Battle of the Viruses: Aripo Virus Induced Superinfection Exclusion of Mosquito-Borne Viruses

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Entomology

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December 15th 2020
Blacksburg, Virginia

Keywords: Flavivirus, Superinfection Exclusion, Insect-specific Flavivirus, Aripo Virus

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Academic Abstract

Flaviviruses are a single-stranded, positive sense, RNA virus that affect around 400 million people annually. Flaviviruses are transmitted by arthropod vectors, the most common vector being the mosquito. Currently, many mosquito control strategies are in use, these control strategies are diverse in both efficiency and cost. However, developing new vector control strategies is becoming increasingly important, due to climate changing affecting vector population distribution and the current limitations conventional control strategies face. Although many different control strategies exist, there is limited research pertaining to reducing viral infection in the vector. Reducing the transmission capabilities of vectors could help relieve the disease burden felt around the world. Aripo virus (ARPV), an insect-specific flavivirus, has ushered in opportunities to discover a novel approach to arbovirus control. The exclusionary effects of ARPV were explored as a means to eventually understanding superinfection exclusion (SIE) and utilizing it as a calculated defense against mosquito-borne disease. Aripo virus was evaluated for its SIE potential *in vitro* and experiments were performed to explore the possible mechanisms underlying SIE. Aripo virus showed significant exclusion against the flaviviruses tested, as well as an alphavirus. Additionally, West Nile virus was unable to adapt and overcome SIE barriers over 9 serial passages. Lastly, ARPV was superinfected with chimeric viruses to asses replication kinetics, and possible exclusionary bias

was seen with non-structural genes. These data show ARPV is capable of reducing viral titer, as well as possible leads into understanding the underlying mechanism of SIE, a critical step in utilizing SIE as a strategy to combat vector-borne disease.

KEY WORDS Flavivirus, Superinfection Exclusion, Insect-specific Flavivirus, Aripo Virus

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General Audience Abstract

Mosquitoes all around the globe spread disease causing pathogens. Infection with viral pathogens such as West Nile virus, Zika virus, and Japanese encephalitis virus can cause lifelong health effects or even death. Risk for increased spread of mosquito-vectored disease is on the rise. The continued effects of climate change, increasing temperatures away from the equator, and increased encroachment into natural areas for urban development, is opening the door for new infectious diseases spread by mosquitoes. There are many ways to curb the effects that these viral diseases can have on humans, including vaccines or mosquito repellents, and even eliminating mosquito populations is helping to reduce the effects of these important diseases. Sadly, no one method can solve the problem. The methods of dealing with mosquito transmitted disease are likely to be most effective when done in combination. The more countermeasures that attempt to stop the spread of disease, the greater dent in reducing the spread of disease. In this dissertation, we dive into the realm of stopping disease transmission before humans become a factor. If we can limit mosquito infections, then the disease has no way of reaching humans. An approach to controlling mosquito infections could lie within viruses themselves. Aripo virus, an insect-specific virus, is interesting in that it does not affect vertebrates, only mosquitoes. When Aripo virus infects mosquito cells at the same time as deadly viruses, the deadly viruses do not accumulate in the mosquito cells nearly as much.

Within mosquito cells, Aripo virus was able to reduce the levels of West Nile virus, St. Louis encephalitis virus, Japanese encephalitis virus, Zika virus, and Mayaro virus. If the underlying concept of the method of Aripo virus induced exclusion of deadly viruses could be further understood, it could be used to reduce the spread of mosquito-related viral diseases.

Acknowledgements

I would like to deeply thank Dr. Jonathan Auguste for giving me the opportunity to study under his guidance. Because I did not come from a virology background, I had a lot to learn in order to work in your lab. Your patience and instruction allowed me to grow and develop as a graduate researcher and as an individual. Although your hands-off approach to mentoring was difficult at times, I believe it led me to become more independent, as well as confident in my own ability. Though hands-off, your kindness and understanding were ever-present and allowed me to work freely knowing I could reach out to you for help. Thanks to you I feel confident in taking on the challenges that my future holds.

I would also like to thank the members of my committee. Dr. Gillian Eastwood, sadly we did not spend enough time talking about all things science and the adventures you must have had doing your research. Nonetheless, your knowledge of vectors and medical entomology was crucial to my final understanding of my research and my overall development as a medical entomologist. Dr. James Weger-Lucarelli, you were an invaluable asset to have at my disposal. You are incredibly gifted when it comes to all things science and your quick-witted banter was nothing less than brilliant. Having you across the hall was extremely impactful and I am glad to have had your assistance through my time as a graduate student.

For the faculty and staff in the entomology department, thank you for the kindness you have shown throughout my entire time at Virginia Tech. I am beyond grateful for the friendships I made. Emily Webb, you are an amazing friend to have, thank you for your help and friendship. Dr. Sally Paulson and Dr. Jake Bova, if not for you both I would have never joined the

department in the first place, for that I thank you. Dr. Kevin Chan, Dr. Ben McMillan, and Brandi Benedict, thank you for always being willing to help me through the ups and downs of science.

Thank you to everyone at the Centers of Disease Control and Prevention: Division of Vector-borne Disease Branch. Dr. Aaron Brault, you and your researchers, as well as the staff, were nothing but kind and helpful. I learned so much from my time there and I would not trade that time for anything.

Lastly, thank you to my friends and family for supporting me through my entire journey. Thank you, Sunni and Regi Carver, for all your love and encouragement. A big thank you to my girlfriend, Hanna Green, for ensuring that my sanity was intact and my work was designed beautifully.

Thank you.

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction and Literature Review

1.1 Flaviviridae

Flaviviridae is a family of viruses that is commonly trafficked by arthropod vectors, such as ticks and mosquitoes. These RNA viruses are found nearly globally and flavivirus infection can result in disease ranging from mild illness to debilitating hemorrhagic or encephalitic disease. Many viruses within the genus have no licensed vaccines or therapeutics and continue to affect upwards of 400 million people every year[1,2]. Most affected world regions are found in tropical and subtropical areas, and are often developing nations and remote regions with inadequate or inaccessible healthcare. The most common outcomes of flavivirus infection include headache, myalgia, and fever, additionally, some flaviviruses will cause encephalitic complications, most often associated with the elderly or immunocompromised[3]. Increased urbanization and anthropogenic disturbances to natural habitats have increased the contact network between humans and enzootic vectors of zoonotic diseases[4,5]. The most common mosquito vectors for flaviviruses include *Aedes* spp. and *Culex* spp.; these mosquitoes are found globally and many species within the genera are considered urban mosquitoes due to adaptability for easy colonization of developed towns or cityscapes[5–7]. The smooth integration of these mosquito species into developing areas can present extreme exposure of the vectored flaviviruses to human populations, especially areas that lack mosquito control programs.

1.1.1 Genome structure

Flaviviruses are single-stranded, positive sense, encapsidated RNA viruses. Virions are typically 50nm in diameter and consist of three structural proteins and 7 non-structural proteins[8]. The flaviviruses genome is translated along a single open reading frame (ORF) in the following sequence: 5'-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'[8]. There are three structural proteins at the 5' end, including the capsid (C), membrane (prM), and Envelope (E). Following the structural proteins are the non-structural proteins (NS1-NS5), some highly conserved (NS1, NS3, and NS5) while others are not (NS2 and NS4 A/B)[9]. The non-structural proteins are involved in virus replication, including an RNA-polymerase (NS5). Of the structural proteins, the E protein mediates virion binding to target cell receptors, while the prM proteins chaperone the E protein throughout the binding pathway[8].

1.1.2 Phylogenetic Classifications of Flaviviruses

Flaviviruses make up a large phylogenetic genus broadly organized by vector-virus-host relationships[10]. *Flavivirus* ecology is incredibly diverse and complex; most flaviviruses are transmitted by arthropods, ergo arboviruses, while others have no known vector, or are carried by insects but cannot be transmitted to animals (insect-specific flaviviruses; ISFV). No-known-vector (NKV) flaviviruses are divided into groups based on relation to the vertebrate host animal (e.g. rodents or bats). Arboviruses can be further divided into two clades, tick-borne flaviviruses (TBFV) and mosquito-borne flaviviruses (MBFV). Within these clades there are further classifications; TBFV can be divided into pathogenic flaviviruses, associated mainly with *Ixodes* spp., and apathogenic viruses. Additionally, MBFVs divide into groups based on the mosquito vector interactions of the viruses, a *Culex* spp. group and an *Aedes* spp. group.

Flavivirus taxonomy is constantly expanding and flowing with advancements in analytical methods and updates as a result of virus discovery efforts[10].

1.1.3 Zika Virus

Zika virus (ZIKV), like many arboviruses, was discovered during research programs funded by the Rockefeller foundation from 1914 to 1970[11]. ZIKV was first isolated from rhesus macaque monkeys in 1947 and the first reported human case of Zika infection was in Nigeria in 1954[11,12]. The first ZIKV vector was identified as *Aedes africanus* in 1948[11]. In 2007, Micronesia was the first region to report an outbreak of Zika fever[13]. Following Micronesia, the islands of French Polynesia had an epidemic in 2013 and 2014[14]. Zika first landed in the Americas in 2015, in Brazil[15]. The full genome of ZIKV was sequenced in 2007 leading to the phylogenetic classification of ZIKV within the mosquito-borne flavivirus group and the Spondweni serogroup. In 2016, the World Health Organization classified ZIKV a global health emergency[16].

Common clinical symptoms of ZIKV infection include fever, rash, arthralgia, myalgia, conjunctivitis and fatigue[13,17]. More severe complications of ZIKV infection include onset of Guillain-Barre syndrome, congenital neurological complications, and congenital birth defects such as microcephaly[18,19]. Person-to-person spread of ZIKV is possible and has been shown via sexual intercourse and reported cases of perinatal transmission have been documented[20,21]. There are currently no U.S Food and Drug Administration (FDA) licensed antiviral treatments or vaccines available for ZIKV. Like the majority of arboviruses, prevention of disease can be achieved via prevention of mosquito bites and vector control strategies.

ZIKV is nearly distributed almost globally throughout tropical and subtropical regions. The main reservoir host for ZIKV is suspected to be non-human primates, as ZIKV antibodies

have been found in multiple monkey species in Asia and Africa[11,22–24]. Other animals have been shown to have ZIKV antibodies (rats, goats, rodents, and sheep) but there is little evidence that these animals can serve as reservoir hosts[24–26]. The primary vectors for ZIKV are *Aedes* spp. mosquitoes; *Aedes* mosquitoes play a critical role in the transmission of ZIKV via their presence in the sylvatic and urban cycles of transmission. In addition to reservoir hosts such as primates, ZIKV is thought to be able to maintain itself within a mosquito population through vertical transmission, albeit at low rates[27,28]. Areas with competent vectors, primarily *Aedes aegypti* and *Ae. albopictus*, are at risk of possible ZIKV circulation. Imported cases of Zika virus have been frequently reported and raise the risk of persistence and epidemic transmission[29,30]. Currently there are no countries experiencing a ZIKV outbreak but with temperatures rising and populations of *Aedes* mosquitoes moving to new environments ZIKV still remains a global threat[31,32].

1.1.4 West Nile Virus

In the early 1900s West Nile virus (WNV) was first isolated from an ill patient in Uganda[33]. Until the 1990s, WNV outbreaks were observed in Israel and Africa but were minimal and infrequently reported[34–36]. However, the introduction of WNV to the Americas lead to its notoriety. In 1999, New York City reported its first cases of WNV and by 2000, Washington D.C. and 12 states had reported cases of WNV[37,38]. By 2003, WNV reached the Pacific coast, and in 2006, other regions of North America and the Caribbean were reporting cases[39,40].

West Nile virus disease ranges in its clinical symptoms. WNV is a member of the Japanese encephalitis serogroup with St. Louis encephalitis, and other flaviviruses. Most human infections are mild or asymptomatic, however, less often WNV can manifest into febrile

symptoms such as fever and myalgia, and to severe disease such as encephalitis or death[41,42]. Roughly 13,000 cases were reported to the CDC between 2005 and 2009, and 35% of infections resulted in severe forms of neuroinvasive disease[41–43]. The severe form of the disease can become encephalitic and progress into a poliomyelitis-like syndrome with severe long-term effects[41,42]. Most individuals with encephalitic symptoms will develop coarse tremors[41–43]. Elderly individuals are more prone to severe disease and death following WNV infection. Current options for WNV treatment are limited to supportive therapies, as there are no FDA approved vaccines or antivirals. However, four (4) vaccines for WNV are available for equids[44–46]. Many WNV vaccines fail to enter phase three clinical trials, possibly due to unknown market potential. Like ZIKV, reducing exposure to vectors of WNV is the primary defense against infection.

West Nile virus is maintained in a sylvatic cycle by *Culex* mosquitoes and the most common reservoir are birds, within the United States the American Robin[47,48]. Humans do not act as an amplification hosts and are considered dead-end hosts because inadequate viremia is achieved to subsequently infect feeding mosquitoes. Even though *Culex* mosquitoes are accepted as the main vector for WNV, there have been cases of *Aedes* spp. mosquitoes infected with WNV and vertically transmitting the virus [49, 50]. *Aedes* mosquitoes pose an increased risk of humans acquiring a WNV infection *because Aedes* mosquitoes are known to commonly feed on mammals. West Nile virus exists with a global distribution; with expanding host and vector ranges and a lack of vaccines WNV remains a threat to populations globally.

1.1.5 St. Louis Encephalitis Virus

The first case of St. Louis encephalitis virus (SLEV) was discovered in 1933 when the virus was isolated from the brain of a patient with encephalitic disease in St. Louis, Missouri[51]. There have been multiple well-documented St. Louis encephalitis cases in the United States since Paris, Louisiana in 1933 through Fresno, California in 2020[52,53].

