94: 318-325.

Mécharles, S., C. Herrmann, P. Poullain, T. H. Tran, N. Deschamps, G. Mathon, A Landais, S. Breurec, & A. Lannuzel. 2016. Acute myelitis due to Zika virus infection. Lancet. 387: 1481.

Melnick, J. L., J. R. Paul, J. T. Riordan, V. H. Barnett, N. Goldblum, & E. Zabin. 1951. Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. Proc. Soc. Exp. Biol. Med. 77: 661-665.

Mercado-Reyes, M., J. Acosta-Reyes, E. Navarro-Lechuga, S. Corchuelo, A. Rico, E. Parra, N. Tolosa, L. Pardo, M. González, J. Martin-Rodriguez-Hernández, L. Karime-Osorio, M. Ospina-Martinez, H. Rodriguez-Perea, G. Del Rio-Pertuz, & D. Viasus. 2019. Dengue, chikungunya and Zika virus coinfection: results of the national surveillance during the Zika epidemic in Colombia. Epidemiol. Infect. 147: e77.

Miner, J. J., A. Sene, J. M. Richner, A. M. Smith, A. Santeford, N. Ban, J. Weger-Lucarelli, F. Manzella, C. Rückert, J. Govero, K. K. Noguchi, G. D. Ebel, M. S. Diamond, & R. S. Apte. 2016. Zika virus infection in mice causes panuveitis with shedding of virus in tears. Cell Rep. 16: 3208-3218.

Mlakar, J., M. Korva, N. Tul, M. Popović, M. Poljšak-Prijatelj, J. Mraz, M. Kolenc, K. R. Rus, T. V. Vopotnik, V. F. Vodušek, A. Vizjak, J. Pižem, M. Petrovec, & T. A. Županc. 2016. Zika virus associated with microcephaly. N. Engl. J. Med. 374: 951-958.

Molaei, G., A. Farajollahi, J. J. Scott, R. Gaugler, & T. G. Andreadis. 2009. Human bloodfeeding by the recently introduced mosquito, *Aedes japonicus japonicus*, and public health implications. J. Am. Mosq. Control Assoc. 25: 210-214.

Monlun, E., H. Zeller, B. Le Guenno, M. Traoré-Lamizana, J. P. Hervy, F. Adam, L. Ferrara, D. Fontenille, R. Sylla, & M. Mondo. 1993. Surveillance of the circulation of arbovirus of medical interest in the region of eastern Senegal. Bull Soc Pathol Exot. 86: 21-28.

Moser, L. A., B. T. Boylan, F. R. Moreira, L. J. Myers, E. L. Svenson, N. B. Fedorova, B. E. Pickett, & K. A. Bernard. 2018. Growth and adaptation of Zika virus in mammalian and mosquito cells. PLoS Negl Trop Dis. 12: e0006880.

Mukhopadhyay, S., R. J. Kuhn, & M. G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. Nat. Rev. Microbiol. 3: 13-22.

Musso, D., C. Roche, E. Robin, T. Nhan, A. Teissier, & V. M. Cao-Lormeau. 2015A. Potential sexual transmission of Zika virus. Emerging Infect. Dis. 21: 359-361.

Musso D., C. Roche, T. X. Nhan, E. Robin, A. Teissier, & V. M. Cao-Lormeau. 2015B. Detection of Zika virus in saliva. J. Clin. Virol. 68: 53-55.

Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, & M. Layton. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. N. Engl. J. Med. 344: 1807-1814.

Nayar, J. K. & D. M. Sauerman. 1975. The effects of nutrition on survival and fecundity in Florida mosquitoes. Part 2. Utilization of blood meal for survival. J. Med. Entomol. 12: 99-103.

O'Donnell, K. L., M. A. Bixby, K. J. Morin, D. S. Bradley, & J. A. Vaughan. 2017. Potential of a northern population of *Aedes vexans* (Diptera: Culicidae) to transmit Zika virus. J. Med. Entomol. 54: 1354-1359.

Oehler, E., L. Watrin, P. Larre, I. Leparc-Goffart, S. Lastere, F. Valour, L. Baudouin, H. Mallet, D. Musso, & F. Ghawche. 2014. Zika virus infection complicated by Guillain-Barré

syndrome – case report, French Polynesia, December 2013. Euro Surveill. 19: 20720.

Okia, N. O., P. V. George, P. M. Tukei, G. W. Kafuko, M. Lule, E. Sekyalo, A. Mukuye. 1971. Arbovirus survey in wild birds in Uganda. East Afr Med J. 48: 725-731.

Okuda, K., A. de Souza Caroci, P. E. Ribolla, A. G. de Bianchi, & A. T. Bijovsky. 2002. Functional morphology of adult female *Culex quinquefasciatus* midgut during blood digestion. Tissue Cell. 34: 210-219.

Ouellette, E. M., H. L. Rosett, N. P. Rosman, & L. Weiner. 1977. Adverse effects on offspring of maternal alcohol abuse during pregnancy. N. Engl. J. Med. 297: 528:530.

Olson, J. G., T. G. Ksiazek, D. J. Gubler, S. I. Lubis, G. Simanjuntak, V. H. Lee, S. Nalim, K. Juslis, & R. See. 1983. A survey for arboviral antibodies in sera of humans and animals in Lombok, Republic of Indonesia. Ann Trop Med Parasitol. 77: 131-137.

Pan American Health Organization/World Health Organization. 2017. Zika – Epidemiological Update. Washington, D.C.: Pan American Health Organization/World Health Organization. Retrieved February 24, 2019 from: https://www.paho.org/hq/dmdocuments/2017/2017-aug-25-phe-epi-update-zika-virus.pdf.

Panthier, R. 1968. Epidemiologie du virus West Nile: etude d'un foyer en Camargue. I. Introduction. Ann. Inst. Pasteur. 114: 519-520.

Pealer, L. N., A. A. Marfin, L. R. Petersen, R. S. Lanciotti, P. L. Page, S. L. Stramer, M. G. Stobierski, K. Signs, B. Newman, H. Kapoor, J. L. Goodman, & M. E. Chamberland. 2003. Transmission of West Nile virus through blood transfusion in the United States in 2002. N. Engl. J. Med. 349: 1236-1245.

Pepin, K. M., K. Lambeth, & K. A. Hanley. 2008. Asymmetric competitive suppression between strains of dengue virus. BMC Microbiol. 8: 28.

Perera, R., M. Khaliq, & R. J. Kuhn. 2008. Closing the door on flaviviruses: entry as a target for antiviral drug design. Antiviral Res. 80: 11-22.

Perera-Lecoin, M., L. Meertens, X. Carnec, & A. Amara. 2014. Flavivirus entry receptors: an update. Viruses. 6: 69-88.

Petersen, L. R., & J. T. Roehrig. 2001. West Nile virus: a reemerging global pathogen. Emerging Infect. Dis. 7: 611-614.

Petersen, L. R., D. J. Jamieson, A. M. Powers, & M. A. Honein. 2016. Zika virus. N. Engl. J. Med. 374: 1552-1563.

Phoo, W. W., Y. Li, Z. Zhang, M. Y. Lee, Y. R. Loh, Y. B. Tan, E. Y. Ng, J. Lescar, C. Kang, & D. Luo. 2016. Structure of the NS2B-NS3 protease from Zika virus after self-cleavage. Na Commun. 7: 13410.

Pierson, T. C. & M. S. Diamond. 2012. Degrees of maturity: the complex structure and biology of flaviviruses. Curr Opin Virol. 2: 168-175.

Platonov, A. E., L. S. Karan, T. A. Shopenskaia, M. V. Fedorova, N. M. Koliasnikova, N. M. Rusakova, L. V. Shishkina, T. E. Arshba, V. I. Zhuravlev, M. V. Govorukhina, A. A. Valentseva, & G. A. Shipulin. 2011. Genotyping of West Nile fever virus strains circulating in southern Russia as an epidemiological investigation method: principles and results. Zh. Mikrobiol. Epidemiol. Immunobiol. 2: 29-37.

