# Factors affecting the escape of Sindbis virus from the *Aedes aegypti* midgut

by

Alexis Nicole Bair Carpenter

B.S., Truman State University, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

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# Abstract

Mosquito-borne arboviral diseases are a significant and expanding public health threat due to their ability to inflict considerable morbidity and mortality, and the increasing range of their vectors. Each arbovirus is often only able to be transmitted by certain vector species and this specificity, in part, is due to the presence of tissue and immunological barriers in the mosquito. An important tissue barrier that an arbovirus encounters is the midgut escape barrier. If a virus is unable to overcome a midgut escape barrier this means that neither disseminated infection nor transmission can occur. Unlocking the factors that contribute to this barrier could lead to new disease control strategies. In this work, we used the alphavirus Sindbis virus (SINV) and the mosquito vector *Aedes aegypti* to study how viral replication and apoptosis impact viral dissemination from the midgut.

In our first study, we explored how viral midgut replication affects midgut escape. We developed SINV constructs designed to have reduced replication specifically in the midgut cells of *Aedes aegypti* by inserting sequences that are complementary to midgut-specific miRNAs into the 3′ untranslated region (MRE3′miRT) or the structural open reading frame (MRE-ORFmiRT) of the SINV genome. When mosquitoes were fed a blood meal containing one of these viruses or a control virus, nearly all fed with control viruses developed disseminated infection while significantly fewer mosquitoes developed disseminated infection with MRE3’miRT or MRE-ORFmiRT. When mosquitoes did develop infection with one of the midgut replication restricted viruses, titers did not tend to be significantly different compared to controls. We also did not find evidence that a certain threshold titer needed to be reached in the midgut for dissemination to occur. Overall, these results suggest that viral replication within the midgut is an important factor in determining whether a mosquito will develop disseminated infection.

In our next study we aimed to clarify the role of apoptosis in midgut escape. Several studies have shown that midgut apoptosis can negatively impact the ability of a virus to establish disseminated infection. However, contradictory evidence also exists. The goal of this study was to improve upon previous studies by generating a construct that more stably expressed the pro-apoptotic protein Reaper. A previous study showed that a virus that had the *reaper* gene inserted in the duplicated subgenomic promoter region (MRE/rpr) had reduced ability to cause disseminated infection in mosquitoes. However, this effect was short lived as it was found that mutations rapidly rendered Reaper nonfunctional. In order improve stability we inserted the *reaper* gene as an in-frame fusion into the structural open reading frame (ORF) of SINV. This construct, called MRE/rprORF, successfully expressed Reaper protein and induced apoptosis in both cell lines and mosquito midguts. Growth curves in BHK21 and C6/36 cells showed that MRE/rprORF replicated similarly to MRE/rpr. Mosquitoes fed with MRE/rprORF had less midgut and disseminated infection compared to mosquitoes fed with MRE/rpr or a control virus at all timepoints tested. Collectively, these studies show that reduced midgut replication and increased apoptosis negatively impact the establishment of midgut and disseminated infection in *Aedes aegypti*.

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# Dedication

This dissertation is dedicated to the memory of my grandmother, Barbara Jean Bair. I know you would have been proud to see this. I miss you, Mimi!

# Chapter 1 Introduction

## Arboviruses

Arboviruses are viruses that are spread via the bite of an infected arthropod such as a tick or mosquito. The bulk of arboviruses are from the virus families of *Flaviviridae*, *Togaviridae*, *Bunyaviridae* and *Reoviridae*. Arboviral diseases that are spread by mosquitoes are a major public health concern in many areas of the world, including tropical and subtropical areas and their impact may increase as areas affected by arboviral disease are predicted to expand with climate change, allowing disease-spreading species of mosquitos to thrive in areas where they were previously not found (Liu-Helmersson et al., 2016; Ryan et al., 2018). Recent outbreaks of arboviral disease, including the Zika virus (ZIKV) outbreak in the United States, have brought increased attention to the fact that arboviral diseases may emerge or reemerge at any time (Fauci & Morens, 2016). In addition to ZIKV, some of the most concerning arboviruses are dengue virus (DENV), which has been estimated to cause as many as 390 million infections per year and can cause severe and sometimes fatal disease, and chikungunya virus (CHIKV), which carries a risk for causing chronic pain and decreased quality of life post infection (Bhatt et al., 2013; LaBeaud et al., 2011). Few vaccines for arboviral diseases exist and those that do are sometimes limited in their availability or usefulness. For this reason, much of arboviral disease control is aimed at the disease vectors. A major threat to this control is the development of insecticide resistance in mosquitoes, which occurs in many places in the world (Deming et al., 2016; Hernández Ramos, 2020; Tabbabi et al., 2019; Weill et al., 2003). Due to the increasing resistance, we will need to find new and innovative methods of controlling arboviral disease, including preventing the vector itself from being able to transmit the virus. To do this, it is important for scientists to study the pathway of a virus through the vector to identify key points at which it can be stopped in its tracks.

## Virus-vector specificity

Viruses are often limited to a certain host range and arboviruses are no exception. In the United States there are over 200 species of mosquitoes while only about 12 of those are known to be important in disease transmission (CDC, 2020). In natural settings, the reason why some viruses are not transmitted by certain mosquito species can, in part, be due to incompatibilities in geographical range or host-feeding preferences. However, this does not fully explain virus-vector specificity as even when range and behavior align this does not guarantee the ability to transmit the virus and, conversely, experimental infection of certain mosquito species with viruses for which it is not known to be a natural vector can occur. The ability of a mosquito to transmit a particular virus is termed vector competence and it is determined by complex interactions of the genotypes of both the mosquito and virus (Lambrechts et al., 2009). There are a few known factors that may impact vector competence including mosquito immune responses such as the RNA interference (RNAi) pathway and tissue barriers in the mosquito, the latter of which is described in following sections (Franz et al., 2015; Lambrechts et al., 2013). Vector competence is also not static, for example, a single mutation in the CHIKV genome was found to improve the competence of *Aedes albopictus* for this virus (Tsetsarkin et al., 2007, 2011). This adaptation has driven increased outbreaks and geographical spread (Thiberville et al., 2013). Knowing the factors at play in vector competence will be critical for predicting future outbreaks and developing new strategies of arboviral disease control.

## Tissue barriers

A significant driver of virus-vector specificity is the presence of tissue barriers that the virus must overcome in order to be transmitted to a new host (reviewed in Franz et al., 2015). These barriers include the midgut infection barrier (MIB), midgut escape barrier (MEB), salivary

gland infection barrier (SGIB), and salivary gland escape barrier (SGEB) (Fig. 1.1). A successful virus must overcome the midgut infection barrier by having the means to enter the cells of the mosquito midgut epithelium after being ingested in a blood meal. It then must overcome the midgut escape barrier by exiting out of these cells and into other mosquito tissues. Similarly, the virus must overcome the salivary gland barriers by infecting and escaping from these cells to be transmitted in the saliva when they take a blood meal from a new host. If a virus is unable to proceed through all these tissue barriers, then transmission is unable to occur. Therefore, understanding what factors contribute to these barriers could lead to the development of new strategies of arboviral control.

**Midgut infection barrier**

The midgut is the site of blood digestion in the mosquito and is thus the first tissue that a virus is likely to encounter after being ingested by a mosquito. An MIB occurs when a virus is unable to infect mosquito midgut epithelial cells, or the virus is rapidly cleared from these cells before productive infection develops. If a virus is unable to penetrate this barrier, no tissues of the mosquito will become significantly infected.

There are several elements that have been suggested to contribute to this barrier; the peritrophic matrix, which is a fibrous mesh layer that forms around a blood meal after ingestion, has been hypothesized to be one such contributor. If a virus has not successfully adsorbed to or infected the midgut epithelium before the formation of the peritrophic matrix, it is likely that it would be blocked from infection due to the tightly woven nature of the matrix (Houk et al., 1979). Therefore, in principle, incompatibilities between the time it takes for a virus to establish midgut infection and the time it takes for the peritrophic matrix to develop may lead to an MIB.

However, at least one study has shown that eliminating the peritrophic matrix did not strongly affect midgut infection (Kato et al., 2008).

Perhaps one of the most fundamental reasons for a midgut infection barrier is incompatibilities between the virus and receptors on the mosquito epithelial cells. Determining if a vector is incompetent to transmit a virus because it lacks a midgut receptor is complicated by the fact that definitive receptors for many arboviruses have not been identified. Nonetheless, several studies have provided evidence that receptors are a crucial factor in the MIB. For example, one study showed that in a *Culex* mosquito strain that was refractory to western equine encephalitis virus (WEEV) there was only non-specific binding to brush border fragments of the midgut epithelium while specific binding occurred in a susceptible strain (Houk et al., 1990). Another study found that in a mosquito strain that displayed an MIB with DENV, virions bound to a slightly smaller protein (64 kDa) as well as a larger protein (67 kDa) in the midgut while virions in mosquito strains that were susceptible to the virus or had a midgut escape barrier were only found to bind to the 67 kDa protein. The MIB strain also was found to have less of the 67 kDa protein in the midgut (Mercado-Curiel et al., 2008). Several studies examined the differences between strains of Sindbis virus (SINV) that had poor midgut infection rates and strains that had higher rates. It was found that poor infection could be linked to certain amino acid residues in the E2 protein, which is the receptor binding protein, and infection rates could be improved when these regions were substituted with the sequence from the high infection strains (Pierro et al., 2007, 2008). This provides further evidence that receptor incompatibilities could be a primary reason for a midgut infection barrier, but further study will be required for this to be fully understood.

In addition to the inability of a virus to enter midgut cells contributing to this barrier, incompatibilities within the midgut epithelium may lead to clearance of the virus before any significant production of virus occurs, which may also be considered an MIB. This may be related to the immune response in the midgut cells or to incompatibilities in the internal environment or machinery needed for viral replication, the former of which can possibly be overcome by increasing the viral dose. For example, authors of one study found that a SINV strain that had a dose-dependent MIB and MEB in *Aedes aegypti*. Further, they found that impairing the RNAi pathway reduced both of these barriers (Khoo et al., 2010). More studies in this area will be needed to fully understand the many contributing factors to midgut infection barriers.