SLEV belongs to the same serogroup as WNV and Japanese encephalitis, therefore has similar antigenic characteristics and many similar clinical aspects of disease. Like the majority of flavivirus infections, infection begins after being bitten by an infected mosquito, then the virus migrates to the lymph nodes where it replicates and has a chance to cross the blood brain barrier[54]. In most cases the virus never crosses the blood brain barrier and non-specific symptoms such as fever, diarrhea, myalgia and vomiting may be observed[55,56]. Conversely, if the pathogen does cross the blood brain barrier, encephalitic complications may occur along with other disruptions to the central nervous system, such as lymphocytic meningitis or seizures[55]. Developing encephalitic symptoms is very common in elderly patients and the immunocompromised but in healthy young adults' symptoms can remain minimal. There are no effective antivirals or vaccines for SLEV so current care is only supportive therapy. Avoiding exposure to infected mosquitoes is the best defense against SLEV infection.

St. Louis encephalitis virus is a mosquito-borne flavivirus and the main vectors are *Culex* mosquitoes, specifically Cx. *pipiens*, *Cx*. *quinquefasciatus*, and *Cx. tarsalis*[57]. Wild birds, such as pigeons, robins, and blue jays, act as reservoir hosts for the virus[58]. Most cases of SLEV are detected during warmer months of temperate areas when mosquitoes are active but can occur throughout the year in tropical environments. Cases of St. Louis encephalitis range from Canada

down to Argentina, though the majority of cases occur in the United States[51,55]. Both *Culex* mosquitoes and the birds that act as reservoirs for SLEV are commonly found in developed areas thus increasing the risk of transmission into humans[56,57].

1.1.6 Japanese Encephalitis Virus

Records of Japanese encephalitis virus (JEV) date back to 19th century Japan, and subsequent outbreaks have been recorded throughout the 1900s in Japan[59]. During the 1900s, JEV spread throughout SE Asia and neighboring countries in the Pacific. By 1983 it reached as far west as Pakistan[58].

JEV is a major cause of encephalitis in Asia. The pathogenesis of the virus is not as well documented in the early events following transmission but it is thought that infection begins locally in the cells surrounding the bite and then spreads to the brain[60]. Japanese encephalitis virus will infect neurons, microglia, and astrocytes leading to inflammation in the brain[61–63]. Encephalitis is typically the most severe complication caused by infection, encephalitis could lead to neuronal cell death and further complications[62,64]. In some cases, motor neurons in the spinal cord could be affected and lead to acute flaccid paralysis[65]. There are no current therapeutics for JEV infection, but a live-attenuated clinical vaccine exists for JEV and is used in countries where the virus is endemic[66].

Migratory wading and water birds, such as herons, egrets, and ducks act as the main reservoir hosts for JEV[67]. In addition to migratory birds, pigs and possibly bats act as reservoirs for JEV[65]. Furthermore, pigs contribute to a constant circulation of JEV because they will remain year-round while many disease-carrying birds may migrate[68–70]. Horizontal transmission has been documented to occur between pigs contributing to JEV maintenance over winter months

and increasing epidemic potential in warmer months with increased mosquito presence[66-68]. Continued growth of agriculture for ever-growing human populations could contribute to the epidemic potential of JEV. Humans are not able to produce high enough titers of the virus to act as a reservoir so they are considered dead end hosts of JEV. Vectors of JEV are commonly mosquitoes in the *Culex* genera[67]. Due to climate change affecting the geographic range of *Culex* and *Aedes* mosquitoes and the migratory nature of JEV reservoir hosts, the potential of JEV to spread to areas not endemic with JEV is especially concerning. Currently over 2 billion people live in areas endemic with JEV[66]. To date, clinical infections of JEV have been recorded in most regions in Asia, northern parts of Australia and recently been recorded in one African country[71]. Though no clinical cases have been recorded, JEV has been isolated from birds in European countries[72]. Countries not endemic with JEV do not regularly recommend JEV vaccines and therefore have very little protection from transmission other than general mosquito control.

1.2 Chimeric Viruses

Chimeric viruses are viruses that consist of genomic material from two or more viruses. For example, structural genes from one virus can be combined with the nonstructural gene cassette of another virus to create a chimera. For the purposes of this research, chimeric viruses will refer to a flavivirus consisting of some structural proteins (prM and E) from “virus x” and the “virus y” capsid (C) and nonstructural proteins (NS1-NS5).

Chimeric viruses can offer insights into virus-host interactions and many vaccine efforts employ chimeric viruses because the combined genome may generate a different viral-interaction than an otherwise unaltered virus[73–75]. Virus-host interaction studies utilizing

chimeric viruses have shown that viruses with chimeric non-structural components can offer different effects regarding virulence, competence, and replication[74]. The changes in infectivity characteristics of the resultant chimeric virus can facilitate a better understanding of how viruses interact with cells at a structural and nonstructural level.

1.3 Superinfection Exclusion

Viral exclusion has been well documented across many different families of viruses[76–79]. Superinfection exclusion (SIE) occurs when a virus (virus A) infects a cell/host and prevents a secondary virus (virus B) from infecting the same cell/host. Flaviviruses are a notable group of viruses that demonstrate this exclusionary effect[78,80,81]. Because flavivirus infection spans the duration of a vector's lifespan it is possible to have two viruses infect the same host. There are two main types of exclusionary scenarios; heterologous exclusion (two different genera of viruses interacting) and homologous exclusion (two viruses of the same genera interacting). As mosquitoes are vectors for many viruses from different families we are able to see different forms of exclusion in mosquitoes and their cell lines. Superinfection exclusion though well documented, is not well understood. There are many factors that can lead to exclusion and it may not be virus specific. There are a few concepts underlying the guiding principal behind superinfection exclusion. First, the immune system (i.e. innate responses and RNAi) acting as the main component of superinfection exclusion is thought to be a large contributor to homologous exclusion[82]. In addition to the immune system, viral proteins could inhibit the subsequent infection of competing virus, as seen with alphaviruses and the nsp2 protease[83]. However, recent research has highlighted possible flaws in the evidence for the nsp2 protease causing SIE[84]. Lastly, the concept of competition for resources, regarding the use of cell-host

machinery to replicate virus, could also be a contributor to SIE[85]. The competitive dynamics regarding the use of cellular machinery is thought to be much more prevalent with homologous superinfections because viruses of the same family replicate in similar ways while heterologous infections may not replicate at the same sites in the cell (e.g. the nucleus vs the cytoplasm). Currently, the mechanism for superinfection exclusion with flaviviruses is unknown.

1.4 Insect-specific Flaviviruses

Within the family *Flaviviridae*, there are two groups of insect-specific flaviviruses, the classical ISFV group (cISFV) and the dual host ISFV group (dISFV)[10]. The classical group is phylogenetically distinct from other flavivirus groups, unlike dISFV which are affiliated with the mosquito-borne flaviviruses (MBFV). cISFV have been isolated from multiple Dipteran families (midges, sand flies, and mosquitoes) in many regions around the world. The less characterized group, dISFV, sits within the main MBFV clade. There are far fewer dISFV isolated to date and even fewer that are well characterized[10,86]. Due to the phylogenetic resemblance to the mosquito-borne flaviviruses it is thought that dISFV recently lost the phenotypic trait to infect vertebrate cells, the name “dual-host” is to represent the idea that dISFV recently lost capability to infect vertebrate cells[10]. Within the dISFV group there are two sub-divisions. The divisions are based on the theorized loss of the ability to infect vertebrate hosts occurring at separate moments in time. The groups are divided with Nhumirim virus, Barkedji virus, Nounane virus forming one group and Chaoyang virus, Lammi virus, Donggang virus, and Ilomantsi virus form the other group[86]. The group including Nhumirim virus shares a most recent common ancestor with the MBFV clade. None of the ISFV groups (classical or dual host) have been isolated from any vertebrates nor are they able to infect vertebrate cell lines[73,78,86–

[88]. Both groups are thought to be maintained in the population via horizontal and vertical transmission, as demonstrated by Culex flavivirus[81].

1.4.1 Nhumirim Virus

Nhumirim virus (NHUV), a well-characterized ISFV reported by Kenney *et al*. was isolated from *Culex chidesteri* mosquitoes in Brazil[89]. Sequencing of the genome of NHUV shows that it has similar nucleotide sequence identity to Barkedji virus and Nounane virus (65.9 and 56.2% respectively)[89]. As stated previously, NHUV is unable to replicate in vertebrates even though it is very closely related to MBFVs. Analysis of NHUV shows that it has a similar codon usage to MBFVs and it has many conserved structures and sequences associated with viruses in the mosquito-borne flavivirus group[78,89].

Previous work has tested NHUV for SIE effects *in vitro* and *in vivo*[78,80,89]. *Aedes albopictus* cells were challenged with NHUV and WNV, JEV, or SLEV in a co-infection and a delayed secondary infection *in vitro*. Nhumirim virus had an inhibitory effect on all of the viruses at varying efficacies, anywhere from 1.2-6.2 $\log_{10}$ PFU/mL[89]. The effects of the *in vitro* study were promising and lead to an *in vivo* study by Romo *et al.* and Goenaga *et al.* in *Aedes aegypti* mosquitoes, with ZIKV and NHUV, and WNV and NHUV[78,80]. Their studies showed superinfection exclusion *in vivo* had similar effects where NHUV did not eliminate a virus from infection but lowered the level of infection. In a mosquito the reduced degree of infection was able to lower transmission rates of ZIKV and WNV *in vivo*[78,80]. The phylogenetic similarity and similar codon usage patterns could be the driving force underlying the reduced viral transmission in mosquitoes[89].

1.5 Arbovirus Control Strategies

The need for arboviral control strategies is as significant as ever. Climate change has been shown to effect vector ranges and increased involvement of zoonotic cycles mean that arboviral disease could increase over the coming years[90,91]. There are several strategies developed for the defense against arboviral infections. Vaccinations are an incredible defense against viral disease, however, the U.S Food and Drug Administration has only approved the use of three vaccines for arbo-flaviviruses and total population vaccination is an extremely difficult task due to immunocompromised persons and phobia surrounding vaccines. Additionally, vaccinating possible reservoir hosts for viruses is expensive and often not feasible, furthermore, vaccines are unavailable for the vast majority of flavivirus pathogens. Vector population control is an effective strategy, but eliminating entire populations of vectors can be considered an ethical dilemma as well an expensive task. Personal protection from virus-spreading vectors is cost efficient and simple to do, however, many repellents cannot be used on infants and complete protection from biting vectors is a difficult task for everyday life. Lastly, the restriction of viral infection within the vector itself with methods like *Wolbachia* and genetic modification is costly and, in many cases, are not adequately supported with sufficient research evidence to be effectively implemented in the field. No single strategy is perfect, but when several strategies are implemented together, the risk of acquiring an arboviral disease drops considerably[92].

1.5.1 Flavivirus Vaccines

Vaccines can be an efficient and reliable strategy in the defense against viral pathogens. Developed to bolster the immune system against the virus of interest, vaccines can protect a

population when effectively implemented. However, not everyone can or will use vaccines. The immunocompromised are sometimes unable to receive vaccines due to contraindications, while other individuals may have fears of vaccine-related adverse events or refuse to accept the evidence of vaccine efficacy and safety. In addition to complications for administering a vaccine to a populous, the costs for vaccinating entire populations for multiple diseases can become high. It can cost roughly 3 billion dollars for research and development efforts for a single infectious disease vaccine[93]. Even if an entire population could be vaccinated for a virus, there are only three FDA approved flavivirus vaccines currently approved for human use - yellow fever virus, Japanese encephalitis virus, and dengue virus[94]. Currently, many other flaviviruses have vaccines in development and there are more vaccines available for veterinary use (e.g. WNV and JEV)[45,95]. Although animal vaccines do not directly protect humans these vaccines can eliminate reservoirs for the viruses thus reducing the risk of disease transferred to humans. In conjunction with current endemic diseases, there are emerging diseases that are difficult to predict and anticipate, therefore vaccines are not the best choice for a preemptive defense.

1.5.2 Vector Population Control

Managing mosquito populations can be as easy as dumping out a bucket after a heavy rain or as difficult as genetically modifying millions of mosquitoes at a time. Population control is a great tool to mitigate viral transmission because in the absence of vectors, there is no vector-borne disease. However, there are many hurdles for population control strategies to overcome in order to be as efficient as possible.

Source control techniques for mosquito control include removing any sources of standing water, introducing predators into the environment, and increasing water flow to areas

with stagnant water. Most mosquitoes will selectively lay eggs in still and stagnant water. By checking for stagnant water in easily manipulated areas and removing the water entirely or increasing the flow can lower mosquito populations dramatically. Additionally, introducing native predators of mosquitoes, a form of biocontrol, into areas where standing water cannot be altered can reduce vector populations as well (e.g. fish, predatory insects, etc.)[96,97]. Using these methods can reduce mosquito-borne viruses without dramatically affecting ecosystems and the general population is able to incorporate these methods at the individual and local level. However, introducing predators is not always fool proof, introduction of non-native predators or other invasive species can have major consequences for the ecosystem.