Pompon, J., R. Morales-Vargas, M. Manuel, C. H. Tan, T. Vial, J. H. Tan, O. M. Sessions, P. C. Vasconcelos, L. C. Ng, & D. Missé. 2017. A Zika virus from America is more efficiently transmitted than an Asian virus by *Aedes aegypti* mosquitoes from Asia. Sci. Rep. 27: 1215.

Pond, W. L. 1963. Arthropod-borne virus antibodies in sera from residents of south-east Asia.

Trans. R. Soc. Trop. Med. Hyg. 57: 364-371.

Prow, N. A., J. H. Edmonds, D. T. Williams, Y. X. Setoh, H. Bieledfeldt-Ohmann, W. W. Suen, J. Hobson-Peters, A. F. van den Hurk, A. T. Pyke, S. Hall-Mendelin, J. A. Northill, C. A. Johansen, D. Warrilow, J. Wang, P. D. Kirkland, S. Doggett, C. C. Andrade, A. C. Brault, A. A. Khromykh & R. A. Hall. 2016. Virulence and evolution of West Nile virus, Australia, 1960-2012. Emerging Infect. Dis. 22: 1353-1362.

Pyke, A. T., M. T. Daly, J. N. Cameron, P. R. Moore, C. T. Taylor, G. R. Hewiston, J. L. Humphreys, & R. Gair. 2014. Imported Zika virus infection from the Cook Islands into Australia, 2014. PLoS Curr. 6.

Ralapanawa, D. M., S. A. Kularatne, & W. A. Jayalath. 2015. Guillain-Barre syndrome following dengue fever and literature review. BMC Res Notes. 27: 729.

Rezza, G., L. Nicoletti, R. Angelini, R. Romi, A. C. Finarelli, M. Panning, P. Cordioli, C. Fortuna, S. Boros, F. Magurano, G. Silvi, P. Angelini, M. Dottori, M. G. Ciufolini, G. C. Majori, & A. Cassone. 2007. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet. 370: 1840-1846.

Ribeiro, J. M., J. J. Sarkis, P. A. Rossignol, & A. Spielman. 1984. Salivary apyrase of *Aedes aegypti*: characterization and secretory fate. Comp. Biochem. Physiol. B. 79: 81-86.

Richard, V., T. Paoaafaite, & V. M. Cao-Lormeau. 2016. Vector competence of French Polynesian *Aedes aegypti* and *Aedes polynesiensis* for Zika virus. PLoS Negl. Trop. Dis. 10: e0005024.

Rioz, D., P. Boussès, F. Simard, C. Paupy, & D. Fontenille. 2015. Autochthonous chikungunya transmission and extreme climate events in southern France. PLoS Negl. Trop. Dis. 9: e0003854.

Robin, Y., & J. Mouchet. 1975. Serological and entomological study on yellow fever in Sierra Leone. Bull Soc Pathol Exot Filiales. 68: 249-258.

Roth, D., B. Henry, S. Mak, M. Fraser, M. Taylor, M. Li, K. Cooper, A. Furnell, Q. Wong, & M. Morshed. 2010. West Nile virus range expansion into British Columbia. Emerging Infect. Dis. 16: 1251-1258.

Roth, A., A. Mercier, C. Lepers, D. Hoy, S. Duituturaga, E. Benyon, L. Guillaumot, & Y. Souares. 2014. Concurrent outbreaks of dengue, chikungunya and Zika virus infections – an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Sureill. 19: 20929.

Roundy, C. M., S. R. Azar, S. L. Rossi, J. H. Huang, G. Leal, R. Yun, I. Fernandez-Salas, C. J. Vitek, I. A. D. Paploski, U. Kitron, G. S. Ribeiro, K. A. Hanley, S. C. Weaver, & N. Vasilakis. 2017. Variation in *Aedes aegypti* mosquito competence for Zika virus transmission. Emerging Infect. Dis. 23: 625-632.

Salaz-Benito, J. S., & M. De Nova-Ocampo. 2015. Viral interference and persistence in mosquito-borne flaviviruses. J Immunol Res. 2015: 873404.

Saluzzo, J. F., J. P. Gonzalez, J. P. Hervé, & A. J. Georges. 1981. Serological survey for the prevalence of certain arboviruses in the human population of the south-east area of Central African Republic. Bull Soc Pathol Exot Filiales. 74: 490-499.

Saluzzo, J. F., B. Ivanoff, G. Languillat, & A. J. Georges. 1982. Serological survey for arbovirus antibodies in the human and simian populations of the south-east of Gabon. Bull Soc Pathol Exot Filiales. 75: 262-266.

Sardi, S. I., S. Somasekar, S. N. Naccache, A. C. Bandeira, L. B. Tauro, G. S. Campos, & C. Y. Chiu. 2016. Coinfecitons of Zika and chikungunya viruses in Bahia, Brazil, identified by

metagenomic next-sequence sequencing. J. Clin. Microbiol. 54: 2348-2353.

Sarno, M., G. A. Sacramento, R. Khouri, M. S. da Rosário, F. Costa, G. Archanjo, L. A. Santos, N. Nery, N. Vasilakis, A. I. Ko, & A. R. de Almeida. 2016. Zika virus infection and stillbirths: a case of hydrops fetalis, hydranencephaly and fetal demise. PLoS Negl Trop Dis. 10: e0004517.

Schuler-Faccini, L., E. M. Ribeiro, I. M. Feitosa, D. D. Horovitz, D. P. Cavalcanti, A. Pessoa, M. J. Doriqui, J. I. Neri, J. M. Neto, H. Y. Wanderley, M. Cernach, A. S. El-Husny, M. V. Pone, C. L. Serao, & M. T. Sanseverino. 2016. Possible association between Zika virus infection and microcephaly – Brazil, 2015. MMWR Morb. Mortal. Wkly. Rep. 65: 59-62.

Service, M. W. 1997. Mosquito (Diptera: Culicidae) dispersal—the long and the short of it. J. Med. Entomol. 34: 579-588.

Sirbu, A., C. S. Ceianu, R. I. Panculescu-Gatej, A. Vazquez, A. Tenorio, R. Rebreanu, M. Niedrig, G. Nicolescu, & A. Pistol. 2011. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. Euro Surveill. 16: 19762.

Smartt, C. T., T. M. S. Stenn, T. Y. Chen, M. G. Teixeira, E. P. Queiroz, L. S. Dos Santos, G. A. N. Queiroz, K. R. Souza, L. K. Silva, D. Shin, & W. J. Tabachnick. 2017. Evidence of Zika virus RNA fragments in *Aedes albopictus* (Diptera: Culicidae) field-collected eggs from Camaçari, Bahia, Brazil. J. Med. Entomol. 54: 1085-1087.

Smith, D. M., D. D. Richman, & S. J. Little. 2005. HIV superinfection. J. Infect. Dis. 192: 438-444.

Smithburn, K. C. 1952. Neutralizing antibodies against certain recently isolated viruses in the sera of human beings residing in East Africa. J. Immunol. 69: 223-234.

Smithburn, K. C. 1954. Neutralizing antibodies against arthropod-borne viruses in the sera of long-

time residents of Malaya and Borneo. Am J Hyg. 59: 157-163.

Smithburn, K. C., T. P. Hughes, A. W. Burke, & J. H. Paul. 1940. A neurotropic virus isolated from the blood of a native of Uganda. Am. J. Trop. Med. Hyg. 20: 471-472.

Smithburn, K. C., J. A. Kerr, & P. B. Gatne. 1954. Neutralizing antibodies against certain viruses in the sera of residents of India. J. Immunol 74: 248-257.

Smithburn, K. C., R. M. Taylor, F. Rizk, & A. Kader. 1954. Immunity to certain arthropod-borne viruses among indigenous residents of Egypt. Am. J. Trop. Med. Hyg. 3: 9-18.