**Midgut escape barrier**

While the midgut infection barrier can often be attributed to virus-receptor incompatibilities, the MEB is perhaps more puzzling. If a virus encounters an MEB, then it can successfully infect and replicate in midgut epithelial cells, but it fails to reach any other tissues and is unable to be transmitted. For a virus to escape from the midgut, it not only needs to overcome the challenges presented by the midgut epithelium itself such as immune responses, but it also needs to pass through a dense fibrous matrix underlying the midgut, called the basal lamina. The pores in the basal lamina have been determined to be smaller than most arboviruses (Houk et al., 1981). For this reason, there have been several proposals about how exactly a virus is able to overcome this obstacle that deal with both location and mechanism of dissemination. In the former category are two major ideas: 1) Dissemination occurs from the cardia/intussuscepted foregut and 2) Dissemination occurs from the posterior midgut. In addition, there are hypotheses about how the virus can cross the extracellular matrix/basal lamina: 1) That virions can pass

through gaps that are large enough to support this directly or 2) That another tissue such as the tracheae penetrates this layer allowing virions to escape the midgut without needing to navigate the basal lamina (Franz et al., 2015). It is important to note that these ideas are not necessarily mutually exclusive and that it is possible that there are different mechanisms of dissemination in different virus-vector combinations. Another complicating factor is that MEBs are not necessarily an all or nothing phenomenon. For example, in a particular virus-vector combination the midgut escape rate may be poor but there may still be a small proportion of mosquitoes that develop disseminated infection.

As the posterior midgut is the site of blood digestion in mosquitoes and most arboviruses are found to infect this tissue it is generally thought to be the primary site of dissemination (Bowers et al., 1995; Girard et al., 2004; Miller et al., 1989; Salazar et al., 2007). The idea that gaps or pores may exist in the basal lamina that are large enough for viruses to pass through was among the earliest hypotheses of midgut escape (Houk et al., 1981). The distension of the mosquito midgut following blood meal ingestion leads to obvious questions about what this stretching does to the structure of the basal lamina and whether remodeling the basal lamina allows virus escape. Despite this, clear-cut gaps in the basal lamina associated with escaping virions have not been definitively identified. However, an increasing body of evidence suggests that structural changes in the basal lamina may be a possible escape route. For example, modified basal lamina which appears more porous or distorted and is associated with the visceral muscles has been reported after ingestion of virus containing blood meals (Kantor et al., 2018; Romoser et al., 2004). Even so, virus escape from the midgut often takes days, so structural changes would need to persist long enough to allow infection and replication in the midgut. Supporting this idea, Cui et al. found that mesh width of the basal lamina remained expanded

even after the blood meal had been digested (Cui et al., 2019). Some disruption of the basal lamina may be associated with cell degeneration as described by Weaver et al. They found that pathologic changes in the midgut occurred in eastern equine encephalitis virus (EEEV)-infected *Culiseta melanura* including some cellular degeneration which was associated with basal lamina disruption (Weaver et al., 1988). Passage through the basal lamina may lead to viral escape directly into the hemocoel which allows the virus to disseminate to other tissues or the virus may infect another tissue such as the tracheal epithelium before further spread occurs. While gaps in the basal lamina remains a viable and supported hypothesis of midgut escape, further studies will be needed to generate conclusive evidence.

More anterior regions of the digestive system have also been implicated as sites of dissemination. In this hypothesis, the cardia or intussuscepted foregut become infected and then the virus can cross into the hemocoel or into another tissue, such as the tracheae. The cardia is an organ that exists at the junction of the midgut and foregut and contains cells from both types of tissue and is closely associated with tracheae and muscles. It is surrounded by modified basal lamina or matrix which appears more porous and thus may be more permissive for viral escape (Lerdthusnee et al., 1995). The idea that this region might be important for disseminated infection was derived from the observation that when *Culex pipiens* mosquitoes were fed with Rift Valley fever virus (RVFV), most of the mosquitoes that did not develop disseminated infection had virus detected in the midgut, but did not have infection in the intussuscepted foregut, while most mosquitoes with disseminated infection had infected cells in this region (Romoser et al., 1987). Further studies were able to capture images of virions budding from cardial epithelial cells and virions in the basal labyrinth and matrix of these cells (Lerdthusnee et al., 1995). By studying the infection patterns in orally and thoracically infected mosquitoes, the

authors of this study suggested that infection of the cardial cells would lead to spread of the virus to the intussuscepted foregut and possibly to more anterior regions of the gut before dissemination into the hemocoel by utilizing the larger gaps in the matrix (Lerdthusnee et al., 1995). However, this is not a likely route in some virus-vector combinations. For example, DENV-2 was not consistently observed to infect the cardia in *Aedes aegypti* mosquitoes (Salazar et al., 2007) and, while West Nile virus (WNV) antigen was detected in the cardia and intussuscepted foregut of *Culex pipiens quinquefasciatus* mosquitoes, this appearance occurred at approximately the same time as appearance in the salivary glands (Girard et al., 2004). The cardia and intussuscepted foregut may play a critical role in dissemination of some viruses while playing little to no role in others.

Another means of viral escape from the midgut may be via the tracheal system which extends throughout the body of the mosquito and branches can be closely associated with the midgut. Evidence has shown that the tracheae may be a route of midgut escape for viruses in other insects, notably, baculoviruses have been shown to use this route in lepidopteran larvae (Engelhard et al., 1994; Means & Passarelli, 2010). The evidence for arboviruses using this route in mosquitoes does not appear to be quite so conclusive, but nonetheless some studies have indicated that this may be a viable hypothesis. Importantly, several studies have shown that tracheae may penetrate the midgut basal lamina in mosquitoes providing the proximity needed to assist in midgut escape (Romoser et al., 2004, 2005). Infection of tracheal cells also appears to occur in a range of arboviruses such as DENV, Venezuelan equine encephalitis virus (VEEV) and RVFV (Romoser et al., 2004, 2005; Salazar et al., 2007). Some of the most direct evidence for this hypothesis came from one study which concluded that injected VEEV needed to infect tracheal and muscle cells before midgut infection could occur (Romoser et al., 2004). However,

another study using CHIKV found that tracheal cell infection was not a necessary step in viral dissemination (Kantor et al., 2018). There is a possibility that this means of escape may be more relevant in certain virus-vector combinations which may be better understood in the future.

Due to the importance of this tissue barrier in determining vector competence, it is the focus of the studies described in chapters 2 and 3 of this dissertation. Many of the known factors impacting midgut escape are described in later sections of this chapter.

**Salivary gland infection and escape barriers**

After infecting and escaping from the midgut and then infecting and replicating in disseminated tissues, including the fat body and hemocytes, an arbovirus needs to infect and escape from the salivary glands which will allow the virus to be transmitted in the mosquito saliva upon taking its next blood meal. To accomplish this the virus needs to penetrate the basal lamina surrounding the acinar cells of the salivary glands, and it then needs to move from the cells into the central salivary duct. If a virus encounters a SGIB then tissues outside of the midgut will be infected but the salivary glands will not, while if a virus encounters a SGEB then the salivary glands will be infected but virus will not be found in the saliva.

SGIBs have been described for several mosquito-virus combinations including WEEV with *Culex tarsalis* and RVFV and *Anopheles stephensi* (Kramer et al., 1998; Romoser et al., 2005). Viruses encountering this barrier may not be able to penetrate through the basal lamina of the salivary glands or are blocked from entering the glands in some way. It has been proposed that viruses that can overcome this barrier may utilize the midgut trachea for midgut escape (Romoser et al., 2005).

Salivary gland escape presents the final mosquito tissue barrier to transmission. Among others, a SGEB has been described for *Aedes hendersoni* and *Aedes brelandi* and La Crosse virus

(LACV) (Grimstad et al., 1985; Paulson et al., 1989), and *Culex tarsalis* and WEEV (Kramer et al., 1998). The mechanism by which viral escape into the saliva is prevented is not entirely clear, however it may be due in part to antiviral pathways in the salivary glands. Apoptosis has been proposed as one possible pathway because cytopathology in virally infected mosquito salivary glands has been noted (Bowers et al., 2003; Girard et al., 2005, 2007). Girard et al., also found that apoptosis-related cytopathology increased in *Culex* mosquito salivary glands over time while the percentage of saliva samples with detectable WNV decreased (2007), implying that apoptosis is a possible contributor to SGEBs. However, tissue disruption may not always prevent escape. A study found that salivary gland escape of LACV virus in *Aedes hendersoni* mosquitoes could be improved by coinfection with *Plasmodium gallinaceum* (Paulson et al., 1992), although other possibilities besides tissue disruption could explain this result. Additional research on salivary gland barriers will likely bring greater clarity to our understanding of vector competence.

**Factors affecting midgut escape**

Each tissue barrier provides an obstacle to virus transmission and learning how to prevent virus transmission may provide us with insights needed to combat arboviral disease. Regardless of the exact mechanism of midgut escape, the phenomena of MEBs have been noted for decades across a wide swath of arboviruses and mosquito species (Bennett et al., 2002; Boromisa & Grayson, 1991; Hartman et al., 2021; Khoo et al., 2013; Paulson et al., 1989). The presence or absence of a MEB is often not straightforward, as the percentage of mosquitoes exhibiting an MEB can vary widely even in a species in which we know some members can transmit a particular virus. For example, one study that looked at the susceptibility of different *Aedes aegypti* populations in the United States and Mexico to DENV found that in some populations the percentage of mosquitoes with a midgut escape barrier was as high as 43%, while in other

populations, it was found to be as low as 4% (Bennett et al., 2002). Genetic and physical attributes of both the virus and mosquito species involved, as well as environmental factors, are among the many components that play into midgut escape. Midgut escape barriers have been widely studied, and yet there is still much that we do not understand about them. For this reason, factors affecting the MEB are the subject of subsequent chapters of this dissertation. A review of some of the most important known factors are discussed in the following paragraphs.

External factors – temperature

It has been well established that environmental factors can influence whether a virus is able to escape from the midgut of a mosquito. One of the most well studied factors is temperature, but other studies have also shown that other environmental components like insecticide exposure and larval density may also play an important role.

The effect of temperature on midgut escape has been demonstrated in several different mosquito species and virus combinations. It has been found that when adult *Culex pipiens* mosquitoes were infected with WNV and held at higher-than-normal temperatures, midgut escape happened faster and ultimately more mosquitoes developed disseminated infection when compared to mosquitoes held at lower temperatures (Dohm et al., 2002; Richards et al., 2007). This effect has been shown to extend to other combinations of vectors and viruses including in *Aedes albopictus* mosquitoes infected with DENV (Alto & Bettinardi, 2013), *Aedes aegypti* mosquitoes and CHIKV (Mbaika et al., 2016) and *Culex* mosquitoes and St. Louis encephalitis virus (SLEV) (Richards et al., 2009). Several reasons for these results have been suggested including that higher temperature may increase viral replication within the midgut or may cause increased midgut permeability. The effect of temperature is complicated by genetic differences in mosquitoes and viruses. Temperature has been shown to have less of an effect on midgut

escape in some mosquito strains and viral strains while having a greater effect on others (Kilpatrick et al., 2008; Mbaika et al., 2016).