Chemical control strategies for arthropod pests are common all over the world. Chemical interventions are a useful tool for agriculture and vector control. Insecticides are able to eliminate populations very quickly but at times can be a high financial cost[98]. However, insecticides have been shown to have side effects; overuse of chemicals can affect bodies of water creating chemical imbalances affecting the ecosystem within. Certain insecticides, though effective, can affect more than one species of arthropod and thus have collateral damage against very important members of an ecosystem, such as pollinators and natural predators of other pests[99]. Many times, there are unforeseen issues with chemical insecticides that are unknown at the time. For example, DDT was a chemical used in World War Two to control typhus[100]. Public sales of the chemical became available to consumers in the United States; it was then later discovered to have severe effects on wildlife, specifically birds, and is now banned from use[101,102]. Additional dilemmas with insecticide use include the ability for arthropods to develop resistance to overused insecticides. Current insecticides for vector

control are rigorously tested for safety and non-target effects as well as efficacy. Insecticides may not perfect and effects on ecosystems are still observed, but the need for vector control in endemic areas must be solved in the most efficient way possible.

A relatively new form of vector control that has been in headlines is the use of modified mosquitoes to reduce mosquito population sizes. The technique is usually done by genetically modifying or irradiating male mosquitoes to cause sterility[103,104]. This Sterile Insect Technique (SIT) has been used before in America[105]. SIT was used in 1958 to eliminate the primary screwworm, the method was incredibly successful and the primary screwworm was eliminated from the United States and even Mexico[106]. Recently in 2020, SIT was approved for use in Florida to control *Aedes aegypti* mosquitoes. Although the method is extremely efficient there are concerns for unknown side effects because it is a relatively new method being used.

1.5.3 Personal Protective Equipment

For the average modern American, personal protection from mosquitoes is typically associated with camping or sitting outside in the evening and trying to avoid the itchy bumps that mosquito bites cause. However, for people living in places endemic with mosquito-borne viruses, the need for personal protection from vectors is much more serious. Repellents such as diethyltoluamide (DEET) and picaridin are often used to repel mosquitoes and prevent the transmission of disease, and in some areas, insecticide-treated bed nets are used to ensure mosquitoes cannot bite while humans are sleeping. However, repellents wear off without reapplication and bed nets are difficult to maintain; methods of personal protection are not a guarantee but an extreme deterrent for transmission of mosquito-borne viruses.

1.5.4 Vector Immunity to Infection

To effectively prevent or reduce the spread of vector-borne disease, the cycle of the infectious disease must be interrupted. There are many strategies developed to stop the cycle of infection at the ultimate stage, mosquito-human interactions, however a new approach to ending the chain of infection has slowly been gaining traction. Preventing the infection at the stage of the mosquito could be an effective approach to interrupting the cycle of virus transmission.

A promising approach to altering vector competence in mosquitoes are species of *Wolbachia*, an alphaproteobacteria that has been shown to lower viral infection and transmission in *Ae. aegypti*[107,108]. *Wolbachia* has many characteristics that make it a strong candidate for vector control. *Wolbachia* is widespread in insect communities especially those in the order Diptera, *it* is a vertically transmitted symbiont normally transmitted maternally and lastly, *Wolbachia* will sometimes increase fitness advantage thus driving the spread through a population[109]. When mosquitoes carrying *Wolbachia* were released into a community in Indonesia the dengue incidence rate was reduced by 76%[110]. *Wolbachia's* ability to limit viral infection of mosquitoes is a promising lead into interrupting the cycle of infection at the mosquito-level, instead of the more common method of interrupting infection at mosquito-human interaction.

Innate immunity in mosquitoes is robust and research into developing a more resistant immune system in mosquitoes to rid infection at the vector level have been preliminary. An experiment involving the JAK/STAT pathway in mosquitoes to inhibit dengue infection has been done showing that increased expression of certain immune factors can limit infections within

vectors[111]. Increased expression of certain immune pathways and regulatory genes could lead to new ways to regulate mosquito immunity and relieve pressure away from vector control at the population level.

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Chapter 2

Battle of the Viruses: Aripo Virus Induced Superinfection Exclusion of Mosquito-borne Viruses

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2.1 Abstract

Mosquito-borne viruses are a burden to global health. Flaviviruses, debilitating viruses spread by ticks and mosquitoes, are at risk of expanding to new areas due to increasing temperatures worldwide. Many countries, in both the developing world and not, are concerned with the continued effects of arboviral disease. While the research on reducing the spread of arboviral disease is vast, there is limited research on specifically reducing competence of the vectors themselves. Aripo virus (ARPV), an insect-specific flavivirus could lend a hand in discovering a novel approach to arbovirus control. Aripo virus was tested for superinfection exclusion potential *in vitro* by infecting C6/36 cells with ARPV and vertebrate infectious virus in a pre- or co-infection, samples were then taken to assess growth kinetics. Aripo virus, similar to Nhumirim virus, proved to significantly reduce titers of West Nile virus, St. Louis encephalitis

virus, Japanese encephalitis virus, Zika virus, and Mayaro Virus, an alphavirus, during pre- and co-infection studies. West Nile virus was used to attempt to overcome superinfection exclusion barriers through adaptive passaging. WNV, when co-infected with ARPV, was unable to overcome SIE barriers over nine passages. Lastly, C6/36 cells were infected with ARPV and chimeric viruses to further understand ARPV-viral interactions; the chimeric virus interactions showed reductions of chimeric virus growth when in the presence of ARPV. Aripo virus and superinfection exclusion could lead to new techniques to reducing viral load in mosquitoes thus reducing transmission of mosquito-borne viral disease.

KEY WORDS Flavivirus, Superinfection Exclusion, Insect-specific Flavivirus, Aripo Virus

2.2 Introduction

Flaviviridae is a family of viruses that is commonly trafficked by arthropod vectors, such as ticks and mosquitoes. Flaviviruses are single-stranded, positive-sense, enveloped RNA viruses. Virions are typically 50nm in diameter and consist of three structural proteins and seven non-structural proteins. These small RNA viruses are found all over the world, more often in tropical and sub-tropical locations. Flaviviruses make up a large phylogenetic genus broadly organized by vector-virus-host relations; the viruses focused on in this paper are mosquito-borne flaviviruses (MBFV). The MBFV are transmitted by two main genera of mosquito, *Aedes spp.* and *Culex spp.,* these mosquitoes transmit viruses like Zika (ZIKV), West Nile (WNV), and dengue virus. Flavivirus infection can result in a range of disease symptoms, from mild illness to encephalitic debilitation and even death. Many viruses within the genus have no licensed vaccines or therapeutics and continue to affect upwards of 400 million people every year[1,2].

The need for arboviral control strategies is as significant as ever. Climate change has been shown to effect vector ranges and increased involvement of zoonotic cycles mean that arboviral disease could increase over the coming years[3–5]. Arbovirus control comes in many different forms affecting different stages of the transmission cycle. Strategies can include mosquito population control, reducing mosquito-human interaction (repellents), and vaccines to prevent infection of hosts. An often-neglected method of arbovirus control is limiting the infection of mosquitoes. Often times a MBFV requires a reservoir to maintain the virus in an enzootic cycle; practically speaking it is impossible to stop transmission during the enzootic transmission cycle for many viruses due to unknown reservoirs or high costs of reservoir control strategies. However, eliminating infection of mosquitoes is possible. Mass rearing of modified mosquitoes has been done to scale and has been approved for implementation in the united states[6,7]. Methods such as *Wolbachia* have been shown to reduce viral load in mosquito populations but has not had enough research to be implemented globally[8,9]. This paper aims to further research in the process of eliminating mosquito borne disease at mosquito infection.

Dual host insect-specific flaviviruses (ISFV) are within the main MBFV clade of vertebrate infectious flaviviruses(VIF)[10]. ISFV have interesting niche characteristics, the genetic similarities of ISFV to vertebrate infectious flaviviruses, the inability to replicate in vertebrate cells, and the ability to maintain itself in a mosquito population have led to diverse and interesting topics of research[11,12]. The most notable being the ability to lead to superinfection exclusion in mosquito cultures. Superinfection exclusion (SIE) occurs when a virus (virus A) infects a cell/host and prevents a secondary virus (virus B) from infecting the same cell/host. Insect-specific flaviviruses are a notable group of viruses that demonstrate this exclusionary effect.

Superinfection exclusion though well documented, is not well understood. Viruses such as Nhumirim (NHUV) and Nounane virus have shown SIE properties with other flaviviruses in mosquito cell lines[13–15]. Aripo virus (ARPV), a new insect-specific virus isolated from *Psorophora albipes* mosquitoes in Trinidad, phylogenetically lies within the same group of ISFV as NHUV and others listed above and share similar characteristics. In this study we determine the SIE properties of ARPV and compare with NHUV, a similar ISFV known to have robust *in vitro* exclusionary properties.

The goal of this study was to understand ARPV effects on vertebrate infectious viruses (VIV) in mosquito cells. The specific objectives were to study ARPV SIE in the context of four flaviviruses and one alphavirus to determine the degree of exclusion if any in C6/36 cells. Additionally, attempts to mediate a genetic change in WNV to improve growth potential with ARPV in culture. Lastly, we wanted to explore how ARPV interacts with chimeric viruses.

2.3 Materials Methods

2.3.1 Viruses and Cells

Japanese encephalitis virus (JEV), Mayaro virus (MAYV), St. Louis encephalitis virus (SLEV), WNV, ZIKV, and two chimeric viruses (WNVprME/SLEV non-structural and SLEVprME/WNV non-structural) were grown on African green monkey kidney (Vero 76) cells (ATCC: CRL-1587) and harvested when 50% cytopathic effect (CPE) was observed. Aripo and Nhumirim viruses were grown on *Aedes albopictus* (C6/36) cells (ATCC: CRL-1660) and harvested when CPE was observed. Supernatants were collected and clarified by centrifugation for 10 minutes at 4,000 rpm at 4°C. Aliquots were frozen at -80°C until used. ARPV and NHUV were quantified via RT-qPCR using the following primers and probes: (NHUV: forward primer, 5'-

GAATGGCAGTGGAGAGGAGG-3'; reverse primer, 5'-CCTTCCATTACCACGTCCGG-3'; and probe, 5'-FAM-TGCGAGATGGCAGCGGCTCTGT-BHQ1-3' -- ARPV: forward primer, 5'-CGGTGTTCATTGAGGATGAC; reverse primer, 5'-TGATACGTCCAGGTTCGGTA); and probe, 5'-FAM-CGCTGCCTCATGGCAATTCG-BHQ1-3'), while all other viruses were quantified via plaque assay on Vero cells as previously described by Brault et al[16]. C6/36 cells and Vero 76 cells were maintained in CO_2 incubators at 28°C and 37°C respectively with 10% and 5% FBS DMEM (Dulbecco's Modified Eagle Medium, penicillin (100 U/ml), streptomycin (100 µg/ml), 1% non-essential amino acids, and 1% tryptose phosphate broth) respectively.

2.3.2 Superinfection Exclusion Growth Curves

Plates of C6/36 cells were seeded at the same time and density then infected with ARPV or NHUV at a multiplicity of infection (MOI) of 5. Cells were later infected with a vertebrate infectious virus - WNV, MAYV, ZIKV, SLEV, JEV, WNVprME/SLEV, or SLEVprME/WNV at a MOI of 0.1 at the same time as the ISFV infection (co-infection), or 24 hours after the initial infection allowing the ISFV to establish an initial infection (pre-infection). After a one-hour incubation with the vertebrate infectious virus, the monolayers were washed with PBS three times to remove excess virus and fresh maintenance media (5% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml) in DMEM) was added. In addition to the experimental groups, positive controls of the VIV were included, and infections were done with a MOI of 0.1 on uninfected C6/36 cells. Lastly, negative controls consisting of mock infections of C6/36 cells were also performed. For all groups, aliquots of supernatant were collected from triplicate cultures immediately following infection (0 hour) and at subsequent 24-hour intervals for 5 days. For every aliquot taken from the supernatant, an equal amount of media was added to maintain constant volume

throughout infection. Vertebrate infectious viruses were quantified from the supernatants by plaque assay on Vero cells, while ISFVs were quantified by qPCR.

2.3.3 Adaptive Passaging of WNV

An infectious clone-derived WNV (NY99 strain) was used to infect C6/36 cells over the course of 9 serial passages. A passage consists of infection with a one-hour incubation, after incubation the cells were covered with media and the infected cells were left at 28°C for two days. Three groups of C6/36 cells were infected in triplicate, one group containing WNV alone and two groups were infected with ARPV and WNV in a 1:1 ratio. Within the two ARPV-infected groups, one group was infected with ARPV at passage 0 and ARPV was never reintroduced into subsequent passages, while the other group was supplemented with additional ARPV during WNV infection at each subsequent passage. Supernatants were transferred to new cells (100uL or 10uL of the WNV-only group; approximately 4 logs of virus) from each replicate at every passage. WNV replication was quantified at passage 0, 3, 5, 7, and 9 to observe any changes in WNV titers. Passage 9 was used to perform a final growth curve (as described above in the superinfection exclusion section above for co-infection) to determine if WNV replication kinetics changed due to adaptation as a result of being in the presence of ARPV.

2.3.4 Statistics

To determine effects of ISFV on super infection exclusion, a two-way analysis of variance (involving two factors, days post-infection (DPI) and Virus) was conducted followed by Tukey's HSD to compare the different factors. All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of $\alpha = 0.05$.