Song, B., S. Yun, M. Woolley, & Y. Lee. 2017. Zika virus: history, epidemiology, transmission, and clinical presentation. J. Neuroimmunol. 308: 50-64.

Spiglan, I., W. Jasinska-Klingberg, E. Hofshi, & N. Goldblum. 1958. Clinical and laboratory observations in an outbreak of West Nile fever in Israel in 1957. Harefuah. 54: 278-280.

Stadler, K., S. L. Allison, J. Schalich, & F. X. Heinz. 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J. Virol. 71: 8475-8481.

Swaminathan, S., R. Schlaberg, J. Lewis, K. E. Hanson, & M. R. Couturier. 2016. Fatal Zika virus infection with secondary nonsexual transmission. N. Engl. J. Med. 375: 1907-1909.

Tang, H., C. Hammack, S. C. Ogden, Z. Wen, X. Qian, Y. Li, B. Yao, F. Zhang, E. M. Lee, K. M. Christian, R. A. Didier, P. Jin, H. Song, & G. L. Ming. 2016. Zika virus infects human cortical neural progenitors and attenuates their growth. Cell Stem Cell. 18: 587-590.

Taylor, R. M., T. H. Work, H. S. Hurlbut, & F. Rizk. 1956. A study of the ecology of West Nile virus in Egypt. Am. J. Trop. Med. Hyg. 5: 579-620.

Thielman, A. & F. F. Hunter. 2006. Establishment of *Ochlerotatus japonicus* (Diptera: Culicidae) in Ontario, Canada. J. Med. Entomol. 43: 138-142.

Thielman, A. C. & F. F. Hunter. 2007. A photographic key to adult female mosquito species of

Canada (Diptera: Culicidae). Can. J. Arthropod Identif. 4.

Thomas, D. L., T. M. Sharp, J. Torres, P. A. Armstrong, J. Munoz-Jordan, K. R. Ryff, A. Martinez-Quiñones, J. Arias-Berríos, M. Mayshack, G. J. Garayalde, S. Saavedra, C. A. Luciano, M. Valencia-Prado, S. Waterman, & B Rivera-García. 2016. Local transmission of Zika virus – Puerto Rico, November 23, 2015-January 28, 2016. MMWR Morb. Mortal. Wkly. Rep. 65: 154-158.

Tiawsirisup, S., J. R. Kinley, B. J. Tucker, R. B. Evans, W. A. Rowley, & K. B. Platt. 2008. Vector competence of *Aedes vexans* (Diptera: Culicidae) for West Nile virus and potential as an enzootic vector. J. Med Entomol. 45: 425-427.

Tognarelli, J., S. Ulloa, E. Villagra, J. Lagos, C. Aguayo, R. Fasce, B. Parra, J. Mora, N. Becerra, N. Lagos, L. Vera, B. Olivares, M. Vilches, & J. Fernández. 2016. A report on the outbreak of Zika virus on Easter Island, South Pacific, 2014. Arch. Virol. 161: 665-668.

Tsai, T. F., F. Popovici, C. Cernescu, G. L. Campbell, & N. I. Nedelcu. 1998. West Nile encephalitis epidemic in southeastern Romania. Lancet. 352: 767-771.

Turell, M. J., D. J. Dohm, M. R. Sardelis, M. L. O'Guinn, T. G. Andreadis, & J. A. Blow. 2005. An update on the potential of North American mosquitoes (Diptera: Culicidae) to transmit West Nile virus. J. Med. Entomol. 42: 57-62.

Van dan Eeden, S. K., M. R. Karagas, J. R. Daling, & T. L. Vaughan. 1990. A case-control study of maternal smoking and congenital malformations. Paediatr Perinat Epidemiol. 4: 147-155.

Vázquez-Calvo, Á., A. B. Blázquez, E. Escribano-Romero, T. Merino-Ramos, J. C. Saiz, M. A. Martín-Acebes, & N. J. de Oya. 2017. Zika virus infection confers protection against West Nile virus challenge in mice. Emerg Microbes Infect. 6: e81.

Wæhre, T., A. Maagard, D. Tappe, D. Cadar, & J. Schmidt-Chanasit. 2014. Zika virus infection

after travel to Tahiti, December 2013. Emerging Infect. Dis. 20: 1412-1414.

Waggoner, J. J., L. Gresh, M. J. Vargasm B. Ballesteros, Y. Tellez, K. J. Soda, M. K. Sahoo, A. Nuñez, A. Balmaseda, E. Harris, & B. A. Pinsky. 2016. Viremia and clinical presentation in Nicaraguan patients infected with Zika virus, chikungunya virus, and dengue virus. Clin. Infect. Dis. 63: 1584-1590.

Weaver, S. C., F. Costa, M. A. Garcia-Blanco, A. I. Ko, G. S. Ribeiro, G. Saade, P. Y. Shi, & N. Vasilakis. 2016. Zika virus: history, emergence, biology, and prospects for control. Antiviral Res. 130: 69-80.

Weger-Lucarelli, J., C. Rückert, N. Chotiwan, C. Nguyen, S. M. G. Luna, J. R. Fauver, B. D. Foy, R. Perera, W. C. Black, R. C. Kading, & G. D. Ebel. 2016. Vector competence of American mosquitoes for three strains of Zika virus. PLoS Negl. Trop. Dis. 10: e005101.

Whitfield, S. G., F. A. Murphy, & W. D. Sudia. 1973. St. Louis encephalitis virus: an ultrastructural study of infection in a mosquito vector. Virology. 56: 70-87.

Wielanek, A. C., J. D. Monredon, M. E. Amrani, J. C. Roger, & J. P. Serveaux. 2007. Guillain-Barré syndrome complicating a chikungunya virus infection. Neurology. 69: 2105-2107.

Wong, P. S., M. Z. Li, C. S. Chong, L. C. Ng, & C. H. Tan. 2013. *Aedes* (*Stegomyia*) *albopictus* (Skuse): a potential vector of Zika virus in Singapore. PLoS Negl. Trop. Dis. 7: e2348.

Woods, C. G. 2004. Human microcephaly. Curr. Opin. Neurobiol. 14: 112-117.

World Health Organization. 1985. Arthropod-borne and rodent-borne viral diseases. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser. 719: 1-116.

World Health Organization. 2016A. WHO statement on the first meeting of the International Health Regulations (2005) (IHR 2005) Emergency Committee on Zika virus and observed increase in neurological disorders and neonatal malformations. Geneva, Switzerland: World

Health Organization. Retrieved February 24, 2019 from: https://www.who.int/en/news-room/detail/01-02-2016-who-statement-on-the-first-meeting-of-the-international-health-regulations-(2005)-(ihr-2005)-emergency-committee-on-zika-virus-and-observed-increase-in-neurological-disorders-and-neonatal-malformations.

World Health Organization. 2016B. Fifth meeting of the Emergency Committee under the International Health Regulations (2005) regarding microcephaly, other neurological disorders and Zika virus. Geneva, Switzerland: World Health Organization. Retrieved February 24, 2019 from: https://www.who.int/en/news-room/detail/18-11-2016-fifth-meeting-of-the-emergency-committee-under-the-international-health-regulations-(2005)-regarding-microcephaly-other-neurological-disorders-and-zika-virus.

Yin, Y., Y. Xu, L. Su, X. Zhu, M. Chen, W. Zhu, H. Xia, X. Huang, & S. Gong. 2016. Epidemiologic investigation of a family cluster of imported ZIKV cases in Guangdong, China: probable human-to-human transmission. Emerg Microbes Infect. 5: e100.

Yoshida, S., T. Sudo, M. Niimi, L. Tao, B. Sun, J. Kambayashi, H. Watanabe, E. Luo, & H. Matsuoka. 2008. Inhibition of collagen-induced platelet aggregation by anopheline antiplatelet protein, a saliva protein from a malaria vector mosquito. Blood. 111: 2007-2014.