In addition to the temperature at which adult mosquitoes are held, the temperature during larval development may ultimately affect viral midgut escape. One study investigating *Aedes albopictus* and CHIKV found that when larvae were reared at lower temperatures, there were increased rates of dissemination in adults (Westbrook et al., 2010). Conversely, another study found that at low larval densities, increased larval temperature was found to increase the dissemination rate of SINV in adult mosquitoes; however, no difference was seen in dissemination when larval density was high (Muturi et al., 2012). The authors hypothesized that the larval temperature may alter adult mosquito immune gene expression such that mosquitoes reared at low temperatures were poorer vectors. These studies show that there is a need for more research that looks at the how vector competence is affected by different combinations of larval environmental factors.

External Factors – exposure to pesticides

Another environmental factor that may affect midgut escape is exposure to pesticides. An unintended side effect of pesticides may be increased dissemination rates in mosquitoes that are exposed at a sub-lethal level, as this has been shown in several studies. Bifenthrin has been shown to increase dissemination rates of ZIKV in *Aedes albopictus* with a particularly strong effect seen in older mosquitoes (Knecht et al., 2018). However, the same insecticide appears to have little effect on DENV dissemination (Richards et al., 2017). Sub-lethal insecticide exposure may be particularly important for vector competence when larvae are exposed, as larval exposure to malathion has been shown to increase dissemination rates of SINV (Muturi et al., 2011; Muturi & Alto, 2011). A possible reason for this may be that these insecticides cause adult

midgut deformities as has been shown to occur when mosquito larvae are exposed to the insecticide spinosad (Fernandes et al., 2019). This outcome might not just be limited to chemical insecticides because it has shown that sub-lethal exposure of larvae to the bacterial insecticide *Bacillus thuringiensis subsp. Israelensis* also caused increased rates of dissemination of DENV although this was bacterial strain-specific (Moltini-Conclois et al., 2018). It will be increasingly important to consider this possible side effect when treating areas with insecticides.

External factors – larval density/competition

Other larval environmental conditions have also been shown to affect midgut escape, with several studies suggesting a surprising link between larval density and viral dissemination rates in adults. In one study when *Aedes albopictus* mosquito larvae were reared at higher densities, the adult mosquitoes had more disseminated infection; this effect was not seen in *Aedes aegypti* mosquitoes. It is unclear exactly what causes this difference. The authors of this study found that the density treatment negatively correlated with mosquito size and so reasoned that the dissemination rate could be related to smaller mosquitoes being better vectors (Alto et al., 2005). However, another study showed that larval competition between *Aedes albopictus* and *Ochlerotatus triseriatus* led to surviving *Oc. triseriatus* mosquitoes being larger and more likely to develop disseminated infection with LACV virus (Bevins, 2008). This shows that both interspecies and intraspecies competition might ultimately affect vector competence and that this effect might not be entirely related to size.

Mosquito factors – basal lamina thickness/structure

It has been apparent for many years that some mosquito species are incapable or unlikely to transmit certain viruses due to a midgut escape barrier preventing dissemination (Bennett et al., 2005; Paulson et al., 1989; Turell et al., 2008). Many studies have investigated what

mosquito factors contribute to the existence of this barrier, but it is often difficult to separate the mosquito factors from the viral factors because it has been shown that the interaction of the genotypes is important (Lambrechts et al., 2009). Nonetheless, several aspects of mosquito physiology have been implicated in contributing to an MEB. These include physical characteristics of the mosquito such as basal lamina structure as well as mosquito behavior and expression of genes involved in immunity.

The basal lamina is a tightly woven matrix that surrounds the mosquito midgut, and which represents a major obstacle to disseminated infection. For years it has perplexed researchers how viruses manage to pass through this matrix when the measured pore sizes are smaller than the size of most viruses (Houk et al., 1981). Differences in basal lamina structure and thickness have been proposed to contribute to differences in midgut escape rates. One study found that nutritional differences led to mosquitoes of different sizes and having different midgut basal lamina thicknesses (Grimstad & Walker, 1991). They found greater dissemination rates of LACV in the small mosquitoes that had thinner basal laminas and reasoned that this difference may in part explain why some mosquitoes are better vectors. However, other studies have found no association between the thickness of the basal lamina and midgut escape. A study that looked at DENV dissemination in laboratory strains of *Aedes albopictus* with differences in basal lamina thickness found no impact on viral midgut escape (R. E. Thomas et al., 1993). Other studies have found that after blood feeding, perforations appear in the basal lamina that may facilitate midgut escape (Armstrong et al., 2020; Kantor et al., 2018). It remains to be seen if differences in susceptibility to these perforations are a factor in variability in midgut escape.

<u>Mosquito factors – immune gene expression</u>

*RNAi pathway*

The RNAi pathway was first discovered in *C. elegans* where it was unexpectedly found that double stranded RNA could lead to the destruction or translational repression of mRNA with the same sequence (Fire et al., 1998). This was subsequently demonstrated to exist in insects and to be a major contributor to the antiviral response in mosquitoes, including in the midgut (Reviewed in Blair, 2011; Campbell et al., 2008; Sánchez-Vargas et al., 2009). Studies have provided compelling evidence that this pathway can play a major role in whether a mosquito will develop disseminated infection. For example, it has been shown that when the midgut RNAi response was reduced in *Aedes aegypti*, there was more dissemination of SINV (Khoo et al., 2010). Conversely, when mosquitoes were genetically engineered to express inverted repeat RNA derived from DENV-2 in midgut, which triggered an increased RNAi response, there was decreased midgut escape (Franz et al., 2006). While these studies provide compelling evidence of the importance of RNAi, we need to know if there is natural variation in the expression of the components of RNAi and if this can explain why some mosquitoes naturally develop disseminated infection and others do not. Evidence shows that components of RNAi like Dicer-2 can vary in their expression between different strains of mosquito species and that this differential expression may have an impact on the percentage of mosquitoes developing disseminated infection (Bonizzoni et al., 2012; Carvalho-Leandro et al., 2012). Exactly how much variation in the RNAi pathway contributes to midgut escape and vector competence is an area that requires more study.

*Jak/STAT, Toll and IMD pathways*

The antiviral activity in the midgut of mosquitoes does not stop with RNAi, as several other immune pathways have been shown to have antiviral effects and could potentially impact midgut escape. These include the Jak/Stat, Toll and IMD pathways.

The Jak/Stat pathway has been shown to have a role in innate antiviral immunity in *Drosophila* and in mosquitoes (Dostert et al., 2005; Souza-Neto et al., 2009). When a group of researchers engineered mosquitoes to overexpress the Dome and Hop components of the Jak/Stat pathway in the fat body and midgut, they found a lower prevalence of disseminated DENV-2 infection but the prevalence of midgut infection was not altered (Jupatanakul et al., 2017). The role of differential expression of this pathway among mosquito populations with variation in midgut escape rates remains to be seen. However, expression of genes in this pathway have been found to be increased in mosquito strains that are both susceptible and refractory to DENV which may suggest that this pathway alone cannot explain the MEB (Behura et al., 2014).

The Toll signaling pathway is another pathway that has been shown to be important in innate immune defense against a variety of pathogens including gram positive bacteria and fungi (Hoffmann, 2003). It has subsequently been shown to play a role in anti-viral defense in mosquitoes (Ramirez & Dimopoulos, 2010; Sanders et al., 2005; Xi et al., 2008). There is some evidence to suggest that the basal level of activation of this pathway may differ among strains of *Aedes aegypti* as it has been found that the levels of expression of REL1 are different in whole body samples of field-derived mosquitoes versus laboratory strains of mosquitoes and that these mosquito populations differ in their disseminated infection rates with DENV (Carvalho-Leandro et al., 2012). An additional pathway that has been shown to have an antiviral role in *Drosophila* is the IMD pathway (Costa et al., 2009). This pathway has also been shown to be altered in the midgut of virally infected mosquitoes (Sanders et al., 2005). How alterations in these pathways relate to midgut escape specifically should be considered in the future.

*Apoptosis and cell turnover*

Apoptosis is a form of programmed cell death that is highly conserved in animals and has been extensively studied in model organisms such as *C. elegans, Drosophila* and mice (Oberst et al., 2008). Apoptosis is important in development and maintenance of tissues and disruptions in the process can lead to disease (Rudin & Thompson, 2003; Vaux & Korsmeyer, 1999). Importantly for the present study, this pathway has also been known to have an anti-viral role for many years (Clem & Miller, 1993; Clouston & Kerr, 1985). The core mechanisms of apoptosis appear similar in many organisms (Fig. 1.2). The most important actors in the apoptotic pathway are caspases which are a family of proteases that contain cysteine in their active site and are generated in an inactive form called procaspases. In short, in response to activating stimuli, adaptor proteins bind to initiator procaspases which causes aggregation and cleavage at aspartic acid residues. The cleaved and active initiator caspases in turn activate effector caspases which cleave cellular targets and ultimately bring about cell death (Alberts et al., 2002). This process is highly regulated by different proteins, one of the more important families being the IAP or inhibitor of apoptosis family that was first discovered in baculoviruses (Birnbaum et al., 1994; Crook et al., 1993), and subsequently homologs have been found in many organisms including yeast, *C. elegans*, *Drosophila* and *Aedes aegypti* (Bryant et al., 2008; Colin et al., 1996; Fraser et al., 1999; Hay et al., 1995; Uren et al., 1999). These proteins bind to procaspases to prevent activation or bind to caspases to prevent their action. Another group of proteins called IAP antagonists work to prevent the action of IAPs which leads to caspase activation and cell death. In *Drosophila*, the genes *reaper*, *grim*, *sickle* and *hid* encode IAP antagonists. While these proteins are diverse, they all encode an N-terminal IBM or IAP binding motif (Steller, 2008).

More recently, efforts have been taken to understand the apoptotic pathway better in *Aedes aegypti* which has revealed that the core pathway bears striking resemblance to the

pathway in *Drosophila melanogaster* (Fig. 1.2). Annotation of the *Aedes aegypti* genome has uncovered many homologs of known apoptosis-related genes in *Drosophila* (Bryant et al., 2008; Waterhouse et al., 2007). The most important effector caspases in apoptosis in *Aedes aegypti* appear to be CASPS7 and CASPS8 which are homologous to DrICE and Dcp1 in *Drosophila*. The effector caspases are activated by the initiator caspase AeDronc which is activated by the adaptor protein AeArk (Liu & Clem, 2011). AeIAP1 prevents caspase activation and silencing of this gene leads to apoptosis in mosquito cells and mosquitoes (Liu & Clem, 2011; Pridgeon et al., 2008; Wang et al., 2012). The *Aedes aegypti* genome also encodes the IAP antagonists Michelob_x and IMP (Bryant et al., 2008; Wang & Clem, 2011; Zhou et al., 2005).