2.4 Results

2.4.1 ARPV reduced vertebrate infectious virus replication during co-infection and SIE experiments

Insect-specific flaviviruses, such as NHUV, have previously shown SIE effects against several flaviviruses, however, this has not been explored with ARPV[13,15]. The effects of ARPV on other viruses *in vitro* will be tested alongside NHUV, a control ISFV, to determine the SIE

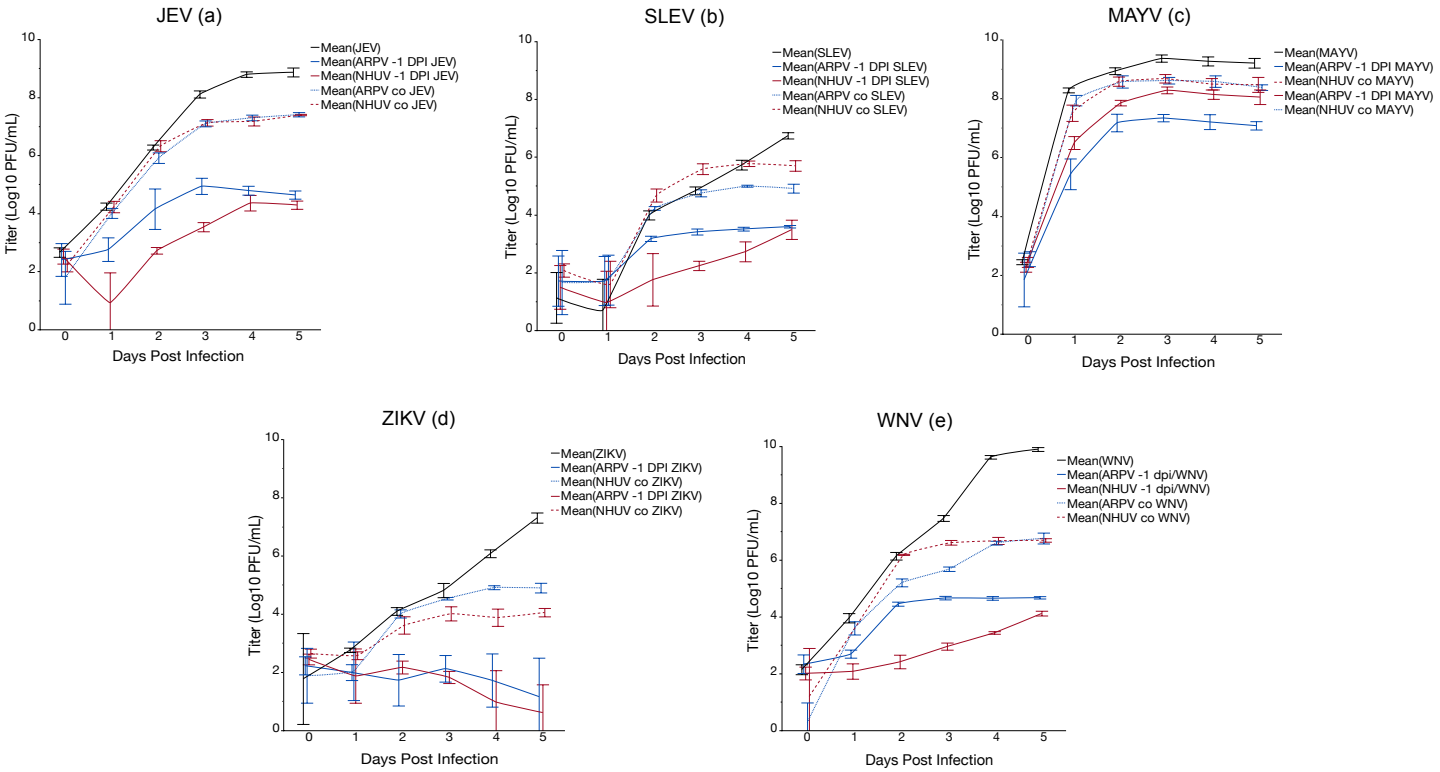

Figure 1 ARPV and NHUV reduce titers of vertebrate infectious virus in C6/36 cells. Growth curves were performed on C6/36 cells pre and co-infected with ARPV/ NHUV (MOI 5) and vertebrate infectious viruses (MOI 0.1) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves were constructed with mean pfu/mL via plaque assay in duplicate. The limit of detection for each group is 10 pfu/mL. Data plotted represents mean values and error bars were constructed using 1 standard deviation away from the mean.

potential of ARPV. C6/36 cells were infected in triplicate with either ARPV or NHUV 24-hours prior (pre-infection) or simultaneously (co-infection) then infected with a VIV to asses exclusionary effects. Both pre- and co-infections showed a reduction in VIV titers with both ARPV and NHUV. Overall, ARPV and NHUV co-infections reduced the ultimate titers of the VIV, but this was further exacerbated during the pre-infection experiments which presented the largest reduction in VIV titers over all viruses tested.

JEV (positive control; figure 1a) titers reached a peak titer of 8.9 logs pfu/ml at 5 DPI, while titers of JEV were significantly lower with ARPV pre-infection and co-infection ($p<0.05$; table 1). Peak titers were estimated at 4.9 logs pfu/ml (3 DPI) and at 7.4 logs pfu/ml (5 DPI) the the ARPV pre-infection and co-infection groups. JEV when pre-infected with NHUV peaked at 4.3 logs pfu/ml (4 DPI) and at 7.4 logs pfu/ml (5 DPI) as a co-infection. Both pre- and co-infection peak titers with NHUV were significantly different from the peak JEV positive control titers ($p<0.05$; table 1). At 5 DPI ARPV co-infection and NHUV co-infection with JEV were not significantly different (table 1). Additionally, JEV pre-infected with ARPV at 5 DPI was not significantly different from NHUV pre-infection at 5 DPI (table 1).

Figure 1b shows the effects of both ARPV and NHUV on SLEV replication *in vitro*. SLEV positive control (SLEVpos) titers reached a peak of 6.7 logs pfu/ml at 5 DPI, while titers of SLEV when pre-infected with ARPV peaked at 3.6 logs pfu/ml (5 DPI) and at 5 logs pfu/ml (4 DPI) during co-infection. Both ARPV pre- and co-infection group peak titers were significantly different from the SLEVpos peak titer ($p<0.05$; table 2). The NHUV pre-infection of SLEV (3.5 logs pfu/ml; 5 DPI) was significantly different from SLEVpos peak titer. However, the NHUV co-infection of NHUV (5.8 logs pfu/ml; 4 DPI) and SLEVpos groups were not significantly different

at their peak titers. At 5 DPI both pre-infections (ARPV and NHUV) showed no significant difference from each other. However, both pre-infected groups were significantly different from the co-infections at 5 DPI, and the co-infections were not significantly different from each other ($p<0.05$; table 2).

Figure 1c shows MAYV positive control (MAYVpos) titers reached a peak titer of 9.3 logs pfu/ml at 3 DPI, while titers of MAYV when pre-infected with ARPV peaked at 7.3 logs pfu/ml (3 DPI) and at 8.6 logs pfu/ml (3 DPI) as a co-infection. MAYV when infected with NHUV as a pre-infection peaked at 8.2 logs pfu/ml (3 DPI) and at 8.7 logs pfu/ml (3 DPI) during co-infection. All peak titers for both ARPV and NHUV pre- and co-infection groups against MAYV were significantly different from the MAYVpos peak titer, except for the MAYV co NHUV peak titer which was not significantly different from the peak MAYVpos titer ($p<0.05$; table 3). At 5 DPI MAYV pre-infected with ARPV was significantly different from the rest of the 5 DPI groups (MAYVpos, both co-infections, and the NHUV pre-infection). The co-infections and NHUV pre-infection were significantly different from the MAYVpos, but not statistcally different from eachother ($p<0.05$; table 1).

ZIKV (positive control; figure 1d) titers reached a peak titer of 7.3 logs pfu/ml (5 DPI). ZIKV titers were significantly lower when pre- or co-infected with ARPV ($p<0.05$; table 4). Pre-infection with ARPV peaked at 2.1 logs pfu/ml (3 DPI), while co-infection peaked at 4.9 logs pfu/ml by 4 DPI. Additionally, NHUV pre- or co-infections with ZIKV were significantly lower than the peak titer of the ZIKVpos group. ZIKV peaked at 2.2 logs pfu/ml (2 DPI) as a NHUV pre-infection and at 4.1 logs pfu/ml (5 DPI) as a co-infection. ZIKV co-infected with ARPV at 5 DPI was at a significantly higher titer than NHUV co-infection at 5 DPI. However, both ARPV and

NHUV when pre-infected with ZIKV were not significantly different at 5 DPI but were significantly different from both co-infections and the positive control at 5 DPI.

Figure 1e shows the SIE effects of ARPV and NHUV on WNV *in vitro*. WNV positive control titers achieved a peak titer of 9.9 logs pfu/ml at 5 DPI, while titers of WNV when infected with ARPV were significantly reduced and peaked at 4.7 logs pfu/ml (3 DPI) as a pre-infection and at 6.8 logs pfu/ml (5 DPI) during co-infection ($p<0.05$; table 5). WNV when infected with NHUV peaked at 4.1 logs pfu/ml (5 DPI) as a pre-infection and at 6.7 logs pfu/ml (5 DPI) during co-infection, both of which were significantly different from the WNV positive control. Both ARPV and NHUV when co-infected with WNV had similar titers at 5 DPI and were not statistically significant. Lastly, WNV pre-infected with ARPV at 5 DPI was not significantly different to NHUV pre-infection at 5 DPI.

Evaluating ISFV replication kinetics during the five-day infection periods is important for understanding reciprocal exclusionary effects with the VIVs. Our results suggest that both ISFVs replicated throughout the duration of infection, and achieved comparable peak titers among all experimental groups. Our results suggest that ISFV was present throughout the infection. Figure

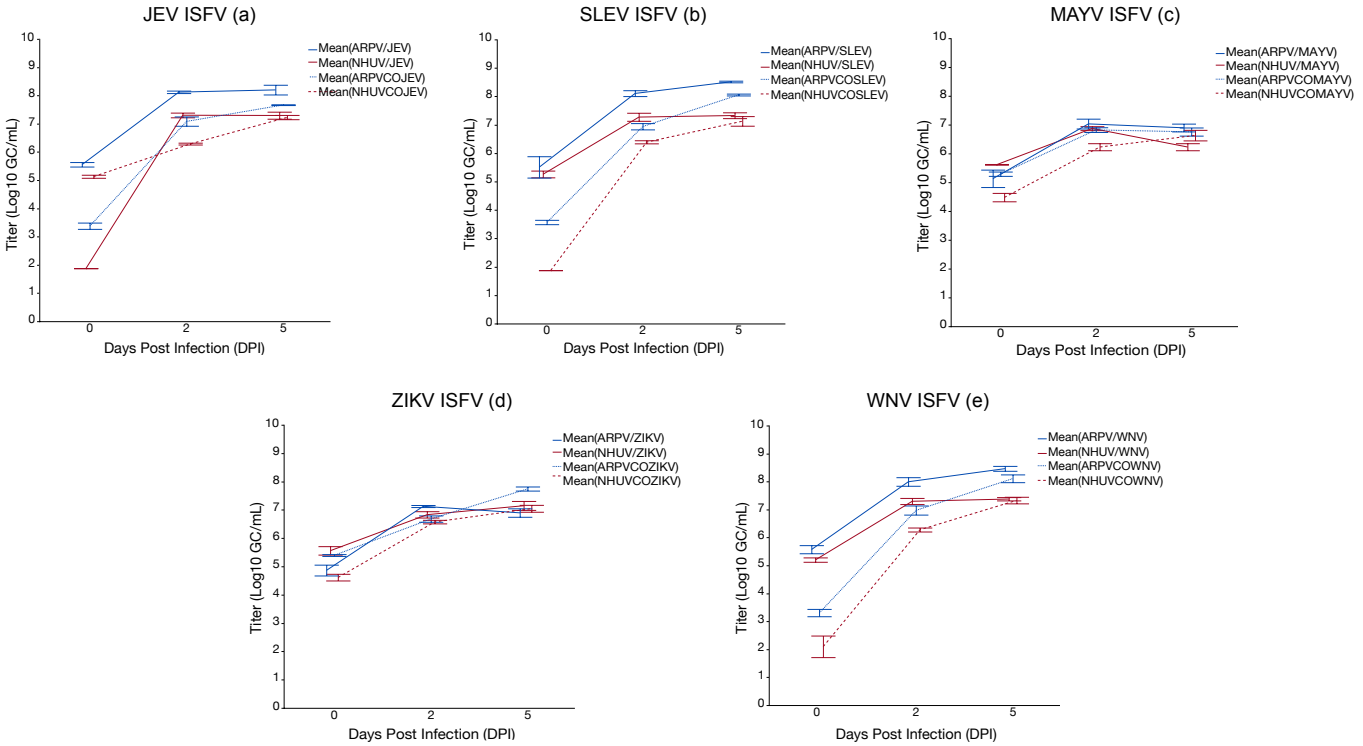

Figure 2 ARPV and NHUV titers in C6/36 cells when superinfected with vertebrate infectious virus. Growth curves were performed on C6/36 cells superinfected with vertebrate infectious viruses (MOI 0.1) and ARPV/ NHUV (pre-infection (-1 DPI) and co-infection (0 DPI); MOI 5) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves were constructed with mean GC/mL via qPCR in triplicate. Data plotted represents mean values and error bars were constructed using 1 standard deviation away from the mean.

2a-e shows RT-qPCR quantified titers in genome copies/mL for ARPV and NHUV from the *in vitro* experiments indicated in figure 1a-e. ISFV replication kinetics were therefore similar

among all experimental groups tested. ARPV titers at 5 DPI were approximately ~8 Log_{10} genome copies/mL (GC/mL), while NHUV titers were approximately ~7 Log_{10} GC/mL.