Yuki, N., & Hartung, H. 2012. Guillain-Barré syndrome. N. Engl. J. Med. 366: 2294-2304.

Yun, S., & Y. Lee. 2014. Japanese encephalitis. Hum Vaccin Immunother. 10: 263-279.

Yun, S. I., B. H. Song, J. C. Frank, J. G. Julander, I. A. Polejaeva, C. J. Davies, K. L. White, & Y. M. Lee. 2016. Complete genome sequences of three historically important, spatiotemporally distinct, and genetically divergent strains of Zika virus: MR-766, P6-740, and PRVABC-59. Genome Announc. 4: e00800-e00816.

Zambrano, H., J. J. Waggoner, C. Almeida, L. Rivera, J. Q. Benjamin, & B. A. Pinsky. 2016. Zika

virus and chikungunya virus coinfections: a series of three cases from a single center in Ecuador. Am. J. Trop. Med. Hyg. 95: 894-896.

Zammarchi, L., G. Stella, A. Mantella, D. Bartolozzi, D. Tappe, S. Günther, L. Oestereich, D. Cadar, C. Muñoz-Fontela, A. Bartoloni, & J. Schmidt-Chanasit. 2015. Zika virus infections imported to Italy: clinical, immunological and virological findings, and public health implications. J. Clin. Virol. 63: 32-35.

Zanluca, C., V. C. Melo, A. L. Mosimann, G. I. Santos, C. N. Santos, & K. Luz. 2015. First report of autochthonous transmission of Zika virus in Brazil. Mem. Inst. Oswaldo Cruz. 110: 569-572.

Zdenek, H. & J. Halouzka. 1999. West Nile fever – a reemerging mosquito-borne viral disease in Europe. Emerging Infect. Dis. 5: 643-650.

Zhang, Y., K. Kaufmann, P. R. Chipman, R. J. Kuhn, & M. G. Rossmann. 2007. Structure of immature West Nile virus. J. Virol. 81: 6141-6145.

Appendices

Appendix I: Reagents and Solutions

3.2% CMC Solution

For 1L, add 32 g of powdered CMC sodium salt (Catalog No. C4888-500G, Sigma) to 500 mL of distilled water. Bring the total volume up to 1 L using distilled water. Mix solution using a sterile magnetic stir bar at 100˚C until fully dissolved. Autoclave solution at 121˚C for 10 minutes.

2% FBS Medium

For 1 L, combine 20 mL of FBS (Catalog No. F1051, Sigma) with 980 mL of 1X Dulbecco's Modified Eagle's Medium (DMEM; Catalog No. D6546, Sigma).

1:1 Carboxymethyl Cellulose (CMC) Solution / 2% Fetal Bovine Serum (FBS) Overlay

Combine equal volumes of CMC solution and 2% FBS medium. Mix overlay using a sterile magnetic stir bar until fully homogenized.

1:1 Penicillin / Streptomycin / L-Glutamine (PSG) Solution

Combine equal volumes of 100X penicillin/streptomycin (Catalog No. 15140122, GIBCO) and 100X L-glutamine (Catalog No. 25030081, GIBCO).

10% Sucrose Solution

For 1 L, add 100 g of sucrose (Catalog No. S0389-1KG, Sigma) to 500 mL of distilled water. Bring the total volume up to 1 L using distilled water. Mix solution using a sterile magnetic stir

bar until fully dissolved.

C6/36 Culture Medium

For 1 L, combine 20 mL PSG, 100 mL FBS, and 880 mL Minimum Essential Medium Eagle (MEME; Catalog No. M0643-10X1L, Sigma). Filter sterilize (0.2 µm) medium.

DPBS

For 1 L, add 9.6 g of powdered DPBS (Catalog No. D5652-50L, Sigma) to 1 L of distilled water. Filter sterilize (0.2 µm) following preparation and autoclave at 121˚C for 20 minutes.

Crystal Violet Staining Solution

For 1L, add 10 g of powdered crystal violet (Catalog No. C0775, Sigma) to 300 mL of 100% ethanol. Add 200 mL of formaldehyde and bring the total volume to 1 L using Dulbecco's Phosphate-Buffered Saline. Mix solution using a sterile magnetic stir bar for 30 minutes.

VERO E6 Culture Medium

For 1L, combine 20 mL PSG, 100 mL FBS, and 880 mL DMEM.

Appendix II: Viral Titer Calculations

$$\frac{\text{PFU}}{\text{mL}} = \frac{(\text{Mean Number of Plaques})}{(\text{Dilution Factor})(\text{Volume of Inoculum})}$$

West Nile Virus (WN-NY99) Strain

$$\frac{\text{PFU}}{\text{mL}} = \frac{\left(\frac{186 + 173 + 178)}{3}\right)}{(10^{-5})(0.4\text{mL})} = 44750000\,\frac{\text{PFU}}{\text{mL}}$$

Zika Virus (PRVABC59) Strain

$$\frac{\text{PFU}}{\text{mL}} = \frac{\left(\frac{99 + 84 + 86)}{3}\right)}{(10^{-3})(0.4\text{mL})} = 224167\,\frac{\text{PFU}}{\text{mL}}$$

Appendix III: Volume of Inoculum Calculations

$$\text{Volume of Inocolum} = \left(\frac{(\text{Multiplicity of Infection})(\text{Number of Cells})}{(\frac{\text{PFU}}{\text{mL}})} \right)$$

West Nile Virus (WN-NY99) Strain

$$\text{Volume of Inoculum} = \left(\frac{(0.01)(3.52\times10^5 \text{cells})}{44750000 \frac{\text{PFU}}{\text{mL}}} \right) \left(\frac{1000\mu\text{L}}{1\text{mL}} \right) = 0.07866 \frac{\mu\text{L}}{\text{well}}$$

Zika Virus (PRVABC59) Strain

$$\text{Volume of Inocolum} = \left(\frac{(0.01)(3.52\times10^5 \text{cells})}{224167 \frac{\text{PFU}}{\text{mL}}} \right) \left(\frac{1000\mu\text{L}}{1\text{mL}} \right) = 15.7026 \frac{\mu\text{L}}{\text{well}}$$

Appendix IV: qRT-PCR Materials and Protocols

West Nile Virus

Primers and Probes:

WNV Generic (Gen)-Forward	5'-CAGACCACGCTACGGCG-3'
WNV Gen-Reverse	5'-CTAGGGCCGCGTGGG-3'
WNV Gen-Probe (FAM)	5'-TCTGCGGAGAGTGCAGTCTGCGAT-3'
WNV Envelope (E)-Forward	5'-TCAGCGATCTCTCCACCAAAG-3'
WNV E-Reverse	5'-GGGTCAGCACGTTTGTCATTG-3'
WNV E-Probe (FAM)	5'-TGCCCGACCATGGGAGAAGCTC-3'

Master Mix Formula:

iTaq Universal Probe One-Step Master Mix (Catalog No. 1725131, Bio-Rad)	12.5 µL
Nuclease-Free Water	6.5 µL
WNV-Forward Primer [10 µM]	0.25 µL
WNV-Reverse Primer [10 µM]	0.25 µL
WNV-Probe [10 µM]	0.15 µL
iTaq-RT	0.3 µL

Thermocycling Conditions:

Cycle 1	50˚C for 30 minutes
Cycle 2	95˚C for 10 minutes
Cycle 3 (x40)	90˚C for 15 seconds
	60˚C for 60 seconds

Zika Virus

Primers and Probes:

ZIKV 1-Forward	5'-TTGGTCATGATACTGCTGATTGC-3'
ZIKV 1-Reverse	5'-CCTTCCACAAAGTCCCTATTGC-3'
ZIKV 1-Probe (FAM)	5'-CGGCATACAGCATCAGGTGCATAGGAG -3'
ZIKV 2-Forward	5'-TTGGTCATGATACTGCTGATTGC-3'
ZIKV 2-Reverse	5'-TTGGTCATGATACTGCTGATTGC-3'
ZIKV 2-Probe (FAM)	5'-AGCCTACCTTGACAAGCAGTCAGACACTCAA -3'

Master Mix Formula:

iTaq Universal Probe One-Step Master Mix	12.5 µL
Nuclease-Free Water	4.5 µL
ZIKV-Forward Primer [10 µM]	1.0 µL
ZIKV-Reverse Primer [10 µM]	1.0 µL
ZIKV-Probe [10 µM]	0.5 µL
iTaq-RT	0.5 µL

Thermocycling Conditions:

Cycle 1	50˚C for 30 minutes
Cycle 2	95˚C for 15 minutes
Cycle 3 (x40)	94˚C for 15 seconds
	60˚C for 60 seconds

Appendix V: Mosquito Infection qRT-PCR Data

Table AV.1: Positive vector competence results for blood-infected wild-caught mosquitoes. Cycle threshold (CT) values obtained from the qRT-PCR using the ZIKV 1 P/P set for wild-caught mosquitoes whose bodies tested positive for the presence of ZIKV following oral exposure to an infected blood meal.