Apoptosis is known to be an anti-viral pathway which has implications for vector competence in mosquitoes. However, it has also been hypothesized that this cell death may create an opening through which viruses may escape. Some evidence suggests that midgut apoptosis varies among mosquitoes with differing levels of midgut escape. For example, midguts in a WNV refractory *C. pipiens pipiens* strain showed evidence of apoptosis (Vaidyanathan & Scott, 2006). Also, expression of caspase genes and other genes critical to apoptosis have been shown to be increased in *Aedes aegypti* mosquito strains that are refractory to DENV or strains that show different percentages of mosquitoes displaying a midgut escape barrier (Ocampo et al., 2013; Serrato et al., 2017). Experiments in manipulating the process of apoptosis have also suggested that this pathway may affect midgut escape. For example, when SINV was engineered to express the reaper gene, the virus rapidly lost the inserted gene which suggests that expression of the proapoptotic gene was severely detrimental to the viruses' survival (O'Neill et al., 2015). However, not all evidence suggests that apoptosis is detrimental to viral dissemination. A study which reduced apoptosis by knocking down the gene *Aedronc* found that viral dissemination was

reduced (Wang et al., 2012). Interestingly, one group has hypothesized that these apparently contradictory results may be explained by the role that Aedronc plays in autophagy which may support viral replication (Eng et al., 2016). The same study also found that when AeIAP1 was silenced, which increases apoptosis, dissemination was found to be increased. However, high mosquito mortality with this treatment demonstrated that widespread apoptosis was induced in the mosquito and this likely reduced the structural integrity of the midgut (Wang et al., 2012).

An active area of research is how the balance of apoptosis and cell generation in the midgut affects midgut escape. A recent study found that DENV-susceptible mosquitoes had slower generation of new cells in the midgut (Taracena et al., 2018). This study, however, only looked at infection in the midgut and did not study disseminated infection, so this would need to be studied further to determine a link to midgut escape specifically.

Mosquito factors – behavior

A factor which is only starting to be considered a piece in the puzzle of midgut escape is the role of mosquito feeding behavior. While at the surface it may seem that the two are unrelated, recent evidence suggests otherwise. In laboratory studies of vector competence, mosquitoes are often given a single infectious blood meal, however, this does not reflect the natural behavior of mosquitoes. It has been shown that mosquitoes will take multiple blood meals during one gonadotrophic cycle. In one study 61% of *Aedes aegypti* mosquitoes in the laboratory imbibed a second blood meal, often within 24 hours, and 50% of wild-caught mosquitoes showed evidence of multiple blood meals (Scott et al., 1993). Recently, researchers have presented evidence that this behavior may improve viral dissemination from the midgut. The findings laid out in Armstrong et al. indicate that when *Aedes aegypti* mosquitos receive an infectious blood meal containing ZIKV and then a subsequent non-infectious blood meal, the

number of mosquitoes developing a disseminated infection increases. Similar results were seen in *Aedes aegypti* and DENV, *Aedes aegypti* and CHIKV and *Aedes albopictus* and ZIKV (Armstrong et al., 2020). Using a similar feeding regime, Kantor et al. examined the results of a second non-infectious feeding by electron microscopy and found that after this blood meal, CHIKV virions could be found outside of the midgut and could be seen on the basal lamina on the side of the hemocoel. Meanwhile in mosquitoes fed with only a single infectious blood meal, virions were only seen in the strands of the basal lamina (Kantor et al., 2018). In the future, other aspects of mosquito behavior should be investigated in relation to midgut escape including volume of the blood meal and number of blood meals imbibed.

Viral factors – midgut replication

The exact role of virus replication in the midgut in midgut escape has been debated and it is still unclear as to whether midgut replication is necessary or if an intercellular dissemination route exists. Several studies have documented the appearance of viruses in the hemolymph at timepoints before they could have had time to replicate (Boorman, 1960; Miles et al., 1973). This led to the hypothesis that viruses may be able to move between the cells of the midgut. Further evidence for this came from a study in which red blood cells from a blood meal could be detected in the hemocoel after feeding (Weaver et al., 1991). Also supporting this idea are experiments in which nanoparticles of similar sizes to arboviruses were fed to mosquitoes and could move beyond the midgut (Dong et al., 2017; Paquette et al., 2015). However, several other studies indicate that replication is often necessary for midgut escape. Therefore, midgut replication may not be required for dissemination in all cases.

Studies in which the RNAi pathway in the midgut was manipulated to enhance or reduce virus replication showed that there was a corresponding decrease or increase, respectively, in

disseminated infection (Franz et al., 2006; Khoo et al., 2010). Additionally, when GFP-expressing VEEV pseudovirus particles that were only capable of a single round of infection were used to orally infect mosquitoes it was found that GFP expression was limited to the midgut (Romoser et al., 2004).

Some researchers have hypothesized that viruses may need to reach a threshold level to escape from the midgut and several studies have provided evidence for this. Studies done with WEEV in *Culex* mosquitoes and ZIKV in European *Aedes albopictus* mosquitoes determined that a certain midgut level must be achieved for escape to occur (Kramer et al., 1981; Vazeille et al., 2019). However, other studies have refuted this idea, including research using DENV in *Aedes aegypti* (Bosio et al., 1998; Dickson et al., 2014). Research has shown that virus midgut replication level is an important component of midgut escape; however, it may play less of a role or no role in some cases.

Viral factors – viral diversity and replication error rate

It has been well documented that the midgut represents a significant bottleneck to arboviral diversity (Ciota et al., 2012; Forrester et al., 2012; Smith et al., 2008). One study estimated that in mosquitoes fed a high dose of VEEV, the number of viruses infecting the midgut was around 1200 while the number of viruses escaping the midgut was only around 50 (Forrester et al., 2012). These studies bring up an important question: is having high viral diversity an advantage in overcoming this bottleneck? Most arboviruses are RNA viruses which are known to have high mutation rates (Drake & Holland, 1999). Researchers have been interested in the question of whether decreasing this mutation rate leads to less diversity and thus decreased ability to overcome the challenges of midgut escape, and conversely if there is an advantage to increasing mutation rate. One study showed that a mutation which increased the

fidelity of CHIKV replication led to decreased titers in disseminated tissues, but a similar number of mosquitoes developed disseminated infection when compared to wild type infection (Coffey et al., 2011). One possible conclusion that can be drawn from this study is that decreased diversity may have led to a decrease in the number of virions able to disseminate from the midgut. Another study using high fidelity replication mutants of VEEV found significantly decreased dissemination rates (Lane et al., 2019). Interestingly, the same study found that low fidelity mutants which increased the mutation rate and viral diversity also decreased the dissemination rate (Lane et al., 2019). This can possibly be attributed to the increased accumulation of detrimental mutations.

Studies done without the use of mutator variants have also implicated the importance of diversity in dissemination. For example, one study found that SLEV that had been serially passaged in C6/36 cells displayed reduced genetic diversity compared to unpassaged virus and when this was fed to mosquitoes there was a reduction in disseminated infection (Ciota et al., 2007). Taken together, these studies suggest that changes which affect viral diversity within the midgut may alter the ability of virions to escape the midgut.

Viral factors – co-infection

In nature, some mosquito species may populate areas where several or many different disease-causing arboviruses, parasites, and bacteria also circulate. In mosquitoes that are co-infected with a combination of disease-causing agents, there is a need to know how these complex interactions affect midgut dissemination.

Filarial worms can cause serious disease in humans and animals, and like arboviruses, they require an insect vector to complete their life cycle. These nematodes circulate in parts of Asia, Africa, and South America (Simonsen et al., 2013) which may also host endemic

arboviruses. Studies have shown that with several viruses, mosquito ingestion of microfilariae can enhance arboviral dissemination from the midgut (Turell et al., 1984, 1987; Zytoon et al., 1993). The reason for this has been thought to be that the microfilariae puncture holes in the mosquito midgut which allows more rapid and enhanced escape into the hemocoel. This is supported by a study that found that dissemination rates of CHIKV were increased in mosquitoes that were co-infected with *Dinofilaria immitis* microfilariae and found holes in the midgut epithelium produced by the microfilariae (Zytoon et al., 1993). Recently, it has been found that viral dissemination may not be enhanced by simply escaping through these holes but rather that viruses may be transported across the midgut epithelium by the microfilariae. When *Brugi malayi* microfilariae were incubated with EEEV or VEEV and then extensively washed and used to infect mosquitoes, many mosquitoes became infected with the viruses (Vaughan & Turell, 2017). This suggests that the viruses may attach to or in some other way be transported by the microfilariae. The concern is that this may lead to more people with complicated infections with both parasites and viruses and that ignoring the issue of parasites may compromise the effort to eliminate arboviral disease.

While co-infection with filarial worms increased arbovirus dissemination, arbovirus co-infection with other arboviruses seems to have a neutral or negative effect on dissemination. Concurrent exposure of *Aedes aegypti* mosquitoes to varying combinations of CHIKV, ZIKV and DENV-2 resulted in little difference in dissemination compared to singly infected mosquitoes (Göertz et al., 2017; Rückert et al., 2017). Similarly, sequential exposure to CHIKV and ZIKV did not affect dissemination rates, however transmission rates were affected (Magalhaes et al., 2018). There appear to be instances of arbovirus co-infections having a negative impact on dissemination as SINV was found to lower infection and dissemination rates

of DENV-4 in *Aedes albopictus* mosquitoes (Muturi & Bara, 2015). Interestingly, mosquito infection with insect specific flaviviruses may also have a negative effect on virus dissemination. The insect specific virus cell fusing agent virus was found to reduce dissemination rate and dissemination titer of DENV-1 and dissemination titer of ZIKV in *Aedes aegypti* (Baidaliuk et al., 2019). Culex flavivirus was also found to affect dissemination of WNV at 7 days post infection (dpi), however this difference dissipated by 14 dpi (Bolling et al., 2012). Viral co-infection, particularly with insect specific viruses, could be important to better understand in the future.

Viral factors – dose

An MEB can be either affected or unaffected by viral dose. A dose-dependent barrier can be overcome by increasing the dose of the virus to a level that may or may not be possible to attain in natural settings. Rather than some fundamental incompatibility between the virus and vector, a dose-dependent barrier may be due to a factor such as the mosquito immune response, which may be overwhelmed by a larger dose of virus. Khoo et al. supported this idea in a study which implicated the RNAi pathway in contributing to a SINV dose-dependent MEB in *Aedes aegypti* (Khoo et al., 2010). The ability of higher initial doses of virus to overcome a MEB has also been shown in WEEV and *Culex tarsalis* (Kramer et al., 1981; Mahmood et al., 2006), ZIKV and *Aedes aegypti* (Tesla et al., 2018), and CHIKV and *Aedes aegypti* (Merwaiss et al., 2020). Understanding if a barrier is dose-dependent and the range of viral titers a vector may encounter in a natural blood meal is important to understanding its overall vectorial capabilities.