2.4.2 WNV was unable to revert to wildtype growth potential when passaged with ARPV

Previous SIE studies have shown that WNV, when competing with a WNV-replicon, was able to overcome the SIE barrier and revert to wildtype growth potential[17]. In this study, WNV

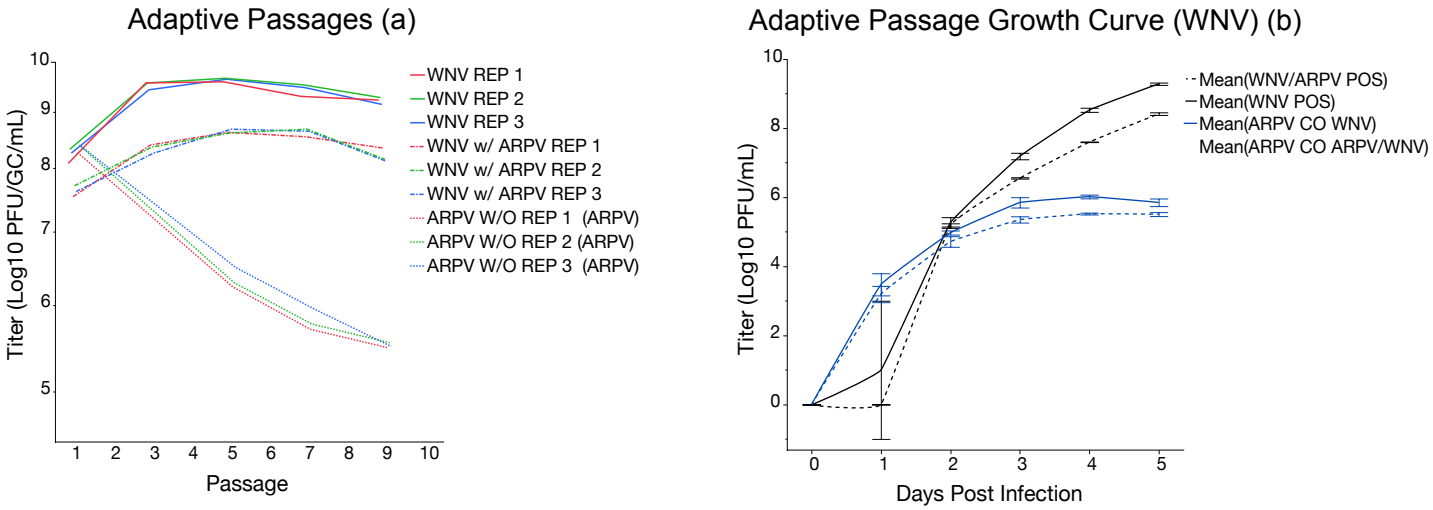

Figure 3 Adaptive passaging of WNV in C6/36 cells co-infected with ARPV. WNV titer at each passage (a), C6/36 cells were simultaneously infected with WNV and ARPV in a 1:1 ratio or only WNV and subsequently passaged in three isolated passages. ARPV was measured with qPCR while WNV was measured via plaque assay and measurements were taken at every other day. (b) Passage 9.1 from all groups was incubated for 5 days to form a growth curve to show WNVs potential to return to its normal growth potential. ARPV (MOI 5) was co-infected with the passage 9.1 WNV positive and WNV/ARPV isolates (MOI 0.1) to form the growth curve. Growth curves were constructed with mean pfu/mL via plaque assay in triplicate. Data plotted represents mean values and error bars were constructed using 1 standard deviation away from the mean.

was passaged with ARPV to encourage *in vitro* adaptation, which could potentially overcome the ARPV-induced SIE barriers. Exploring the adaptive potential of VIVs is essential for

understanding the utility of this strategy and related strategies as a countermeasure for reducing vector competence.

WNV replication, titers were measured via plaque assay on Vero cells. The titers for WNV that had not been exposed to ARPV (referred to as naïve-WNV) averaged 1 log higher than the WNV strain passaged with ARPV (referred to as neo-WNV). Figure 3a shows the serial passaging titers of naïve-WNV and neo-WNV. WNV reached an average peak titer of 9.5 Log_{10} pfu/ml, and neo-WNV reached an average peak titer of 8.5 Log_{10} pfu/ml. ARPV persisted throughout the 9 serial passages but reduced in titer with each passage, dropping to 5.5 Log_{10} GC/ml at passage 9.

Within the same study, WNV infections were repeatedly supplemented with additional ARPV at each subsequent passage. The WNV group then was undetectable by passage 3 (data not shown). The pressure exerted by repeatedly adding ARPV may have overwhelmed the cells, or WNV may have been unable to replicate and was eliminated beyond our limit of detection, the results are inconclusive.

In order to determine if neo-WNV was able to overcome ARPV exclusionary pressures, we next evaluated the replication kinetics of the passage 9 WNV to assess its growth in the presence of ARPV (figure 3b). Our SIE studies performed with the passage 9 supernatant showed that neo-WNV did lose fitness and reached a peak titer of 8.4 Log_{10} pfu/ml (5 DPI) while naïve-WNV reached a peak of 9.3 logs pfu/ml (5 DPI). When the supernatants were co-infected with ARPV, neo-WNV reached a peak titer of 5.6 Log_{10} pfu/ml (5 DPI) while naïve-WNV reached a peak of 6 Log_{10} pfu/ml (5 DPI), showing no significant difference between groups. Altogether the data suggests that WNV was unable to overcome the SIE barrier caused by ARPV *in vitro*.

2.4.3 Chimeric virus interactions with ISFV could be based on non-structural proteins

In order to asses sequence specificity and its potential relationship with SIE, we utilized chimeric viruses, specifically SLEV (prM and E proteins) with WNV non-structural proteins, and

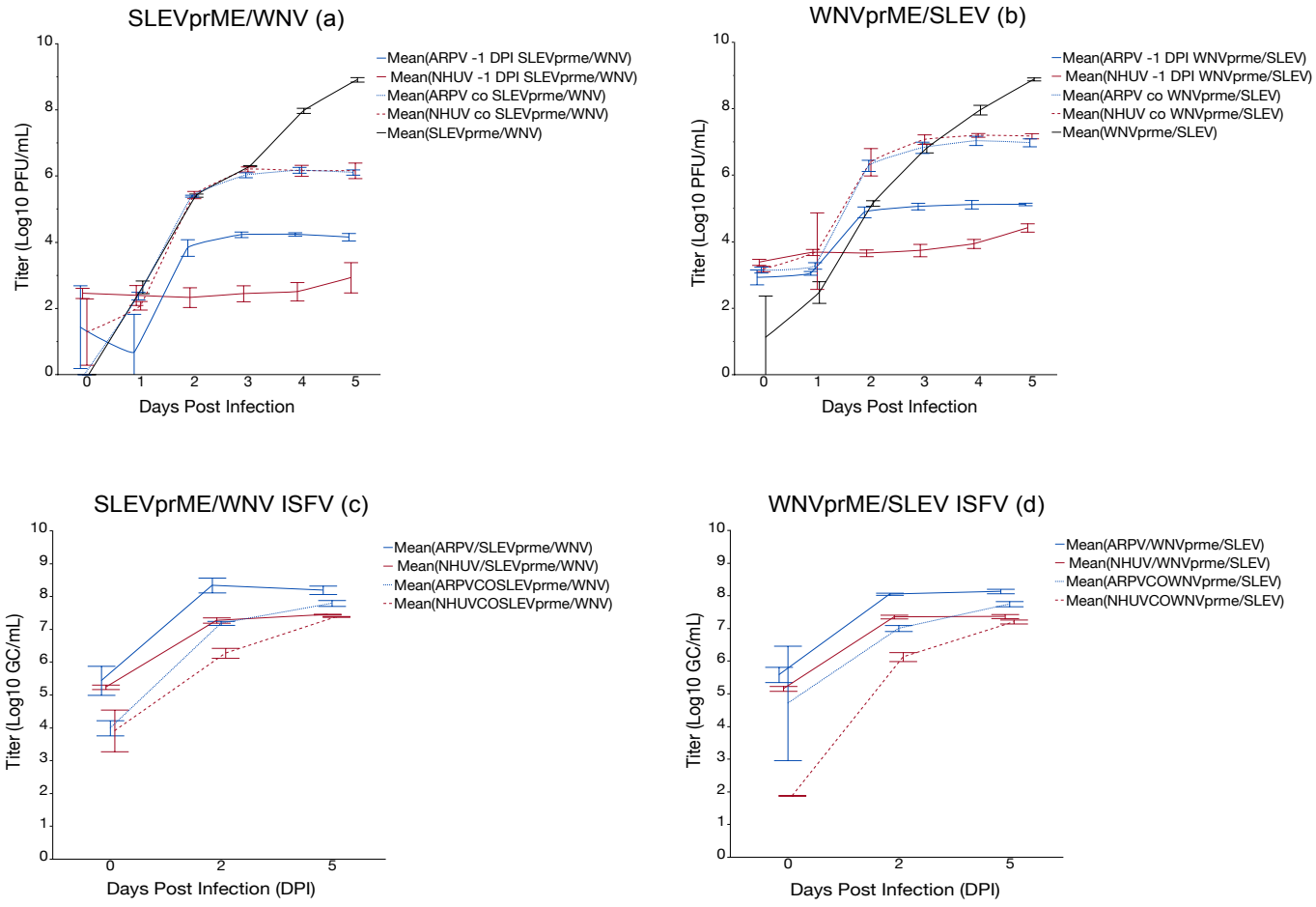

Figure 4 (a,b) ARPV reduces titers of chimeric vertebrate infectious virus in C6/36 cells. Growth curves (a,b,c,d) performed on C6/36 cells superinfected with vertebrate infectious viruses (MOI 0.1) and ARPV/ NHUV (pre-infection (-1 DPI) and co-infection (0 DPI); MOI 5) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves of the VIF chimeras (a,b) were constructed with mean pfu/mL via plaque assay in duplicate the limit of detection for each group was 10 pfu/mL. ARPV. Data plotted represents mean values and error bars were constructed using 1 standard deviation away from the mean.

vice versa. In addition to understanding sequence specificity, chimeric viruses could show a possible bias in how ISFV SIE affects virus gene sequences, such as structural or non-structural

genes. Following the same methods as the pre- and co-infection experiment described above, for both ARPV and NHUV, we explored replication kinetics among these to chimeric viruses. Both chimeric viruses (figure 4a-b) shared similar SIE growth trends with the parental viruses shown in figure 1. The WNVprME/SLEV chimera pre-infected with ARPV showed a significant decrease in growth at 5 days and a smaller but still significant reduction in growth during co-infection. For WNVprME/SLEV and NHUV, the co-infection produced a significant reduction in viral titers compared to the positive control, and the pre-infection of NHUV showed an even larger reduction, both of which were similar to the ARPV pre- and co-infection groups. SLEVprME/WNV and WNVprME/SLEV showed similar trends and responses to ISFV induced SIE. Both co-infections with NHUV or ARPV had significant reductions in viral titer at 5 DPI, while the pre-infection with ISFV had the greatest impact on viral titer by 5 DPI. RT-qPCR showed both ISFVs replicated efficiently to high titers throughout the experiment. (figure 4 c-d).

2.5 Discussion

There are many strategies developed to reduce the transmission of mosquito-borne flavivirus disease, but few of those strategies target the penultimate stage, mosquito- viral interactions. The experiments conducted in this study were designed to examine the impact of ARPV on other mosquito-borne viruses and to understand the specificity and degree of exclusionary effects. ARPV offers an approach to vector control down the road and aid in development of a possible means to an end of vector borne disease.

ARPV showed a significant exclusionary effect on the replication of all viruses tested, including MAYV, an alphavirus. Additionally, NHUV, the control ISFV, showed significant exclusionary effects across all viruses tested. This data trend is in agreeance with testing done

by Kenney et al[18]. A particularly interesting notable exclusionary effect was that of MAYV, an alphavirus. Superinfection exclusion has been documented with viruses in the same family before[19,20], however, within mosquitoes, superinfection by viruses from two differing families usually result in a co-infection rather than exclusion[21]. Although MAYV titers were reduced during ARPV superinfection, the alphavirus is still at a relatively high titer (7 logs pfu/mL), therefore the results could indicate a co-infection of the viruses rather than exclusion like we see with the flaviviruses. Continued experiments exploring the SIE effects on alphaviruses caused by ISFV infection should be done to further understand the results from this experiment.

ARPV showed major differences in SIE effect based on initial infection time of the ISFV. Pre-infection reduced titers significantly more across all viruses tested. Interestingly, although co-infections were not as effective as pre-infection, it reduced titers across all viruses as well. Co-infection SIE alludes to the possibility that competition for resources among viruses can lead to the differences seen in virus titers. Competition for resources could also offer some explanation to the exclusion of MAYV. Alphaviruses, like flaviviruses, replicate in the cytoplasm of the cell, therefore, some of the cellular machinery used by the vertebrate infectious viruses could be competed for by the ISFVs[22]. Studies have shown that WNV during superinfection is excluded due to competition for host cell machinery by one virus over the other[17,23,24]. This could be the cause of ARPV-based SIE within C6/36 cells. Additionally, previous studies of NHUV have mentioned similar codon usage patterns between NHUV and other flaviviruses[18]. The similar codon usage between flaviviruses could lead into the idea that SIE is seen because of the competition between codons. Because ARPV is closely related to NHUV, the codon usage

patterns could be similar and offer another source of competition regarding ARPV induced superinfection among VIFs. Further analysis of codon usage in ARPV could shed more light on the subject in the future. However, competition-based exclusion may not be the only cause of SIE. Figure 1 shows pre-infection having a greater exclusionary effect across all viruses compared to co-infection and may indicate a presence of an immunity-based exclusion in addition to competition-based exclusion. In order to further understand the effects ARPV and other ISFV have on mosquito innate-immunity, a transcriptomic study should be done in the future.