Mosquito Sample	CT Value		
	Body	Legs/Wings	Saliva
Aedes vexans	22.13	N/A	N/A
Aedes vexans	26.30	N/A	N/A
Aedes vexans	25.96	N/A	N/A
Coquillettidia perturbans	24.48	N/A	N/A

Table AV.2: Positive vector competence results for blood-infected colony mosquitoes. Cycle threshold (CT) values obtained from the qRT-PCR using the ZIKV 1 P/P set for colony mosquitoes that tested positive for the presence of ZIKV following oral exposure to an infected blood meal.

Mosquito Sample	CT Value		
	Body	Legs/Wings	Saliva
Aedes aegypti	25.90	N/A	N/A
Aedes aegypti	32.18	N/A	N/A
Aedes aegypti	35.34	N/A	N/A
Aedes aegypti	31.19	35.35	35.79
Aedes aegypti	18.74	N/A	N/A
Aedes aegypti	17.94	N/A	N/A
Aedes aegypti	19.40	N/A	N/A
Aedes albopictus	31.91	N/A	N/A
Aedes albopictus	33.22	33.52	29.54
Aedes albopictus	35.14	32.01	29.54
Aedes albopictus	36.26	N/A	N/A

Table AV.3: Positive vector competence results for sugar-infected wild-caught mosquitoes. Cycle threshold (CT) values obtained from the qRT-PCR using the ZIKV 1 P/P set for wild-caught mosquitoes that tested positive for the presence of ZIKV following oral exposure to an infected sugar meal.

Mosquito Sample	CT Value		
	Body	Legs/Wings	Saliva
Culex pipiens	27.37	N/A	N/A
Culex pipiens	35.07	N/A	N/A
Culex pipiens	36.70	N/A	N/A
Culex pipiens	34.99	N/A	N/A
Culex pipiens	34.56	37.71	34.49

Appendix VI: Cellular Infection qRT-PCR Data

Table AVI.1: Dual flavivirus infection results in VERO E6 cells using the WNV Gen P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV Gen P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in VERO E6 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	18.14	5.79	5.60	0.26
	DAY 2 B	19.62	5.31		
	DAY 2 C	18.36	5.71		
WNV/ZIKV SI	DAY 2 A	17.96	5.84	5.85	0.01
	DAY 2 B	18.00	5.83		
	DAY 2 C	17.91	5.86		
ZIKV/WNV SI	DAY 2 A	28.92	2.30	2.05	0.22
	DAY 2 B	30.12	1.91		
	DAY 2 C	30.04	1.93		
WNV-ZIKV CI	DAY 3 A	16.25	6.40	6.16	0.20
	DAY 3 B	17.27	6.07		
	DAY 3 C	17.41	6.02		
WNV/ZIKV SI	DAY 3 A	16.85	6.20	6.40	0.19
	DAY 3 B	15.66	6.59		
	DAY 3 C	16.21	6.41		
ZIKV/WNV SI	DAY 3 A	24.25	3.81	3.66	0.34
	DAY 3 B	25.91	3.27		
	DAY 3 C	23.98	3.90		
WNV-ZIKV CI	DAY 4 A	17.37	6.04	6.06	0.03
	DAY 4 B	17.21	6.09		
	DAY 4 C	17.30	6.06		
WNV/ZIKV SI	DAY 4 A	17.70	5.93	6.12	0.21
	DAY 4 B	17.23	6.08		
	DAY 4 C	16.43	6.34		
ZIKV/WNV SI	DAY 4 A	22.29	4.44	4.12	0.30
	DAY 4 B	24.15	3.84		
	DAY 4 C	23.45	4.07		
WNV-ZIKV CI	DAY 5 A	13.60	7.26	6.86	0.34
	DAY 5 B	15.51	6.64		
	DAY 5 C	15.33	6.70		
WNV/ZIKV SI	DAY 5 A	12.57	6.71	6.73	0.05
	DAY 5 B	15.37	6.68		
	DAY 5 C	15.05	6.79		
ZIKV/WNV SI	DAY 5 A	19.46	5.36	5.27	0.17
	DAY 5 B	19.40	5.38		
	DAY 5 C	20.35	5.07		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			20.45		
STANDARD CURVE TRENDLINE			y = -3.090x + 36.019		

Table AVI.2: Dual flavivirus infection results in VERO E6 cells using the WNV E P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV E P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in VERO E6 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	15.67	6.79	6.34	0.64
	DAY 2 B	19.10	5.61		
	DAY 2 C	16.17	6.62		
WNV/ZIKV SI	DAY 2 A	15.50	6.85	6.92	0.07
	DAY 2 B	15.27	6.93		
	DAY 2 C	15.08	6.99		
ZIKV/WNV SI	DAY 2 A	25.96	3.25	2.81	0.38
	DAY 2 B	27.66	2.66		
	DAY 2 C	28.05	2.54		
WNV-ZIKV CI	DAY 3 A	13.93	7.37	7.14	0.22
	DAY 3 B	15.03	7.01		
	DAY 3 C	15.03	7.01		
WNV/ZIKV SI	DAY 3 A	14.39	7.23	7.32	0.12
	DAY 3 B	13.71	7.46		
	DAY 3 C	14.25	7.28		
ZIKV/WNV SI	DAY 3 A	22.96	4.28	4.30	0.41
	DAY 3 B	24.08	3.89		
	DAY 3 C	21.68	4.72		
WNV-ZIKV CI	DAY 4 A	13.82	7.43	7.36	0.13
	DAY 4 B	13.76	7.45		
	DAY 4 C	14.44	7.21		
WNV/ZIKV SI	DAY 4 A	14.77	7.10	7.24	0.12
	DAY 4 B	14.07	7.34		
	DAY 4 C	14.27	7.27		
ZIKV/WNV SI	DAY 4 A	21.08	4.93	4.97	0.10
	DAY 4 B	21.17	4.90		
	DAY 4 C	20.65	5.08		
WNV-ZIKV CI	DAY 5 A	10.98	8.40	8.05	0.31
	DAY 5 B	12.60	7.85		
	DAY 5 C	12.47	7.89		
WNV/ZIKV SI	DAY 5 A	12.32	7.94	7.94	0.04
	DAY 5 B	12.21	7.98		
	DAY 5 C	12.42	7.91		
ZIKV/WNV SI	DAY 5 A	16.20	6.61	6.50	0.16
	DAY 5 B	16.31	6.57		
	DAY 5 C	17.06	6.31		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			19.18		
STANDARD CURVE TRENDLINE			$y = -2.905x + 35.393$		