## Alphaviruses

Understanding vector competence requires detailed knowledge of both mosquito and virus. One group of arboviruses are the alphaviruses which are a genus of medically significant arboviruses in the *Togaviridae* family. This group includes several viruses which can cause significant disease in humans and animals. One subgroup of alphaviruses, called New World Alphaviruses, includes the Eastern, Western and Venezuelan equine encephalitis viruses, and is associated with potentially severe encephalitic disease. These viruses often cause several infections each year in the United States with sporadically higher numbers (*ArboNET Disease Maps*). Though infection is rare, cases can be serious or even fatal, particularly in vulnerable groups such as children. For example, the CDC found that between 2003 and 2012, Eastern equine encephalitis virus was responsible for 30 cases of neuroinvasive viral infection in children and 10 of those children died from the disease, which is a case fatality rate of 33% (Gaensbauer et al., 2014).

Not all alphaviruses are typically associated with neuroinvasive disease. Old World alphaviruses such as CHIKV, Ross River virus (RRV), and SINV are more often associated with fever and joint pain. One particularly troublesome virus in this group is CHIKV which has caused recent and sometimes large-scale outbreaks in Asia, Africa, Indian Ocean islands and the Americas (Khongwichit et al., 2021; Magalhaes et al., 2017; WHO, 2006) and can be associated with long-term pain and decreased quality of life after acute infection (de Andrade et al., 2010). This alarming spread has been attributed to mutations in CHIKV which has made it more transmissible by *Aedes albopictus* mosquitoes which are widespread even in areas with temperate climates (Vega-Rua et al., 2014; Vega-Rúa et al., 2020).

Currently, there are no specific treatments for alphaviruses, so treatment is only supportive in nature. There are also currently no approved vaccines, although several CHIKV vaccines are in clinical trials (Chen et al., 2020; Wressnigg et al., 2020). The lack of vaccines means that disease control is primarily targeted at reducing human exposure to the mosquito vector.

## Alphavirus virion structure and genome

Alphaviruses are small, enveloped, icosahedral viruses with T=4 symmetry (Fuller, 1987). Embedded in the envelope are viral glycoproteins E1 and E2 which form heterodimers and then associate in trimers to form spike proteins (Vogel et al., 1986). These glycoproteins are membrane spanning and the cytoplasmic domain of the E2 glycoprotein interacts with a hydrophobic pocket on the capsid protein (Skoging et al., 1996). E2 is generated from cleavage of a precursor protein called pE2, resulting in mature E2 as well as another protein called E3. E3 appears to be retained in the mature viral particle of some alphaviruses, while it is released in others (Li et al., 2010; Voss et al., 2010). Another protein called 6K is also found in the membrane but in small amounts. Its functions are not yet fully defined, but it appears to be involved in protein processing, glycoprotein translocation, trafficking to the membrane, budding and membrane permeabilization (Sanz et al., 2007). More recently, it has been discovered that ribosomal frameshifting during translation of 6K leads to the generation of another protein called TF (Firth et al., 2008; Snyder et al., 2013). The function of TF has not been fully explored but it has been suggested to play a role in viral assembly or exit from the cell (Snyder et al., 2013). The core of the virus is composed of the capsid which encloses the RNA genome. The capsid protein interacts with the genomic RNA at several places which facilitates selective packaging and viral assembly (Brown et al., 2020).

The alphavirus genome is made up of a single strand of positive sense RNA which contains between 11 and 12 kilobases (Fig. 1.3). Other features of the genome include a 5' cap and a 3' poly(A) tail (E. G. Strauss et al., 1984; E. G. Strauss & Strauss, 1986). These features allow the genome to act as a messenger RNA and be directly translated. The 5' end of the genome contains the nonstructural genes (nsP1, nsP2, nsP3 and nsP4) used in replication while the 3' end of the genome contains the structural genes (capsid, E3, E2, 6K, TF and E1). The nonstructural proteins are translated from the full-length genome while the structural proteins are translated from a subgenomic RNA that is generated from a negative strand copy of the genome (J. H. Strauss & Strauss, 1994). Both the nonstructural and structural proteins are expressed as large polyprotein precursors, which undergo cleavage to yield the final protein products.

## The alphavirus replication cycle

### Attachment and receptors

Most alphaviruses cycle between vertebrate and insect hosts and thus need to be able to attach to a variety of cell types under many different conditions. As such, many attachment factors and receptors have been identified; however, it is not always clear if these factors serve as entry receptors, where attachment is required for entry to the cell, or if they simply facilitate bringing the virus in proximity to entry receptors. It has been suggested that alphaviruses may first loosely attach to a cell via proteins or other factors and then move around on the cell surface until the receptor needed for entry is located (Kononchik et al., 2011; Wessels et al., 2007). While there is still much to be learned about what entry and attachment receptors alphaviruses use in the various species and cell types that they infect, several of the more well studied examples are discussed in the following paragraphs.

One proposed receptor for alphaviruses is heparan sulfate (HS) which is a glycoaminoglycan commonly found on cell surfaces including mammalian cells and insect cells. Early studies found that removal of HS from cells resulted in less SINV binding to cell surfaces in two mammalian cell lines (Byrnes & Griffin, 1998). However, the importance of this receptor in natural infections was quickly challenged as it was found that viral passage in mammalian cell lines leads to adaptation of the virus to this receptor via a mutation in the E2 protein (Gardner et al., 2013; Klimstra et al., 1998; Silva et al., 2014). Nonetheless, while dependence on or an increase in infectivity with HS has not been demonstrated in many natural alphavirus strains, there is some evidence that its role in alphavirus attachment and entry should be considered. For example, one study showed that a naturally occurring strain of North American EEEV utilized HS as an attachment factor and that this led to increased replication in neural tissues (Gardner et al., 2011).

A more recently observed alphavirus receptor is Mxra8, which is an adhesion molecule that is found in mammals, birds, and amphibians. This receptor has been shown to be important for a number of alphaviruses including CHIKV, RRV, Mayaro virus and O'nyong'nyong virus (Zhang et al., 2018). Anti-Mxra8 antibodies were found to prevent CHIKV infection in several human cell types and mutating Mxra8 in mice alleviated some CHIKV symptoms (Zhang et al., 2018, 2019). However, this receptor has not yet been shown to play a role in infection of mosquitoes, as an insect orthologue has not been discovered.

C-type lectins such as DC-SIGN and L-SIGN may play a particular role in the entry of some alphaviruses into important cell types. DC-SIGN, for example, has been found to be expressed on dendritic and macrophage cells (Soilleux et al., 2002), which play an important role in alphaviral pathogenesis (Gardner et al., 2008; Ryman & Klimstra, 2008). One study found

that these lectins can be used by SINV for attachment to these cells and, importantly, that SINV generated from mosquito cells was able to more efficiently infect cells expressing these C-type lectins than virus generated from mammalian cell lines, presumably due to differences in carbohydrate processing (Klimstra et al., 2003). This could potentially lead to viruses being primed to use different receptors in different hosts.

Natural resistance-associated macrophage protein or NRAMP is another molecule which has been demonstrated to be of importance for entry of an alphavirus. NRAMP is an iron transporter and has been identified in insects (NRAMP) and has an orthologue in mammals (NRAMP2). It was demonstrated that NRAMP was required for infection of *Drosophila* with SINV; and that this protein was required at the entry stage because when this was bypassed, replication occurred normally (Rose et al., 2011). NRAMP2 was also shown to be important for SINV infection in mice and Vero cells (Martinez & Kielian, 2016; Rose et al., 2011). To date, the only alphavirus that has been found to use NRAMP as a receptor is SINV.

**Entry**

In enveloped viruses, there are several ways that a virus can enter a cell including direct membrane fusion or endocytosis. In addition, endocytosis may occur using several different pathways including clathrin-dependent and independent mechanisms. The mode of entry of alphaviruses into cells is variable and dependent on both virus and host cell type, however, the accepted primary mode of alphaviral entry is receptor-mediated clathrin-dependent endocytosis. In this pathway, clathrin and other proteins work to bend the cell membrane which causes a vesicle to pinch off into the cytosol of the cell and leads to the formation of endosomes (reviewed in Kaksonen & Roux, 2018). This pathway has been shown to be used by a range of alphaviruses and in both mammalian and insect cells (Hua et al., 2013; DeTulleo & Kirchhausen,

1998; Hoornweg et al., 2016; Kolokoltsov et al., 2006). However, alternative endocytic pathways have also been shown to be important. In one experiment in which expression of the clathrin heavy chain was knocked down, CHIKV was still found to enter cells (Bernard et al., 2010). CHIKV was also found to enter cells via macropinocytosis (Hua et al., 2019). Also, WEEV and VEEV were found to use caveolae-mediated endocytosis to cross the blood brain barrier (Salimi et al., 2020). Alternatively, some research has shown that alphaviruses may enter cells in a manner that bypasses endocytosis altogether. One study found that infection can occur at the cell surface and that membrane fusion may not always be necessary (Paredes et al., 2004). Another study found that after SINV attached to cells, complete virions lost their RNA and became empty protein shells. The authors suggest that infection may occur through a pore complex (Vancini et al., 2013).

**Endosomal fusion and uncoating**

After endocytosis the enveloped alphavirus enters the cytoplasm enclosed in an endosome. For the genome to be released to begin the processes of translation and replication, fusion with the endosomal membrane must occur to release the nucleocapsid and then the nucleocapsid must break down to release the genome. As the endosome matures, the internal pH lowers which has been shown to be a requirement for virus fusion with the endosomal membrane (Helenius et al., 1980). This pH drop causes the E1-E2 heterodimers to change their conformation and a part of E1 called the fusion peptide becomes exposed and is inserted into the membrane (Ahn et al., 1999; Gibbons et al., 2003; Gibbons & Kielian, 2002). E1 forms a homotrimer in the membrane and this results in the release of the nucleocapsid into the cytoplasm (Gibbons & Kielian, 2002; Wahlberg et al., 1992). A low pH is not the only requirement for endosomal fusion; the lipid composition of the membrane may also play a

critical role. Studies have shown a dramatic reduction in viral fusion when cholesterol is depleted (Lu et al., 1999; Phalen & Kielian, 1991). Other studies have shown that sphingolipids are required in the target membrane (Moesby et al., 1995; Nieva et al., 1994).

Once the nucleocapsid is free in the cytoplasm it then needs to disassemble to expose the genome for translation and replication. It has been suggested that viral E1 proteins may form ion-permeable pores that allow protons to move from the endosome into the viral particle (Wengler et al., 2004; Wengler & Wengler, 2002). This lower pH may assist in priming the nucleocapsid for disassembly. After fusion with the endosomal membrane, the nucleocapsid then interacts with the 60S subunit of the ribosome which completes the breakdown process (Singh & Helenius, 1992; Wengler et al., 1992; Wengler & Wengler, 1984).