When passaged with ARPV, WNV showed no adaptation for normal growth potential, and an inability to evolve and overcome superinfection exclusionary barriers. The inability of WNV in regaining normal growth potential could be a result of inadequate time for adaptation, since 9 serial passages represent a limited number of passages for mutations to overtake a population, although adaptation to SIE changes have been seen in WNV in only seven passages[17]. Nonetheless, these data could highlight the fact that some exclusionary barriers are within highly conserved areas of the genome, as seen in alphaviruses with the nsp2 structure[22]. However, when comparing to previous studies for flaviviruses, SIE has been linked to the less conserved regions of the genome, such as the NS4a-b and 2k structures[17,23,24]. The relation of SIE to the less conserved regions of the genome could explain why cISFV do not have the same level of SIE that dual host ISFV have on VIF. Because cISFV are more removed from the MBFV clade of viruses the likelihood that the less conserved regions have been altered is much higher than when compared to the dual host clade.

ARPV had a similar trend across most of the viruses tested, VIV when pre-infected with ARPV began to plateau at 2 days post infection (Figure 1). The trend could be attributed to ARPVs phenotypic trait of causing intense CPE 2-3 days into an infection of C6/36 cells, explaining the plateau at 2 DPI in VIV titer pre-infection and a plateau in ISFV titer shown in Figure 2. The role of CPE in cells infected with ARPV could be a driving force for ARPVs ability to cause SIE in C6/36 cells. Further testing of cell mortality during infection could be done to further understand the role of CPE and cell death of SIE in C6/36 cells. Additionally, ARPV should be tested *in vivo* to measure the effects of ARPV on mosquito morbidity and mortality, as well as the SIE effects.

Previous studies suggest that RNA synthesis is a potential site resulting in SIE. In both alphaviruses and flaviviruses, mutations to structures with roles in RNA synthesis have reduced the effects of SIE *in vitro*[17,24–27]. In WNV, it has been shown that the 2k peptide has direct role in RNA synthesis and has an effect on SIE[17,24]. In alphaviruses, the nsp2 structure could have a role in viral RNA minus strand synthesis and has been shown to affect SIE[25]. Conversely, the research backing the hypothesis of nsp2 protease having a role in SIE has been shown to have inconclusive results[27]. However, the hypothesis of RNA synthesis being a point of contention in a superinfection is still very possible. Since alphaviruses and flaviviruses replicate in the cytoplasm and SIE is possibly affected by RNA synthesis, the exclusion generated by ARPV and NHUV in MAYV and flaviviruses could be attributed to inhibiting RNA synthesis. Additionally, SIE of the chimeric viruses, SLEVprme/WNV and WNVprme/SLEV, shows similar trends with the non-structural parental virus of the chimera. The similarities of growth and SIE effects between the chimeras and their non-structural counterparts lead to the hypothesis that non-structural

regions are affected by ISFV to induce SIE. The hypothesis of non-structural components factoring into SIE aligns with the belief that RNA synthesis is a critical step in the SIE mechanism.

2.6 Conclusions

Aripo virus has shown it is able to exclude several different flaviviruses and an important emerging alphavirus during superinfection scenarios such as pre- or co-infections. Pre-infection has a larger exclusionary effect than co-infection for both ARPV and NHUV, this could be the result of two possible mechanisms, (i) competition for resources in host cells, and (ii) possibly immunity-based exclusion. Our study attempting to facilitate adaptation in the face of SIE showed that WNV was unable to adapt and overcome ARPV-induced SIE over the nine passages done. Further studies are needed to more clearly determine the efficacy and utility of ARPV-induced SIE as a potential route to inhibiting transmission of arboviral disease. In addition to determining ARPV's SIE effects against arboviruses *in vivo,* experiments aimed at understanding effects of ARPV on mosquito morbidity and mortality are needed. Once SIE in both mosquito cells and live mosquito models is observed to be effective at reducing viral transmission, determining the mechanism underlying the restriction is the next step. Further exploration of the mechanism underlying SIE, such as experiments focusing on RNA synthesis should be performed, possibly using single-cell analysis of viral kinetics[27].

Identifying the mechanism underlying SIE could be the key step to eventually utilizing SIE to combat transmission of disease at the level of vector infection. By knowing the mechanism, the genome of a mosquito could be altered to express genes that induce SIE and prevent infection of the mosquito. Mosquito-borne disease is a concern around the globe and

discovering new strategies to reduce the burden of disease is imperative for the protection of billions at risk of mosquito-borne pathogens.

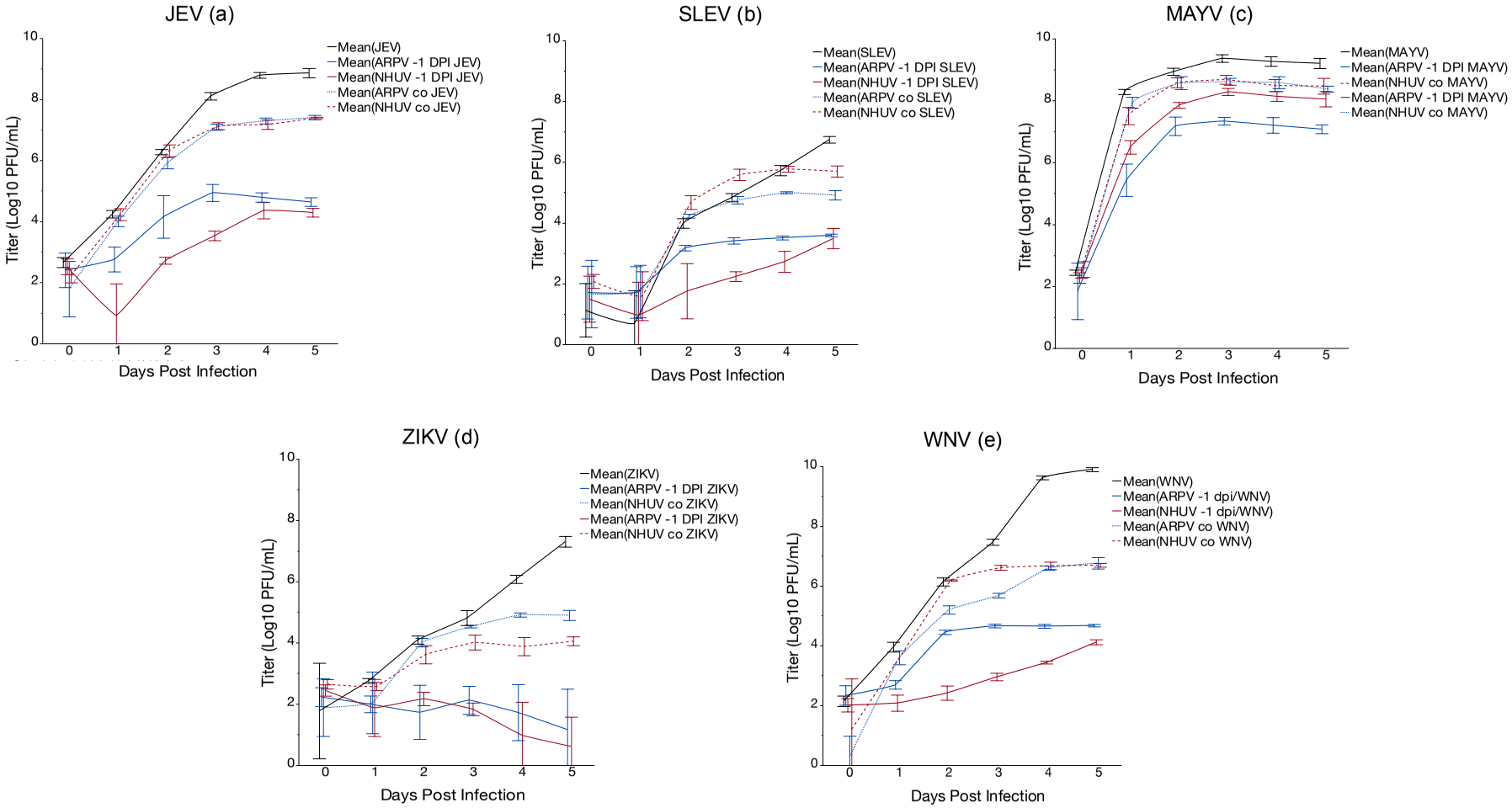

Figure 5 ARPV and NHUV reduce titers of vertebrate infectious virus in C6/36 cells. Growth curves were performed on C6/36 cells pre and co-infected with ARPV/ NHUV (MOI 5) and vertebrate infectious viruses (MOI 0.1) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves were constructed with mean pfu/mL via plaque assay in duplicate. The limit of

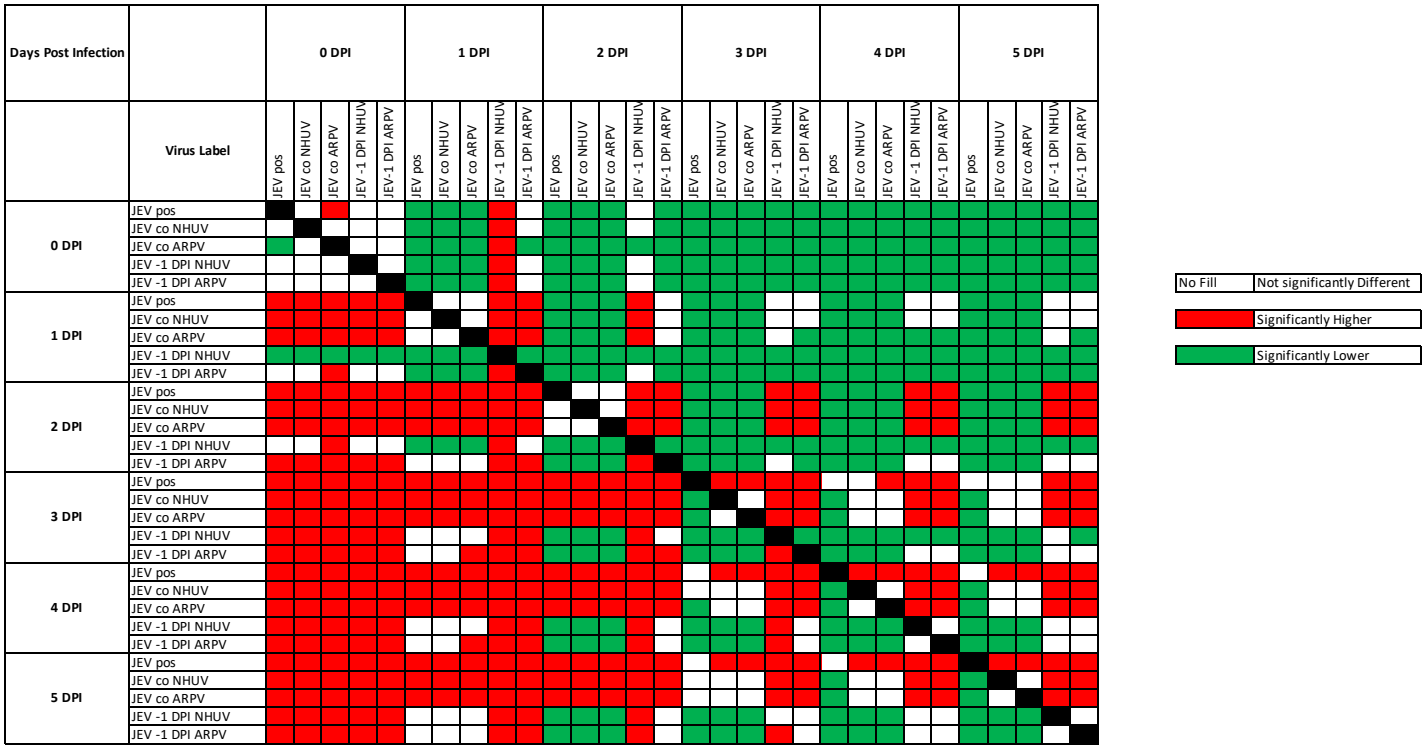

Table 1 Statistically significant values among experimental groups across all timepoints for Japanese encephalitis virus. Data presented in table 1 reflects the equivalent experiment shown in figure 1a, but instead indicates statistically significant comparisons. Red boxes indicate the row titer is significantly higher than the compared column titer, green boxes indicate the row titer is significantly lower, and white boxes indicate no significant difference between the row and column titers (Tukey's HSD test; $P > 0.05$). Mean based on three replications. *All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of $\alpha = 0.05$.*