Table AVI.3: Dual flavivirus infection results in VERO E6 cells using the ZIKV 1 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 1 P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in VERO E6 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	19.16	3.98	3.86	0.12
	DAY 2 B	19.99	3.75		
	DAY 2 C	19.62	3.85		
WNV/ZIKV SI	DAY 2 A	25.65	2.15	2.06	0.18
	DAY 2 B	25.58	2.17		
	DAY 2 C	26.73	1.85		
ZIKV/WNV SI	DAY 2 A	18.85	4.07	4.12	0.15
	DAY 2 B	19.09	4.00		
	DAY 2 C	18.07	4.29		
WNV-ZIKV CI	DAY 3 A	17.12	4.56	4.47	0.17
	DAY 3 B	18.15	4.27		
	DAY 3 C	17.06	4.58		
WNV/ZIKV SI	DAY 3 A	23.96	2.63	2.89	0.24
	DAY 3 B	22.93	2.92		
	DAY 3 C	22.24	3.11		
ZIKV/WNV SI	DAY 3 A	16.27	4.80	4.73	0.16
	DAY 3 B	17.17	4.55		
	DAY 3 C	16.10	4.85		
WNV-ZIKV CI	DAY 4 A	18.69	4.12	4.31	0.18
	DAY 4 B	17.93	4.33		
	DAY 4 C	17.42	4.48		
WNV/ZIKV SI	DAY 4 A	25.85	2.09	2.21	0.20
	DAY 4 B	24.62	2.44		
	DAY 4 C	25.89	2.08		
ZIKV/WNV SI	DAY 4 A	16.53	4.73	4.82	0.13
	DAY 4 B	16.39	4.77		
	DAY 4 C	15.67	4.97		
WNV-ZIKV CI	DAY 5 A	17.27	4.52	4.47	0.05
	DAY 5 B	17.45	4.47		
	DAY 5 C	17.65	4.41		
WNV/ZIKV SI	DAY 5 A	25.10	2.31	2.10	0.20
	DAY 5 B	26.49	1.91		
	DAY 5 C	25.91	2.08		
ZIKV/WNV SI	DAY 5 A	16.22	4.81	4.85	0.04
	DAY 5 B	15.95	4.89		
	DAY 5 C	16.13	4.84		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			25.12		
STANDARD CURVE TRENDLINE			y = -3.540x + 33.264		

Table AVI.4: Dual flavivirus infection results in VERO E6 cells using the ZIKV 2 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 2 P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in VERO E6 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	17.46	4.22	4.08	0.23
	DAY 2 B	18.76	3.81		
	DAY 2 C	17.57	4.19		
WNV/ZIKV SI	DAY 2 A	23.84	2.21	2.16	0.06
	DAY 2 B	23.89	2.19		
	DAY 2 C	24.21	2.09		
ZIKV/WNV SI	DAY 2 A	16.80	4.43	4.37	0.06
	DAY 2 B	17.19	4.31		
	DAY 2 C	17.00	4.37		
WNV-ZIKV CI	DAY 3 A	16.26	4.60	4.45	0.13
	DAY 3 B	17.05	4.35		
	DAY 3 C	16.93	4.39		
WNV/ZIKV SI	DAY 3 A	21.69	2.89	2.94	0.08
	DAY 3 B	21.22	3.04		
	DAY 3 C	21.68	2.89		
ZIKV/WNV SI	DAY 3 A	15.60	4.81	4.89	0.20
	DAY 3 B	15.80	4.75		
	DAY 3 C	14.62	5.12		
WNV-ZIKV CI	DAY 4 A	16.96	4.38	4.42	0.03
	DAY 4 B	16.77	4.44		
	DAY 4 C	16.80	4.43		
WNV/ZIKV SI	DAY 4 A	22.65	2.58	2.57	0.23
	DAY 4 B	21.96	2.80		
	DAY 4 C	23.44	2.33		
ZIKV/WNV SI	DAY 4 A	15.23	4.93	4.99	0.05
	DAY 4 B	15.02	5.00		
	DAY 4 C	14.91	5.03		
WNV-ZIKV CI	DAY 5 A	16.70	4.46	4.47	0.11
	DAY 5 B	16.32	4.58		
	DAY 5 C	17.02	4.36		
WNV/ZIKV SI	DAY 5 A	22.38	2.67	2.29	0.45
	DAY 5 B	25.14	1.80		
	DAY 5 C	23.25	2.39		
ZIKV/WNV SI	DAY 5 A	14.17	5.26	5.21	0.07
	DAY 5 B	14.27	5.23		
	DAY 5 C	14.61	5.13		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			29.95		
STANDARD CURVE TRENDLINE			y = -3.163x + 30.822		

Table AVI.5: Dual flavivirus infection results in C6/36 cells using the WNV Gen P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV Gen P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in C6/36 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	32.01	1.98	2.09	0.11
	DAY 2 B	31.64	2.10		
	DAY 2 C	31.31	2.20		
WNV/ZIKV SI	DAY 2 A	31.85	2.03	1.65	0.04
	DAY 2 B	30.88	2.33		
	DAY 2 C	31.74	2.07		
ZIKV/WNV SI	DAY 2 A	32.95	1.70	2.09	0.11
	DAY 2 B	33.16	1.63		
	DAY 2 C	33.17	1.63		
WNV-ZIKV CI	DAY 3 A	27.96	3.22	2.91	0.28
	DAY 3 B	29.80	2.66		
	DAY 3 C	29.12	2.86		
WNV/ZIKV SI	DAY 3 A	27.00	3.51	3.33	0.20
	DAY 3 B	28.28	3.12		
	DAY 3 C	27.50	3.36		
ZIKV/WNV SI	DAY 3 A	31.14	2.25	2.34	0.08
	DAY 3 B	30.79	2.36		
	DAY 3 C	30.62	2.41		
WNV-ZIKV CI	DAY 4 A	26.14	3.77	3.44	0.31
	DAY 4 B	28.14	3.16		
	DAY 4 C	27.36	3.40		
WNV/ZIKV SI	DAY 4 A	25.05	4.10	3.95	0.22
	DAY 4 B	26.36	3.70		
	DAY 4 C	25.20	4.06		
ZIKV/WNV SI	DAY 4 A	29.73	2.68	2.80	0.11
	DAY 4 B	29.04	2.89		
	DAY 4 C	29.21	2.84		
WNV-ZIKV CI	DAY 5 A	25.39	4.00	3.91	0.33
	DAY 5 B	24.79	4.18		
	DAY 5 C	26.87	3.55		
WNV/ZIKV SI	DAY 5 A	24.16	4.37	4.38	0.04
	DAY 5 B	24.02	4.42		
	DAY 5 C	24.28	4.34		
ZIKV/WNV SI	DAY 5 A	28.34	3.10	3.50	0.36
	DAY 5 B	26.03	3.80		
	DAY 5 C	26.72	3.59		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			25.34		
STANDARD CURVE TRENDLINE			$y = -3.284x + 38.524$		

Table AVI.6: Dual flavivirus infection results in C6/36 cells using the WNV E P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV E P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in C6/36 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	29.94	1.88	2.00	0.17
	DAY 2 B	29.76	1.94		
	DAY 2 C	29.02	2.19		
WNV/ZIKV SI	DAY 2 A	30.16	1.80	1.80	0.21
	DAY 2 B	30.77	1.59		
	DAY 2 C	29.56	2.01		
ZIKV/WNV SI	DAY 2 A	31.03	1.50	1.53	0.06
	DAY 2 B	31.08	1.48		
	DAY 2 C	30.77	1.59		
WNV-ZIKV CI	DAY 3 A	25.92	3.26	2.86	0.36
	DAY 3 B	27.92	2.57		
	DAY 3 C	27.39	2.75		
WNV/ZIKV SI	DAY 3 A	26.72	2.99	2.99	0.22
	DAY 3 B	27.35	2.77		
	DAY 3 C	26.08	3.21		
ZIKV/WNV SI	DAY 3 A	29.77	1.94	2.07	0.12
	DAY 3 B	29.28	2.10		
	DAY 3 C	29.10	2.17		
WNV-ZIKV CI	DAY 4 A	24.31	3.82	3.18	0.55
	DAY 4 B	27.25	2.80		
	DAY 4 C	26.88	2.93		
WNV/ZIKV SI	DAY 4 A	24.17	3.86	3.84	0.27
	DAY 4 B	25.05	3.56		
	DAY 4 C	23.50	4.09		
ZIKV/WNV SI	DAY 4 A	27.36	2.77	2.65	0.22
	DAY 4 B	28.43	2.40		
	DAY 4 C	27.28	2.79		
WNV-ZIKV CI	DAY 5 A	23.59	4.06	3.80	0.34
	DAY 5 B	24.01	3.92		
	DAY 5 C	25.46	3.42		
WNV/ZIKV SI	DAY 5 A	23.19	4.20	4.09	0.20
	DAY 5 B	24.17	3.86		
	DAY 5 C	23.17	4.21		
ZIKV/WNV SI	DAY 5 A	27.94	2.57	3.33	0.66
	DAY 5 B	24.55	3.73		
	DAY 5 C	24.68	3.69		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			25.07		
STANDARD CURVE TRENDLINE			y = -3.186x + 37.955		