**Replication and synthesis**

As previously described, the viral genome has a 5' cap and a 3' poly(A) tail which mimics mRNA (Fig 1.3). Therefore, the nonstructural proteins at the 5' end can be translated directly from the genome. The nonstructural proteins have functions both individually and as a part of polyprotein intermediates. The function of each nonstructural protein is reviewed in Jose et al, 2009. Briefly, nsP1 functions in the capping of genomic and subgenomic viral RNA, nsP2 has helicase activity as well as protease activity, which is critical for polyprotein processing, and nsP4 acts as the viral RNA-dependent RNA polymerase (RdRP) (Jose et al., 2009). The function of nsP3 is less defined; however, it has been shown to be important in negative strand RNA synthesis (Wang et al., 1994), determining vector specificity (Saxton-Shaw et al., 2013) and in virulence (Park & Griffin, 2009; Tuittila & Hinkkanen, 2003).

Initial translation of the viral genome results in a polyprotein containing nsP1, nsP2, and nsP3 (P123) or, if there is a readthrough of the UGA termination codon, a polyprotein containing

nsP1, nsP2, nsP3 and nsP4 (P1234) (Lit & Rice, 1993). The nsP2 protein, both individually and as a part of the polyprotein, acts as a protease to process the polyprotein into individual proteins as well as functional intermediates (E. G. Strauss et al., 1992). The P3/4 site can be cleaved *in cis* while other sites must be cleaved *in trans*, therefore, in early infection P123 and nsP4 are the dominant products (De Groot et al., 1990). These conditions favor the generation of negative strand copies of the genome (Shirako & Strauss, 1994). Later in infection, the cleaved products start to dominate which favors the switch to synthesis of full-length positive strand genomes as well as the smaller subgenomic RNA using the negative strand copies as a template (J. H. Strauss & Strauss, 1994). The subgenomic RNA is transcribed from a promoter sequence located between the nonstructural and structural open reading frames (Fig 1.3).

The structural proteins are translated as a polyprotein from the subgenomic RNA. The capsid protein has autoproteolytic activity which frees the C-terminal end from the polypeptide (Hahn et al., 1985). The rest of the structural proteins (pE2, 6K, TF and E1) are cleaved from the polypeptide by cellular proteases (Wellink & van Kammen, 1988). pE2 and E1 are then modified in the endoplasmic reticulum where they are glycosylated, and further modifications occur in the Golgi apparatus. Before reaching the plasma membrane, pE2 is cleaved into E2 and E3. The E1-E2 heterodimers are then embedded in the plasma membrane (Jose et al., 2009).

**Assembly and exit**

Alphavirus capsid proteins will self-assemble into a particle similar to the nucleocapsid, called a core-like particle, in the presence of several kinds of nucleic acids (Tellinghuisen et al., 1999; Wengler et al., 1982). An early intermediate in nucleocapsid formation appears to be a capsid protein dimer with a bound nucleic acid (Tellinghuisen & Kuhn, 2000). It is thought that the N-terminal region of the capsid protein is responsible for both this dimerization as well as

nucleic acid binding (Mendes & Kuhn, 2018; Perera et al., 2001). Certain areas in alphavirus genomes have been implicated as packaging signals including nucleotides 945-1076 in SINV and 2761-3062 in RRV, as mutations in these areas reduces the packaging of full-length genomes in the viral particle (Frolova et al., 1997). However, sequences without this signal have been found to be packaged (Frolova et al., 1997; Kim et al., 2011; Volkova et al., 2006). These assembled nucleocapsids then move through the cytoplasm to the plasma membrane.

To complete virus assembly and release, the nucleocapsid interacts with E2 in the plasma membrane where hydrophobic amino acids on E2 interact with a hydrophobic pocket on the surface of the nucleocapsid; this interaction has been shown to be critical for virus budding (Owen & Kuhn, 1997; Suomalainen et al., 1992). The lipid composition of the plasma membrane is also important in this process as cholesterol has been shown to be important in alphavirus exit (Lu et al., 1999). After budding, the virus particle with its newly acquired glycoprotein containing envelope can infect an uninfected cell.

**Sindbis virus**

SINV is the type member of the *Alphavirus* genus. SINV was first isolated in Cairo, Egypt in 1952 (Taylor et al., 1955). It has since been found in Europe, Australia, Asia, and other parts of Africa, where it is transmitted to humans through the bite of mosquitoes, primarily of the *Culex* genus (Adouchief et al., 2016). In nature, SINV is thought to be transmitted between mosquito vectors and birds of many different species with humans acting as accidental dead-end hosts (Buckley et al., 2003; Kurkela et al., 2008; Lundström et al., 2001). Human infection with SINV is often asymptomatic. One study in Finland found that subclinical infection was 17 times more common than clinical infection (Brummer-Korvenkontio et al., 2002). When infection does cause clinical symptoms, they usually include headache, fever, fatigue, rash, and joint pain.

Some patients have persistent joint and muscle pain, but most people recover completely, and complications are rare (Adouchief et al., 2016).

**Sindbis virus genome engineering and transducing systems**

SINV has long been an important model system for studying virus-vector interactions. The advantages of SINV as a model system include that infectious clones of SINV have existed for many years (Lustig et al., 1988; Rice et al., 1987), we have extensive knowledge of the molecular biology of SINV replication, and it poses low risk to humans. Infectious clones are DNA copies of full-length RNA genomes which can be inserted into plasmids, in vitro transcribed, and transfected into cells to generate viral particles. The infectious clone can be manipulated using standard cloning techniques. This opened the door to generating alphavirus transducing systems, which are alphaviruses that are engineered to have the ability to express a foreign gene.

A common way to generate a SINV transducing system is to insert a second copy of the subgenomic promoter followed by cloning sites into an infectious clone which allows expression of the foreign gene in cells that are susceptible to infection with the virus. The first SINV transducing system called pTE/3'2J was derived from a neurovirulent strain of SINV and has an added subgenomic promoter and cloning sites in the 3’ untranslated region of the virus genome, following the structural genes (Hahn et al., 1992; Lustig et al., 1988). However, this virus does not significantly infect mosquito midgut cells making it a poor candidate for foreign gene expression studies in blood-fed mosquitoes. To develop a transducing system that would infect midgut cells, researchers replaced structural coding sequences from TE/3'2J with sequences from a Malaysian isolate of SINV called MRE16, which had undergone only limited passage in tissue culture (Olson et al., 2000; Seabaugh et al., 1998). One chimeric construct called

pMRE/3'2J/GFP which expresses green fluorescent protein (GFP) was found to efficiently infect *Aedes aegypti* midgut cells, however, it was found that there was an issue with the stability of the GFP insert as its expression was primarily limited to the midgut (Olson et al., 2000). Enhanced stability was achieved in a later chimeric construct, 5'dsMRE16ic, which was developed by inserting a duplicated subgenomic promoter between the structural and nonstructural genes, just upstream of the native subgenomic promoter (Pierro et al., 2003). The earlier chimeric constructs based on the neurovirulent TE virus strain are useful for several different applications but do not accurately reflect natural mosquito-virus interactions. The 5'dsMRE16ic system was developed with this in mind as it is based entirely off the genome of MRE16, while retaining the improved stability of the more internal duplicated subgenomic promoter (Foy et al., 2004).

An alternative way to express a foreign gene in SINV without the use of a duplicated subgenomic promoter is to insert the gene directly into the structural open reading frame (ORF). In this strategy the foreign gene is fused to a sequence which causes ribosomal skipping such as the 2A sequence from foot-and-mouth disease virus. This sequence is then inserted in-frame following the capsid sequence. Upon translation, the self-cleaving activity of the capsid will free the N-terminus of the inserted protein while the 2A sequence will induce ribosomal skipping freeing the C-terminus. The result is a foreign protein expressed via the structural ORF that does not impact the normal functioning of the viral structural proteins. An early study using this strategy in SINV showed that it led to improved stability and decreased attenuation compared to expression via a duplicated subgenomic promoter (Thomas et al., 2003). This strategy may be especially crucial in some applications where stability is critical. For example, SINV is being developed as an oncolytic therapy agent and to improve safety, replication in non-target tissues should be limited. One study showed that inserting tissue-specific miRNA target sequences,

which cause tissue-specific attenuation, into the structural genes of SINV led to better attenuation and stability compared to insertion into the 3' UTR, although loss of the transgene still occurred after multiple passages (Kueberuwa et al., 2014). This strategy is also useful for improving stability of genes that are subject to negative selection. In a SINV construct engineered to express the pro-apoptotic gene *reaper* via a duplicated subgenomic promoter, the gene was rapidly lost and thus differences between the percentage of mosquitoes with disseminated infection with the Reaper-expressing virus versus a control quickly dissipated (O'Neill et al., 2015).

## Conclusions

There are many open questions about how alphaviruses, such as SINV, interact with their mosquito hosts and what is needed for dissemination and transmission to occur. The goal of the studies described in the following chapters was to better understand the factors that affect viral midgut escape. These studies focus on replication of SINV in the *Aedes aegypti* midgut and apoptosis in midgut cells. A better understanding of these factors will be important in informing arboviral disease control.