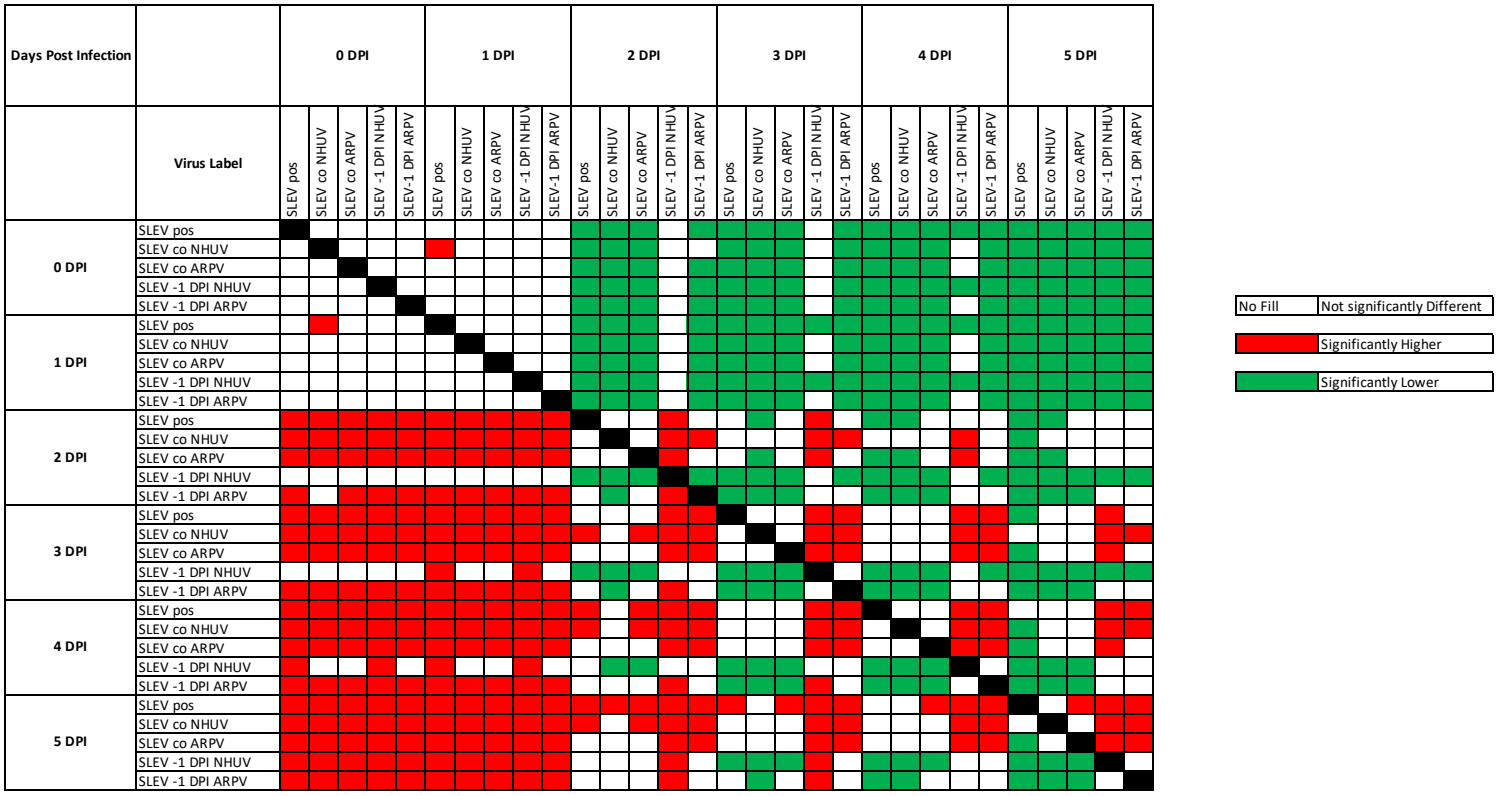

Table 2 Statistically significant values among experimental groups across all timepoints for St. Louis encephalitis virus. Data presented in table 1 reflects the equivalent experiment shown in figure 1b, but instead indicates statistically significant comparisons. Red boxes indicate the row titer is significantly higher than the compared column titer, green boxes indicate the row titer is significantly lower, and white boxes indicate no significant difference between the row and column titers (Tukey's HSD test; $P > 0.05$). Mean based on three replications. *All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of $\alpha = 0.05$.*

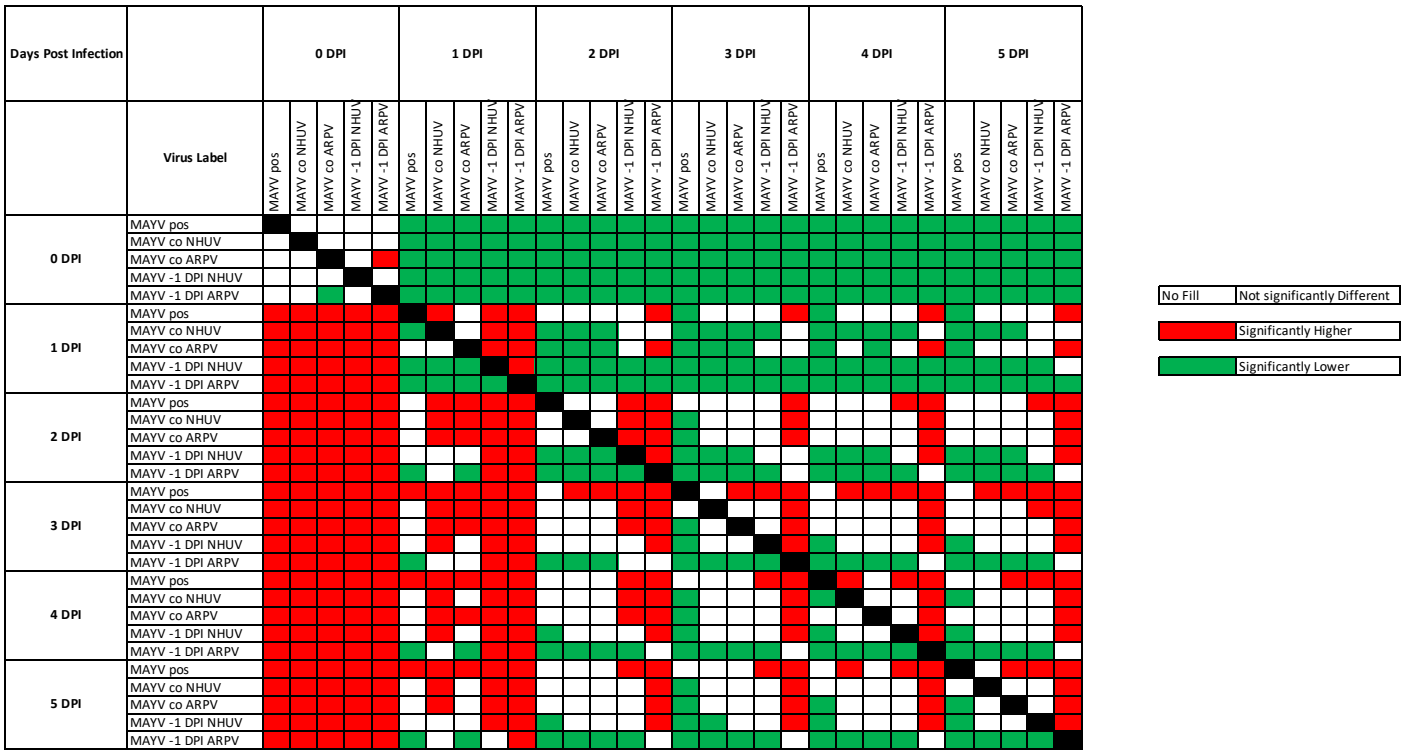

Table 3 Statistically significant values among experimental groups across all timepoints for Mayaro virus. Data presented in table 1 reflects the equivalent experiment shown in figure 1c, but instead indicates statistically significant comparisons. Red boxes indicate the row titer is significantly higher than the compared column titer, green boxes indicate the row titer is significantly lower, and white boxes indicate no significant difference between the row and column titers (Tukey's HSD test; $P > 0.05$). Mean based on three replications. *All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of α = 0.05.*

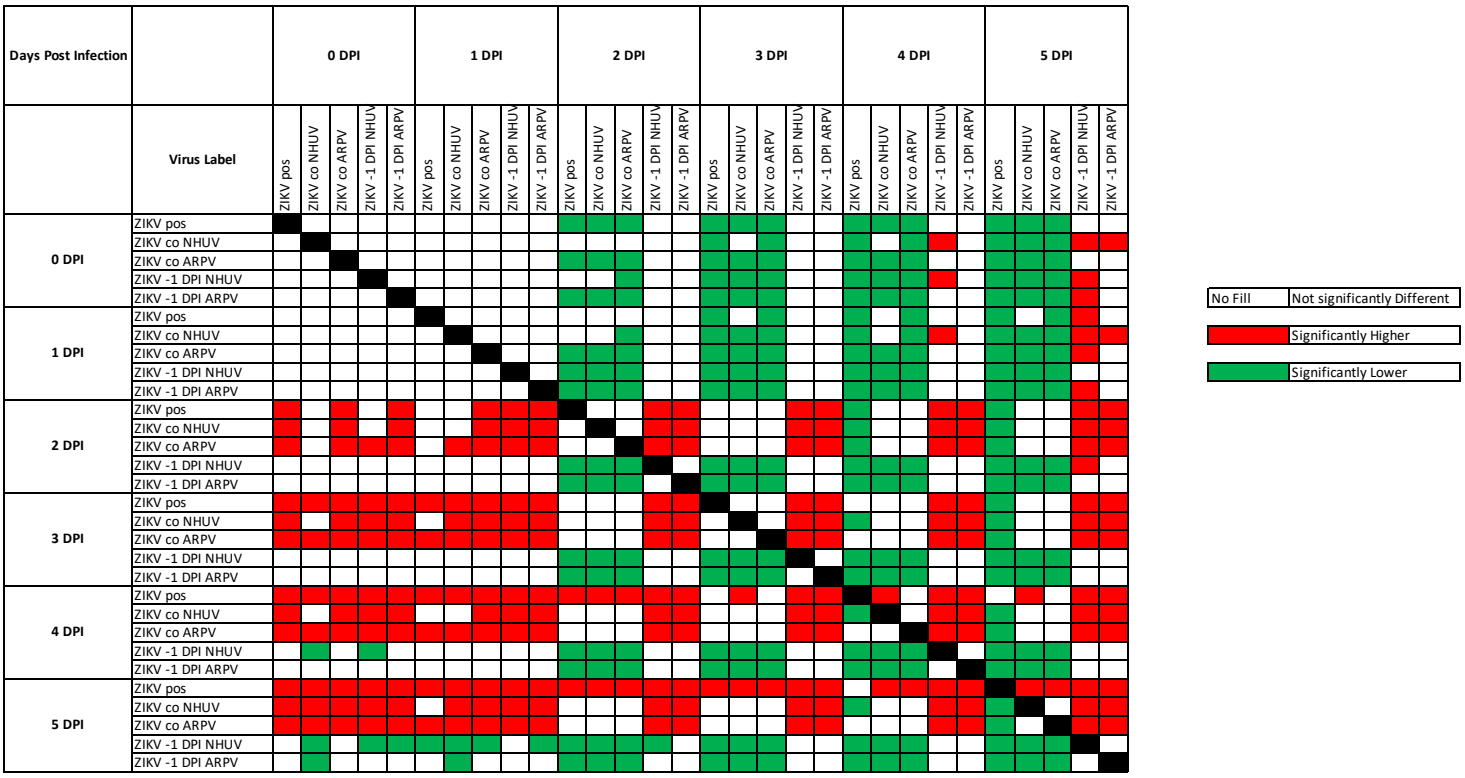

Table 4 Statistically significant values among experimental groups across all timepoints for Zika virus. Data presented in table 1 reflects the equivalent experiment shown in figure 1d, but instead indicates statistically significant comparisons. Red boxes indicate the row titer is significantly higher than the compared column titer, green boxes indicate the row titer is significantly lower, and white boxes indicate no significant difference between the row and column titers (Tukey's HSD test; $P > 0.05$). Mean based on three replications. *All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of $\alpha = 0.05$.*

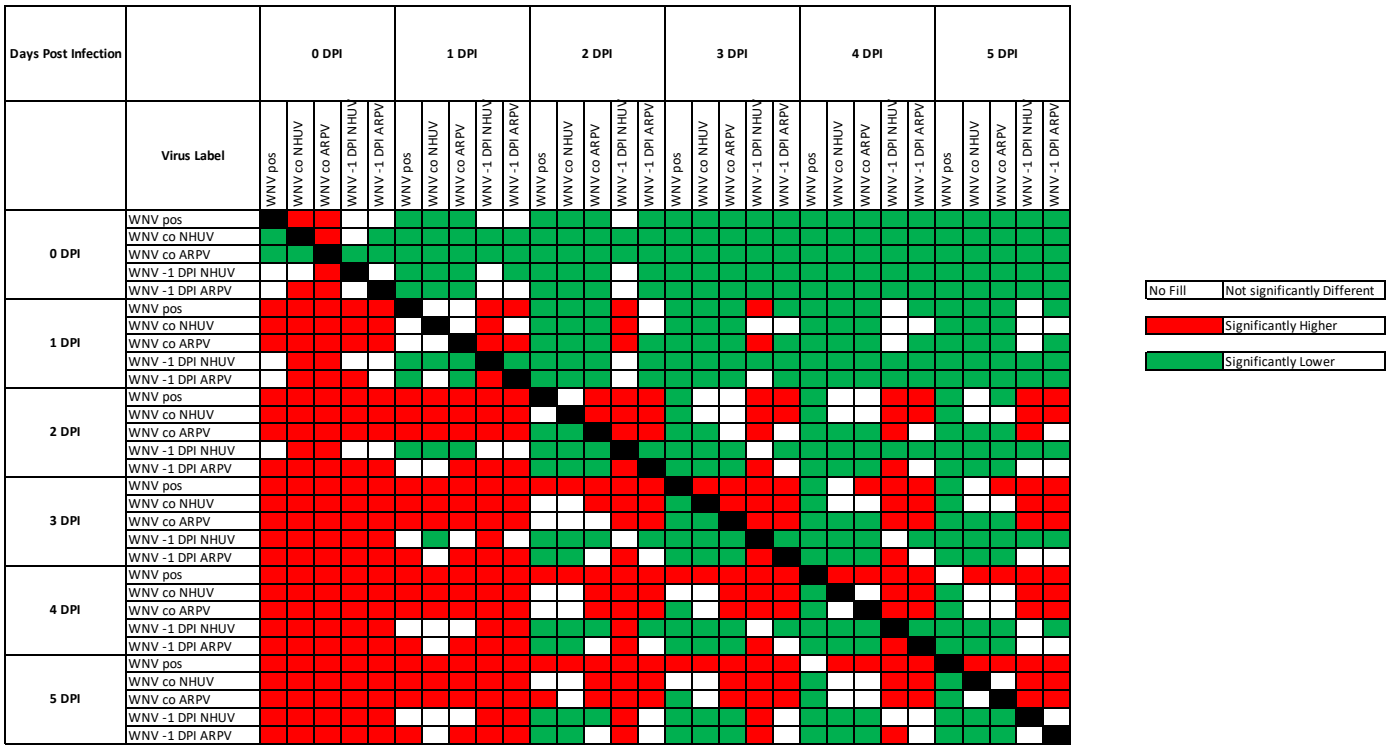

Table 5 Statistically significant values among experimental groups across all timepoints for West Nile virus. Data presented in table 1 reflects the equivalent experiment shown in figure 1e, but instead indicates statistically significant comparisons. Red boxes indicate the row titer is significantly higher than the compared column titer, green boxes indicate the row titer is significantly lower, and white boxes indicate no significant difference between the row and column titers (Tukey's HSD test; $P > 0.05$). Mean based on three replications. *All statistical analyses were performed using JMP Pro v14 (SAS, 2016) at a significance level of α = 0.05.*