Table AVI.7: Dual flavivirus infection results in C6/36 cells using the ZIKV 1 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 1 P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in C6/36 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	26.75	1.60	1.23	0.78
	DAY 2 B	26.28	1.75		
	DAY 2 C	30.98	0.34		
WNV/ZIKV SI	DAY 2 A	29.93	0.65	0.79	0.13
	DAY 2 B	29.10	0.90		
	DAY 2 C	29.40	0.81		
ZIKV/WNV SI	DAY 2 A	24.62	2.24	2.18	0.12
	DAY 2 B	25.26	2.05		
	DAY 2 C	24.57	2.26		
WNV-ZIKV CI	DAY 3 A	23.21	2.66	2.26	0.44
	DAY 3 B	24.30	2.34		
	DAY 3 C	26.14	1.79		
WNV/ZIKV SI	DAY 3 A	23.64	2.54	2.45	0.08
	DAY 3 B	24.12	2.39		
	DAY 3 C	24.01	2.42		
ZIKV/WNV SI	DAY 3 A	22.56	2.86	3.02	0.16
	DAY 3 B	21.47	3.19		
	DAY 3 C	22.01	3.02		
WNV-ZIKV CI	DAY 4 A	22.02	3.02	2.94	0.28
	DAY 4 B	21.48	3.18		
	DAY 4 C	23.33	2.63		
WNV/ZIKV SI	DAY 4 A	22.59	2.85	2.85	0.13
	DAY 4 B	23.04	2.72		
	DAY 4 C	22.17	2.98		
ZIKV/WNV SI	DAY 4 A	19.45	3.79	3.87	0.09
	DAY 4 B	18.88	3.96		
	DAY 4 C	19.20	3.87		
WNV-ZIKV CI	DAY 5 A	18.31	4.13	3.82	0.62
	DAY 5 B	18.01	4.22		
	DAY 5 C	21.76	3.10		
WNV/ZIKV SI	DAY 5 A	19.27	3.84	3.81	0.18
	DAY 5 B	20.03	3.62		
	DAY 5 C	18.83	3.98		
ZIKV/WNV SI	DAY 5 A	17.06	4.51	4.58	0.12
	DAY 5 B	16.38	4.71		
	DAY 5 C	17.04	4.51		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			30.56		
STANDARD CURVE TRENDLINE			y = -3.339x + 32.107		

Table AVI.8: Dual flavivirus infection results in C6/36 cells using the ZIKV 2 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 2 P/P set following the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (ZIKV/WNV SI) in C6/36 cells.

Sample		CT Value	Log Starting Quantity	Mean	St. Deviation
WNV-ZIKV CI	DAY 2 A	26.47	1.67	1.90	0.19
	DAY 2 B	25.47	2.00		
	DAY 2 C	25.42	2.02		
WNV/ZIKV SI	DAY 2 A	28.92	0.87	0.83	0.17
	DAY 2 B	28.59	0.98		
	DAY 2 C	29.60	0.65		
ZIKV/WNV SI	DAY 2 A	24.24	2.40	2.31	0.17
	DAY 2 B	25.15	2.11		
	DAY 2 C	24.24	2.40		
WNV-ZIKV CI	DAY 3 A	22.34	3.03	2.89	0.20
	DAY 3 B	23.48	2.65		
	DAY 3 C	22.49	2.98		
WNV/ZIKV SI	DAY 3 A	22.32	3.03	2.75	0.25
	DAY 3 B	23.43	2.67		
	DAY 3 C	23.80	2.55		
ZIKV/WNV SI	DAY 3 A	22.64	2.93	3.32	0.36
	DAY 3 B	20.46	3.64		
	DAY 3 C	21.21	3.40		
WNV-ZIKV CI	DAY 4 A	21.54	3.29	3.35	0.14
	DAY 4 B	20.84	3.52		
	DAY 4 C	21.65	3.25		
WNV/ZIKV SI	DAY 4 A	21.42	3.33	3.28	0.16
	DAY 4 B	22.12	3.10		
	DAY 4 C	21.18	3.41		
ZIKV/WNV SI	DAY 4 A	18.59	4.26	4.13	0.12
	DAY 4 B	19.32	4.02		
	DAY 4 C	18.99	4.12		
WNV-ZIKV CI	DAY 5 A	18.00	4.45	4.38	0.10
	DAY 5 B	18.08	4.42		
	DAY 5 C	18.54	4.27		
WNV/ZIKV SI	DAY 5 A	19.15	4.07	4.13	0.15
	DAY 5 B	19.32	4.02		
	DAY 5 C	18.43	4.31		
ZIKV/WNV SI	DAY 5 A	16.53	4.93	5.01	0.16
	DAY 5 B	15.73	5.19		
	DAY 5 C	16.58	4.91		
NEGATIVE CONTROL			N/A		
POSITIVE CONTROL			30.75		
STANDARD CURVE TRENDLINE			y = -3.052x + 31.579		

Table AVI.9: Single WNV infection results in VERO E6 cells using the WNV Gen P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV Gen P/P set following the single WNV infection in VERO E6 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	18.08	6.77	6.90	0.11
DAY 2 B	17.28	6.99		
DAY 2 C	17.50	6.93		
DAY 3 A	17.85	6.84	7.14	0.32
DAY 3 B	16.87	7.11		
DAY 3 C	15.55	7.47		
DAY 4 A	17.40	6.96	7.23	0.35
DAY 4 B	16.83	7.12		
DAY 4 C	14.97	7.63		
DAY 5 A	14.16	7.85	7.46	0.34
DAY 5 B	16.45	7.22		
DAY 5 C	16.13	7.31		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		18.22		
STANDARD CURVE TRENDLINE		y = -3.647x + 42.783		

Table AVI.10: Single WNV infection results in VERO E6 cells using the WNV E P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV E P/P set following the single WNV infection in VERO E6 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	17.69	6.63	6.78	0.18
DAY 2 B	17.36	6.74		
DAY 2 C	16.62	6.98		
DAY 3 A	15.22	7.44	7.39	0.08
DAY 3 B	15.63	7.30		
DAY 3 C	15.22	7.44		
DAY 4 A	15.43	7.37	7.60	0.20
DAY 4 B	14.39	7.71		
DAY 4 C	14.33	7.73		
DAY 5 A	13.60	7.97	7.24	0.80
DAY 5 B	15.43	7.37		
DAY 5 C	18.47	6.37		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		16.29		
STANDARD CURVE TRENDLINE		y = -3.060x + 37.975		