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# Chapter 1 figures

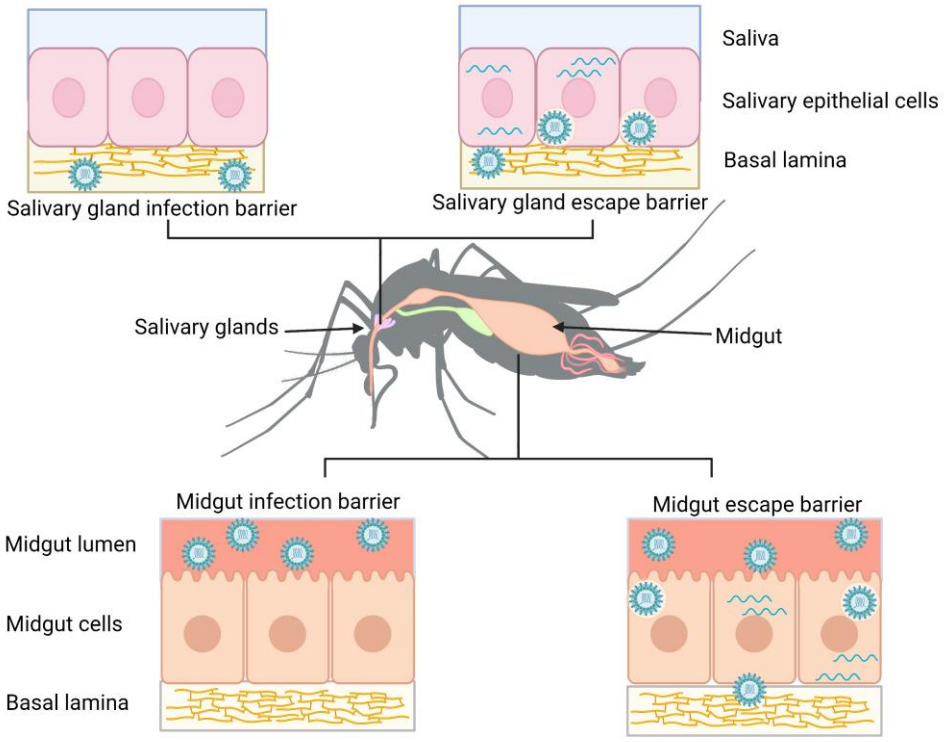


**Figure 1.1 Tissue barriers to arbovirus infection**

A midgut infection barrier (MIB) (bottom left) occurs when viruses are unable to enter midgut epithelial cells. In contrast, a midgut escape barrier (MEB) (bottom right) occurs when viruses are able infect midgut cells but do not disseminate from this tissue. A salivary gland infection barrier (SGIB) (upper left) occurs when the virus fails to infect the cells of the salivary gland while a salivary gland escape barrier (SGEB) (upper right) occurs when the virus is unable to pass into the saliva. Figure created with Biorender.com.

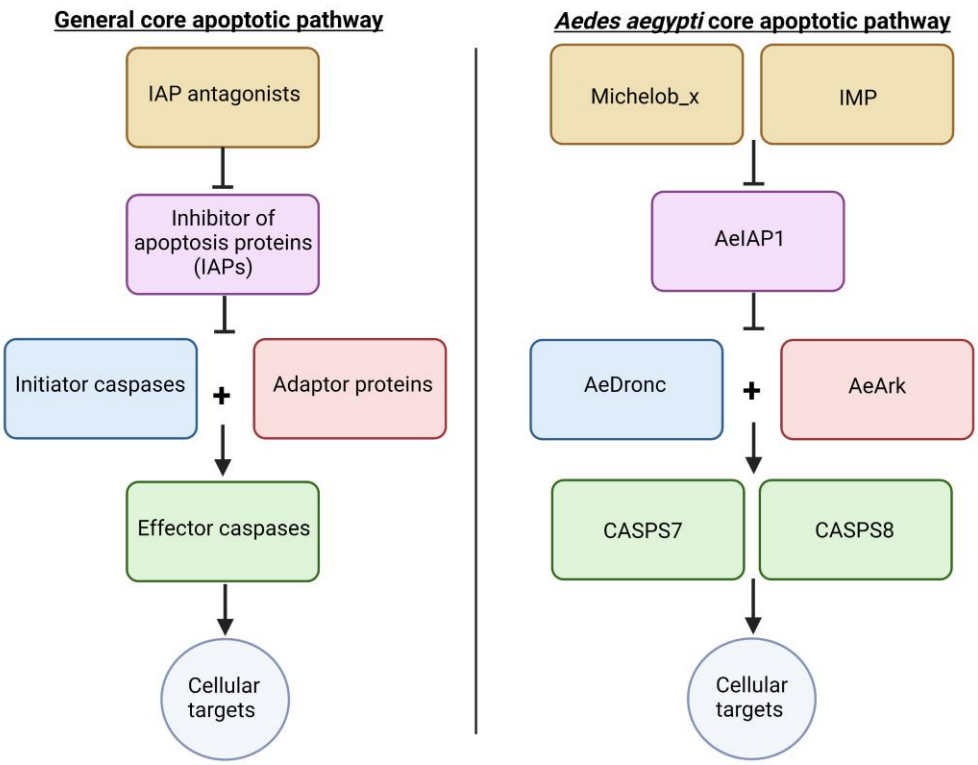


**Figure 1.2 Core apoptotic pathway in *Aedes aegypti***

A general diagram of the core apoptotic pathway is shown on the left while the known components of the *Aedes aegypti* core apoptotic pathway is shown on the right. Figure created with Biorender.com.

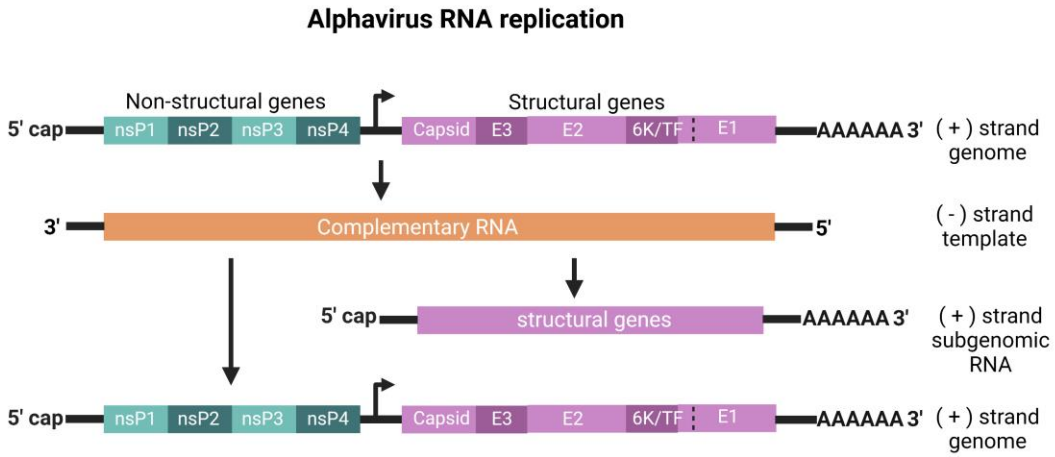


**Figure 1.3 Alphavirus RNA replication**

Schematic of the generalized alphavirus genome and replication steps. Figure created with Biorender.com.

# Chapter 2 Infection of *Aedes aegypti* Mosquitoes with Midgut-Attenuated Sindbis Virus Reduces, but Does Not Eliminate, Disseminated Infection

Carpenter, A., Bryant, W. B., Santos, S. R., & Clem, R. J. (2021). Infection of *Aedes aegypti* Mosquitoes with Midgut-Attenuated Sindbis Virus Reduces, but Does Not Eliminate, Disseminated Infection. *Journal of Virology, 95*(13), 136–157. https://doi.org/10.1128/JVI.00136-21

## Abstract

Arboviruses are transmitted by specific vectors, and the reasons for this specificity are not fully understood. One contributing factor is the existence of tissue barriers within the vector such as the midgut escape barrier. We used microRNA (miRNA) targeting of Sindbis virus (SINV) to study how replication in midgut cells contributes to overcoming this barrier in the mosquito *Aedes aegypti*. SINV constructs were designed to be attenuated specifically in midgut cells by inserting binding sites for midgut-specific miRNAs into either the 3′ untranslated region (MRE3′miRT) or the structural open reading frame (MRE-ORFmiRT) of the SINV genome. Both miRNA-targeted viruses replicated less efficiently than control viruses in the presence of these miRNAs. When mosquitoes were given infectious blood meals containing miRNA-targeted viruses, only around 20% (MRE3′miRT) or 40% (MRE-ORFmiRT) of mosquitoes developed disseminated infection. In contrast, dissemination occurred in almost all mosquitoes fed control viruses. Deep sequencing of virus populations from individual mosquitoes ruled out selection for mutations in the inserted target sequences as the cause for dissemination in these mosquitoes. In mosquitoes that became infected with miRNA-targeted viruses, titers were equivalent to those of mosquitoes infected with control virus in both the midgut and the carcass, and there was no evidence of a threshold titer necessary for dissemination. Instead, it appeared that if infection was successfully established in the midgut, replication and dissemination were largely normal. Our results support the hypothesis that replication is an important factor in allowing SINV to overcome the midgut escape barrier but hint that other factors are also likely involved.

## Importance

When a mosquito ingests an arbovirus during a blood meal, the arbovirus must escape from the midgut of the vector and infect the salivary glands in order to be transmitted to a new

host. We used tissue-specific miRNA targeting to examine the requirement for Sindbis virus (SINV) to replicate in midgut epithelium in order to cause disseminated infection in the mosquito *Aedes aegypti*. Our results indicate that specifically reducing the ability of SINV to replicate in the mosquito midgut reduces its overall ability to establish infection in the mosquito, but if infection is established, replication and dissemination occur normally. These results are consistent with an importance for replication in the midgut epithelium in aiding arboviruses in crossing the midgut barrier

## Introduction

Arboviral diseases continue to cause a significant public health burden worldwide (Murray et al., 2015). Some of the most concerning arboviruses include dengue virus, which has increased 30-fold in incidence in the past 50 years and has been estimated to cause 390 million infections per year (Bhatt et al., 2013; World Health Organization, 2012), as well as the recently re-emerging pathogens Zika virus and chikungunya virus, which can cause severe and sometimes lifelong effects including chronic pain and developmental disability (Paixão et al., 2018). Controlling arboviral infections is difficult because vaccines and antivirals for these viruses are either not available or are limited in their use. These limitations make controlling arboviruses at the level of the vector an attractive alternative.

One possible route of controlling arbovirus infection is to prevent infection of their vectors. While arboviruses can utilize certain mosquito species to replicate and be transmitted to new hosts, most virus-vector combinations do not result in mosquito infection or successful transmission. Unlocking the reasons why most mosquito species do not develop disseminated infection when they ingest an infected blood meal could lead to new control strategies. However, this is a complex undertaking because vector competence varies even amongst populations or

strains within known successful virus-vector species combinations, with the interaction of the genotypes of the mosquito and the virus playing an important role (Lambrechts et al., 2009). The reasons for the differences in vector competence are far from being fully explained, but the presence of tissue barriers likely plays a significant role (reviewed in Franz et al, 2015). One tissue barrier that has been shown to play a key role in vector competence is the midgut escape barrier (Kramer et al., 1981; Paulson et al., 1989; Turell et al., 1984; Weaver et al., 1984). This barrier is encountered when virus is ingested during a blood meal and the mosquito midgut becomes infected, but there is inefficient dissemination of infection to other organs, most importantly the salivary glands. Several aspects of mosquito biology likely contribute to the existence of such a barrier including immune responses such as RNA interference (RNAi) and apoptosis (Ayers et al., 2021; Dong et al., 2017; Khoo et al., 2010; O'Neill et al., 2015), and physical obstacles including the basal lamina that surrounds the midgut (Armstrong et al., 2020; Cui et al., 2019). The basal lamina has a pore size that is too small to allow the passage of viruses (Reddy & Locke, 1990). There is evidence that there are changes to the basal lamina after a blood meal such as the appearance of perforations (Kantor et al., 2018; Reinhardt & Hecker, 1973), changes in the composition of structural components such as collagen IV (Dong et al., 2017) and changes in expression of remodeling enzymes such as matrix metalloproteinases (Kantor et al., 2017). Thus, viral escape may occur during this period of changes to the basal lamina. However, these basal lamina modifications do not explain why there is a midgut escape barrier in some virus-vector combinations.