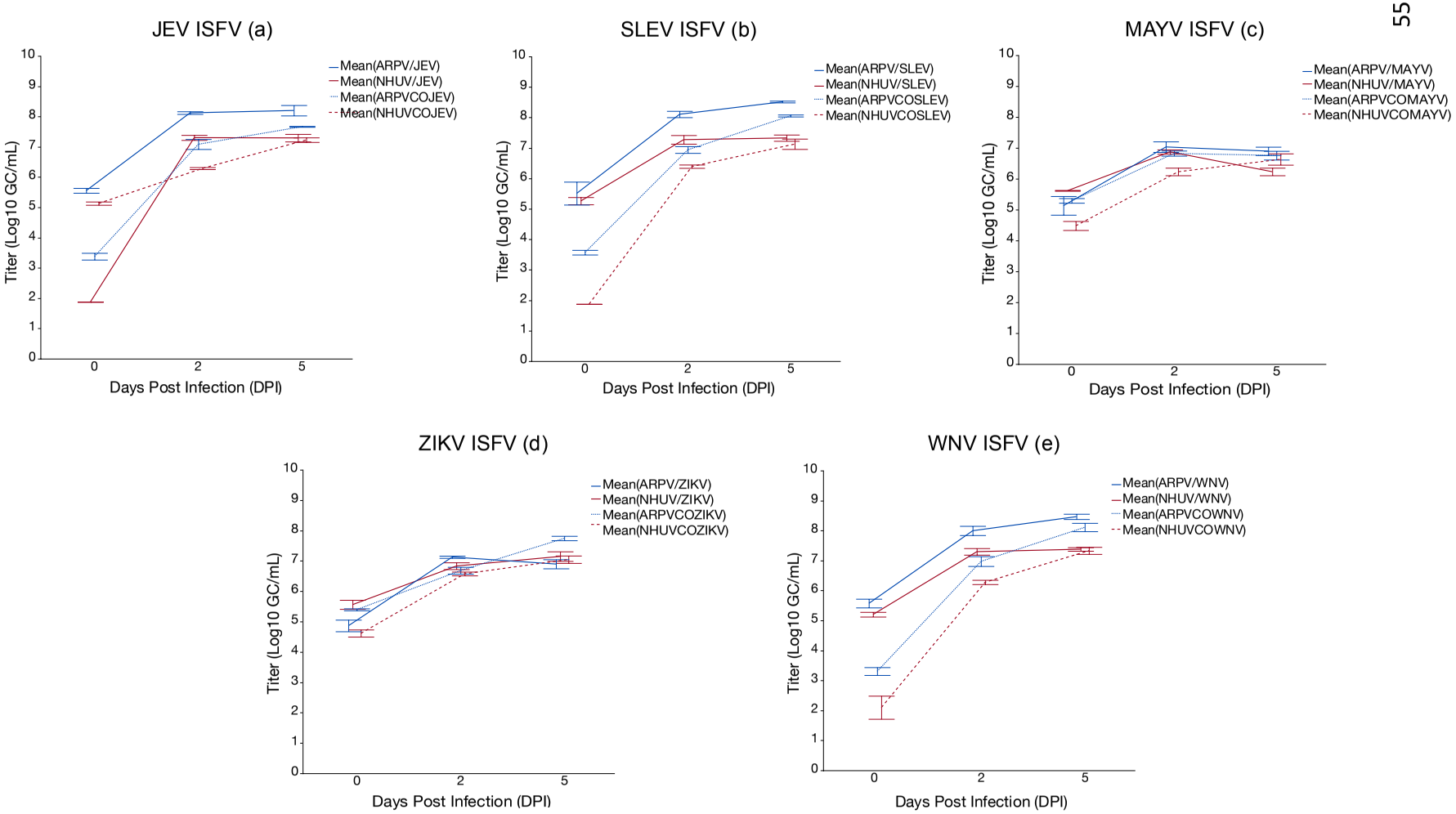

Figure 6 ARPV and NHUV titers in C6/36 cells when superinfected with vertebrate infectious virus. Growth curves were performed on C6/36 cells superinfected with vertebrate infectious viruses (MOI 0.1) and ARPV/ NHUV (pre-infection (-1 DPI) and co-infection (0 DPI); MOI 5) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves were constructed with mean GC/mL via qPCR in triplicate. Error bars are constructed using 1 standard deviation away from the mean.

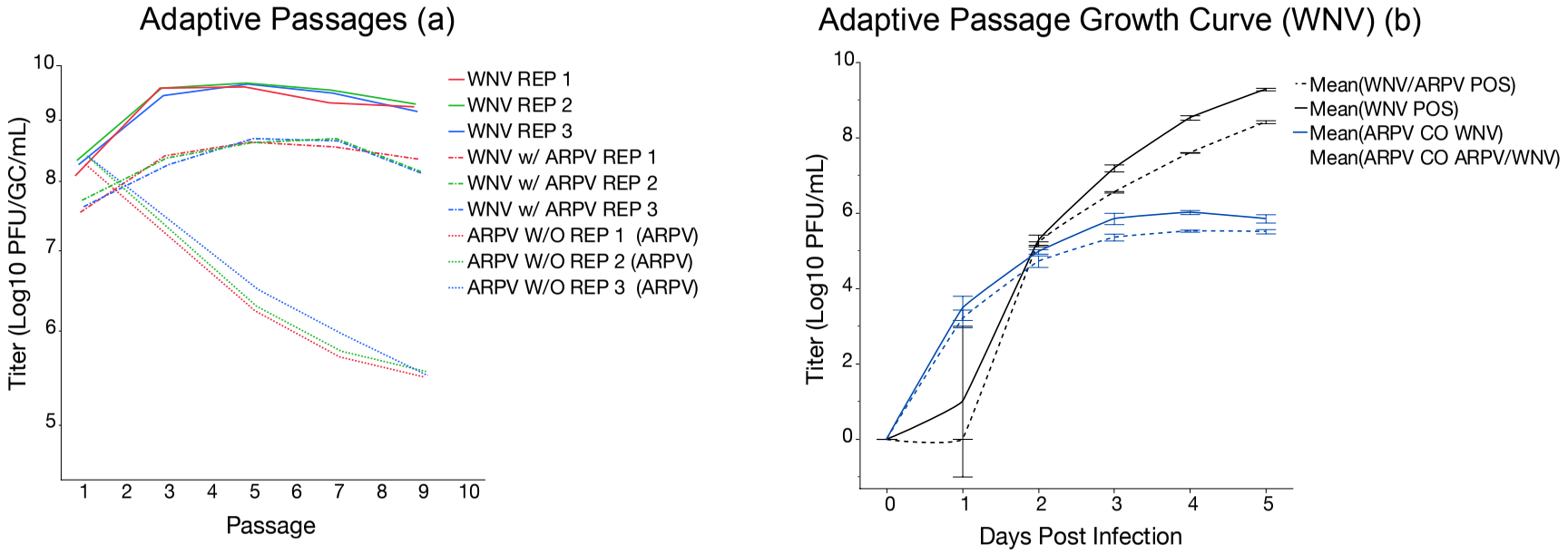

Figure 7 Adaptive passaging of WNV in C6/36 cells co-infected with ARPV. WNV titer at each passage (a), C6/36 cells were simultaneously infected with WNV and ARPV in a 1:1 ratio or only WNV and subsequently passaged in three isolated passages. ARPV was measured with qPCR while WNV was measured vi plaque assay and measurements were taken at every other day. (b) Passage 9.1 from all groups was incubated for 5 days to form a growth curve to show WNVs potential to return to its normal growth potential. ARPV (MOI 5) was co-infected with the passage 9.1 WNV positive and WNV/ARPV isolates (MOI 0.1) to form the growth curve. Growth curves were constructed with mean pfu/mL via plaque assay in triplicate. Error bars are constructed using 1 standard deviation away from the mean.

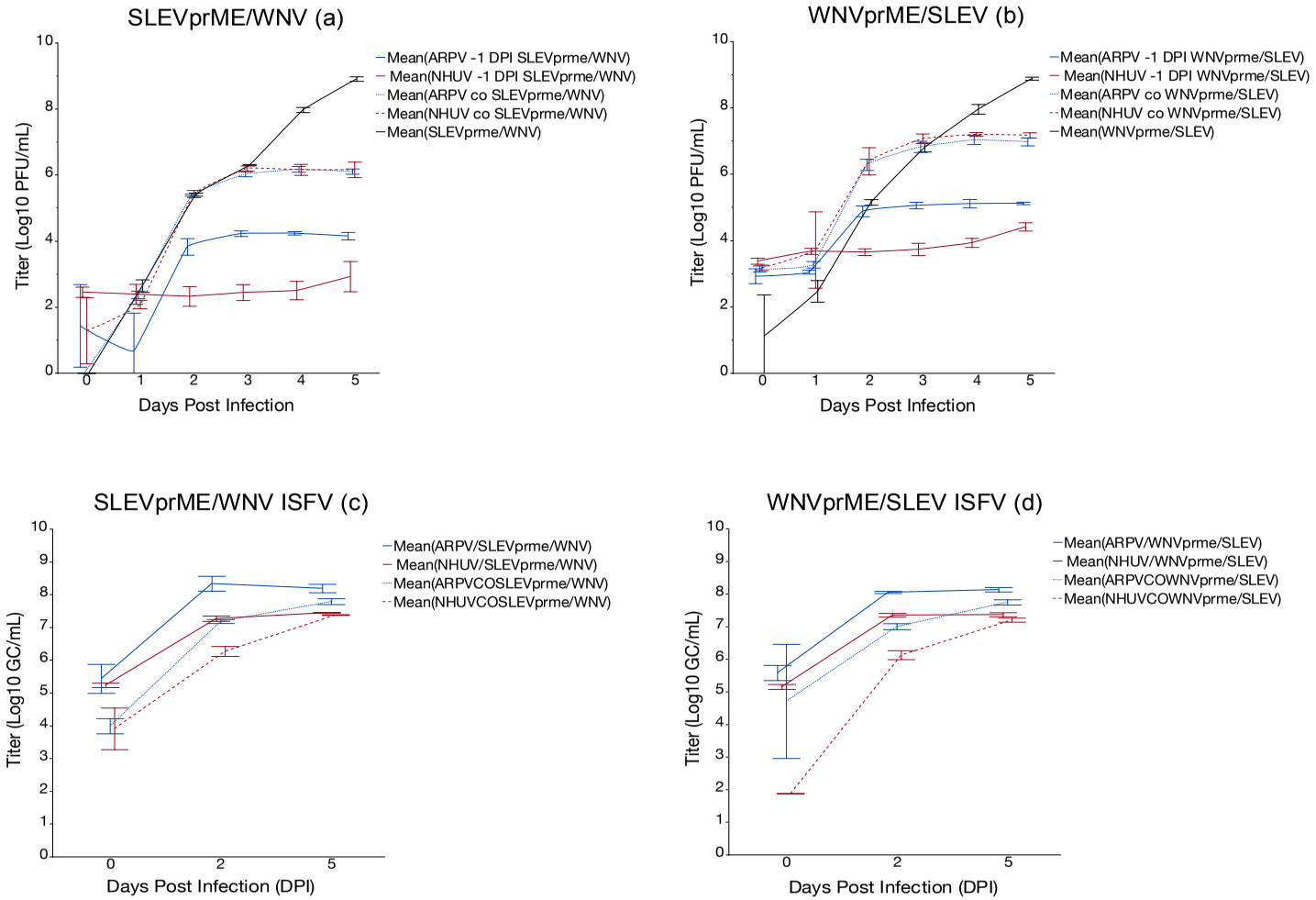

Figure 8 (a,b) ARPV reduces titers of chimeric vertebrate infectious virus in C6/36 cells. Growth curves (a,b,c,d) performed on C6/36 cells superinfected with vertebrate infectious viruses (MOI 0.1) and ARPV/ NHUV (pre-infection (-1 DPI) and co-infection (0 DPI); MOI 5) through 5 days. Inoculations for all viruses were done in triplicate. Growth curves of the VIF chimeras (a,b) were constructed with mean pfu/mL via plaque assay in duplicate the limit of detection for each group was 10 pfu/mL. ARPV and NHUV titers (c,d) growth curves were constructed with mean GC/mL via qPCR in triplicate. Error bars are constructed using 1 standard deviation away from the mean (a,b,c,d).

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