Table AVI.11: Single ZIKV infection results in VERO E6 cells using the ZIKV 1 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 1 P/P set following the single ZIKV infection in VERO E6 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	22.37	4.29	4.54	0.25
DAY 2 B	21.49	4.54		
DAY 2 C	20.57	4.79		
DAY 3 A	19.44	5.11	5.17	0.14
DAY 3 B	19.52	5.08		
DAY 3 C	18.61	5.34		
DAY 4 A	17.06	5.77	5.77	0.12
DAY 4 B	16.59	5.90		
DAY 4 C	17.48	5.65		
DAY 5 A	16.81	5.84	5.87	0.06
DAY 5 B	16.81	5.84		
DAY 5 C	16.44	5.94		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		26.79		
STANDARD CURVE TRENDLINE		y = -3.595x + 37.794		

Table AVI.12: Single ZIKV infection results in VERO E6 cells using the ZIKV 2 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 2 P/P set following the single ZIKV infection in VERO E6 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	23.05	3.67	3.42	0.81
DAY 2 B	21.79	4.07		
DAY 2 C	26.73	2.51		
DAY 3 A	18.54	5.10	5.11	0.06
DAY 3 B	18.68	5.06		
DAY 3 C	18.31	5.18		
DAY 4 A	16.55	5.73	5.64	0.09
DAY 4 B	17.02	5.59		
DAY 4 C	17.01	5.59		
DAY 5 A	18.76	5.03	5.54	0.44
DAY 5 B	16.47	5.76		
DAY 5 C	16.25	5.83		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		17.26		
STANDARD CURVE TRENDLINE		y = -3.155x + 34.642		

Table AVI.13: Single WNV infection results in C6/36 cells using the WNV Gen P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV Gen P/P set following the single WNV infection in C6/36 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	30.51	2.70	2.41	0.80
DAY 2 B	29.57	3.04		
DAY 2 C	33.79	1.51		
DAY 3 A	32.15	2.10	2.81	0.70
DAY 3 B	28.28	3.51		
DAY 3 C	30.20	2.81		
DAY 4 A	27.52	3.78	3.77	0.21
DAY 4 B	27.01	3.97		
DAY 4 C	28.17	3.55		
DAY 5 A	24.14	5.01	4.89	0.12
DAY 5 B	24.79	4.77		
DAY 5 C	24.47	4.89		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		14.87		
STANDARD CURVE TRENDLINE		y = -2.757x + 37.951		

Table AVI.14: Single WNV infection results in C6/36 cells using the WNV E P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the WNV E P/P set following the single WNV infection in C6/36 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	30.75	2.71	2.37	0.90
DAY 2 B	29.81	3.05		
DAY 2 C	34.50	1.35		
DAY 3 A	32.83	1.96	2.74	0.78
DAY 3 B	28.50	3.53		
DAY 3 C	30.67	2.74		
DAY 4 A	27.76	3.79	3.90	0.10
DAY 4 B	27.19	4.00		
DAY 4 C	27.48	3.89		
DAY 5 A	38.37	3.57	4.46	0.78
DAY 5 B	24.32	5.04		
DAY 5 C	25.04	4.78		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		15.08		
STANDARD CURVE TRENDLINE		y = -2.760x + 38.229		

Table AVI.15: Single ZIKV infection results in C6/36 cells using the ZIKV 1 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 1 P/P set following the single ZIKV infection in C6/36 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	31.54	1.27	1.25	0.04
DAY 2 B	31.53	1.27		
DAY 2 C	31.78	1.20		
DAY 3 A	28.60	2.15	2.14	0.03
DAY 3 B	28.77	2.10		
DAY 3 C	28.57	2.16		
DAY 4 A	26.74	2.71	3.01	0.26
DAY 4 B	25.12	3.20		
DAY 4 C	25.43	3.11		
DAY 5 A	22.67	3.93	3.78	0.15
DAY 5 B	23.18	3.78		
DAY 5 C	23.66	3.64		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		30.70		
STANDARD CURVE TRENDLINE		y = -3.330x + 35.773		

Table AVI.16: Single ZIKV infection results in C6/36 cells using the ZIKV 2 P/P set. Cycle threshold (CT) and log starting quantity values obtained from the qRT-PCR using the ZIKV 2 P/P set following the single ZIKV infection in C6/36 cells.

Sample	CT Value	Log Starting Quantity	Mean	St. Deviation
DAY 2 A	27.68	1.28	1.30	0.18
DAY 2 B	28.10	1.14		
DAY 2 C	27.03	1.49		
DAY 3 A	26.02	1.82	1.83	0.14
DAY 3 B	25.55	1.98		
DAY 3 C	26.39	1.70		
DAY 4 A	23.57	2.62	2.87	0.26
DAY 4 B	22.89	2.85		
DAY 4 C	22.00	3.14		
DAY 5 A	21.63	3.26	3.45	0.23
DAY 5 B	20.26	3.71		
DAY 5 C	21.25	3.38		
NEGATIVE CONTROL		N/A		
POSITIVE CONTROL		22.52		
STANDARD CURVE TRENDLINE		y = -3.052x + 31.579		

Appendix VII: Statistical Analyses

Table VII.1: Dual flavivirus infection single factor ANOVA results for VERO E6 cells. Summary of the single factor ANOVA tests ($\alpha = 0.05$) used to detect variance in log starting quantity values associated with the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (SI) in VERO E6 cells. SS, sum-of-squares; df, degrees of freedom; MS, mean squares.

	SS	df	MS	F Ratio	P-value	F-critical
WNV-ZIKV CI						
Between Groups	24.6522292	7	3.5217402	91.12859022	1.0791×10^{-11}	2.6571966
Within Groups	0.61826667	16	0.03863167			
WNV/ZIKV SI						
Between Groups	96.739133	7	13.8198762	429.3553768	5.4263×10^{-17}	3.6571966
Within Groups	0.515	16	0.0321875			
ZIKV/WNV SI						
Between Groups	21/5334625	7	3.07620893	69.72236688	8.521×10^{-11}	2.6571966
Within Groups	0.70593333	16	0.04412083			

Table VII.2: Dual flavivirus infection single factor ANOVA results for C6/36 cells. Summary of the single factor ANOVA tests ($\alpha = 0.05$) used to detect variance in log starting quantity values associated with the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (SI) in C6/36 cells. SS, sum-of-squares; df, degrees of freedom; MS, mean squares.

	SS	df	MS	F Ratio	P-value	F-critical
WNV-ZIKV CI						
Between Groups	17.8787333	7	2.55410476	13.15701101	1.4384×10^{-5}	2.6571966
Within Groups	3.106	16	0.194125			
WNV/ZIKV SI						
Between Groups	28.5635625	7	4.08050893	174.0398335	6.8786×10^{-14}	2.6571966
Within Groups	0.37513333	16	0.02344583			
ZIKV/WNV SI						
Between Groups	19.3506958	7	2.76438512	104.9932629	3.5953×10^{-12}	2.6571966
Within Groups	0.42126667	16	0.02632917			

Table VII.3: Dual flavivirus infection Tukey's test results for VERO E6 cells. Summary of the Tukey's tests used to detect significant differences in log starting quantity values associated with the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (SI) in VERO E6 cells. MS, mean squares; df, degrees of freedom.

Infection	# Groups	# Replicates	MS	df	Q-value	Yardstick Value
WNV-ZIKV CI	8	3	0.03864167	16	4.90	0.56
WNV/ZIKV SI			0.0321875			0.51
ZIKV/WNV SI			0.044120833			0.59

Table VII.4: Dual flavivirus infection Tukey's test results for C6/36 cells. Summary of the Tukey's tests used to detect significant differences in log starting quantity values associated with the WNV and ZIKV co-infection (WNV-ZIKV CI), WNV first, ZIKV second superinfection (WNV/ZIKV SI), and ZIKV first, WNV second superinfection (SI) in C6/36 cells. MS, mean squares; df, degrees of freedom.

Infection	# Groups	# Replicates	MS	df	Q-value	Yardstick Value
WNV-ZIKV CI	8	3	0.194125	16	4.90	1.25
WNV/ZIKV SI			0.02344583			0.43
ZIKV/WNV SI			0.026329167			0.46