A factor that may contribute to the successful escape of a virus from the midgut is the level of virus replication in this tissue. There have been conflicting reports regarding whether replication in midgut cells is necessary for dissemination. It has long been speculated that an

intercellular route through the midgut tissue may exist in certain situations which would hypothetically allow virus to enter the hemocoel without replicating in the midgut epithelium, perhaps by directly accessing a portal such as tracheoles. Evidence for this idea has been described in several studies which include findings that viruses have been found in the mosquito hemolymph within minutes of ingestion, which would not give time for replication and escape from the midgut epithelial cells (Boorman, 1960; Miles et al., 1973), and the observation of "leaky guts" in which host red blood cells and virus were found in the hemocoel of some mosquitoes shortly after ingestion of a blood meal (Weaver et al., 1991). Further supporting the existence of an intercellular route are experiments in which nanoparticles of a similar size to viruses have been found to efficiently transit through the midgut (Dong et al., 2017; Paquette et al., 2015). However, there is also evidence to the contrary which suggests that viral replication in this tissue is necessary to overcome the midgut escape barrier. One such study used *Aedes taeniorhynchus* mosquitoes and Venezuelan equine encephalitis virus replicon particles expressing green fluorescent protein (GFP), which are only capable of a single round of infection, and found that GFP expression was limited to the midgut following oral delivery (Romoser et al., 2004). Another study showed that when *Aedes aegypti* mosquitoes were genetically engineered to have an enhanced RNAi response against dengue virus in the midgut, which reduced midgut replication, the ability of the vector to transmit the virus was diminished (Franz et al., 2006). Similarly, when transgenic *Ae. aegypti* mosquitoes with a diminished RNAi response were orally infected with Sindbis virus (SINV), the mosquitoes had increased viral midgut titers and there were increased rates of viral dissemination (Khoo et al., 2010). An important limitation of these latter studies is that they involved manipulation of the RNAi pathway, which could have unexpected results on mosquito physiology. Nevertheless, these

results suggest that arboviruses may not be able to simply move between the cells of the midgut to reach new tissues but must instead infect and replicate in the midgut epithelium, possibly in order to reach a threshold titer necessary for dissemination, or to gain access to a hypothetical escape portal.

A strategy to limit virus tropism, referred to as microRNA (miRNA) targeting, involves engineering viruses so that they are targeted by tissue-specific miRNAs. In miRNA targeting, short sequences with complementarity to a tissue-specific miRNA are inserted into the genome of an RNA virus, with the goal of reducing viral replication specifically in tissues expressing the miRNA. This technique has been used to develop attenuated virus vaccines, improve oncolytic virus safety and to mitigate risk to researchers working with dangerous pathogens (Barnes et al., 2008; Kelly et al., 2008; Langlois et al., 2013; Ylosmaki et al., 2013). Previous studies have reported differences in the efficiency of miRNA targeting based on the site of insertion of the target sequences. One study with vesicular stomatitis virus showed that the greatest reduction in virus replication occurred when four tandem copies of the miRNA response elements were inserted into the 3′ UTR region (Kelly et al., 2010). Another study using SINV had success using tandem miRNA target sequences inserted into the structural ORF (Kueberuwa et al., 2014). miRNA targeting has also been previously used to prevent flavivirus replication in mosquitoes and ticks. In mosquitoes it was found that inserting complementary miRNA sites in the 3′ UTR had a greater effect at suppressing replication, while in ticks it was found that suppression was equivalent when comparing viruses with miRNA target sites in the 3′ UTR versus the ORF (Tsetsarkin et al., 2015, 2016).

In this study we used miRNA targeting as a method to investigate the requirement for midgut replication. This approach had the important advantage that it did not require enhancing

or disrupting an important host process such as RNAi, which could have unintended effects on other aspects of mosquito physiology. It also lent itself well to our study because of the existence of miRNAs that are specifically expressed in the *Ae. aegypti* midgut (Feng et al., 2018; S. Li et al., 2009; Liu et al., 2014). Using the MRE16 strain of SINV, which retains the ability to cause high rates of disseminated infection in *Ae. aegypti* (Foy et al., 2004; Seabaugh et al., 1998), we inserted target sites for two midgut-specific miRNAs into either the 3′ UTR region or the structural open reading frame (ORF). To our knowledge this study is the first to use miRNA targeting to study arbovirus tissue tropism in mosquitoes. Our results indicate that miRNA targeting in the midgut decreased the ability of SINV to establish midgut infection and disseminate in *Ae. aegypti*, but if the virus managed to establish midgut infection, replication and dissemination occurred normally.

## Results

### The miRNAs 1174 and 1175-5p display midgut-specific expression

Before testing our viral construct in mosquitoes, we wanted to confirm, and further characterize, the midgut expression specificity of miRNAs 1174 and 1175-5p in the *Ae. aegypti* line Orlando, since it had only previously been demonstrated in a different *Ae. aegypti* line (Liu et al., 2014). Thus, we sampled the midguts and remaining carcasses of female Orlando mosquitoes that were either non-fed or had been given a non-infectious blood meal at 10, 24, 48 or 72 hours post-blood meal (hpbm). Pooled tissues (n = 5) were used to examine relative expression levels of *miR1174* and *miR1175-5p* by RT-qPCR. Levels of both miRNAs increased rapidly in the midgut after blood feeding, peaking at 10 hpbm before declining at 24 hpbm. The levels of both miRNAs rose again after 24 hpbm, with *miR1175-5p* being present at higher levels than *miR1174* at 48 and 72 hpbm (Fig. 2.1A). Importantly, both miRNAs exhibited substantially

higher expression levels (10-fold or greater) in midgut versus carcass, both in non-fed and blood-fed mosquitoes at all time points tested (Fig. 2.1B). These results confirmed that expression of miRNAs 1174 and 1175-5p displayed a high degree of midgut specificity both before and after blood feeding.

**miRNA-targeted viruses replicate less efficiently in the presence of cognate miRNAs**

To determine the ability of miRNA targeting to reduce SINV replication, we inserted tandem copies of sequences that were complementary to miRNAs 1174 and 1175-5p in the 3′ UTR region or in-frame within the structural ORF of an infectious clone of MRE-16 (Fig. 2.2). These constructs were named MRE3′miRT and MRE-ORFmiRT, respectively. We also generated control infectious clones (MRE3′control and MRE-ORFcontrol) by inserting a control sequence of the same size into the same sites. This control sequence was derived from the sequence containing the miRNA binding sites, but contained synonymous mutations which would be expected to disrupt miRNA binding (Fig. 2.2C). MRE-ORFmiRT and MRE-ORFcontrol were constructed so that during translation of the structural polyprotein, the peptide encoded by the inserted sequence would be removed without affecting processing of the viral structural proteins (see Materials and Methods for details).

To examine the ability of *miR1174* and *miR1175-5p* to reduce replication of MRE3′miRT or MRE-ORFmiRT, we conducted cell transfection experiments using miRNA mimics in BHK21 cells. In separate experiments with a fluorescently labeled mimic, we observed that transfection efficiency under these conditions was approximately 50% (data not shown). We then infected the cells with either MRE3′miRT or MRE3′control at a multiplicity of infection (MOI) of 0.1 or 0.01 and measured the production of infectious virus by $TCID_{50}$ assay (Fig. 2.3A-B). Replication of MRE3′miRT was reduced at early time points in cells transfected with the midgut

miRNA mimics compared to control mimic- or mock-transfected cells. Specifically, at a MOI of 0.1, a significant difference in titer was observed at 24 hrs post infection (hpi) (2.1 log difference) and 38 hpi (1.1 log difference), but not at 48 hpi (Fig. 2.3A). This inhibitory effect on replication was even more pronounced at MOI 0.01, with average log differences of 1.9, 2.8 and 2.8 at 24, 38 and 48 hpi, respectively (Fig. 2.3B). When this experiment was repeated using MRE-ORFmiRT at MOI 0.01, significant differences were observed at 24 (1.7 log difference) and 38 hpi (2.9 log difference), but not at 48 hpi (Fig. 2.3C). These differences reflect a strong ability of the midgut miRNA mimics to inhibit replication of both MRE3′miRT and MRE-ORFmiRT, especially when considering that only around 50% of the cells were transfected with the mimics. As expected, MRE3′control and MRE-ORFcontrol exhibited no significant differences in replication between any of the transfection treatments (Fig. 2.3).

**Midgut-specific miRNA targeting reduces the ability to orally infect mosquitoes**

We then wanted to determine how the midgut-specific miRNAs would affect replication and dissemination of the miRNA-targeted viruses in mosquitoes. To do this, female mosquitoes were allowed to feed on blood containing MRE3′miRT or MRE3′control, and at 48, 72 or 120 hpbm we dissected mosquito midguts from the remaining carcasses and measured the amount of infectious virus in each source of tissue. At 48 hpbm, 37.7% of the MRE3′miRT infected mosquitoes had a detectable titer in their midgut while 97.1% of control infected mosquitoes had infected midguts (Fig. 2.4A). The percentage of mosquitoes with detectable midgut infection in the MRE3′miRT group decreased over time, to 25.9% at 72 hpbm and 12.5% at 120 hpbm. This decrease was found to be significant between 48 and 120 hpbm by Fisher's exact test ($p=0.003$). The percentage of midgut infection in the control group remained high and there was no significant difference between any of the time points.

There was also a significant difference between the two viruses in the proportion of mosquitoes with detectable virus in the carcass, which indicates disseminated infection (Fig. 2.4A). Seventy-five percent of mosquitoes infected with MRE3′control had disseminated infection by 48 hpbm, compared to only 21.3% of MRE3′miRT-infected mosquitoes. This trend continued over the later time points: at 72 and 120 hpbm, the rate of disseminated infection in the MRE3′miRT group remained around 20% or lower while the control group had 91.4% dissemination at 72 hpbm and 90% dissemination at 120 hpbm.

Roughly similar results were observed when mosquitoes were given blood meals containing MRE-ORFmiRT or MRE-ORFcontrol (Fig. 2.4B), although the degree of reduction in both midgut infection and disseminated infection appeared to be slightly less than with MRE3′miRT. The increased frequency of infection with MRE-ORFmiRT was consistent with the better ability of MRE-ORFmiRT to replicate in the presence of the miRNA mimics compared to MRE3′miRT (Fig. 2.3).

We also determined the levels of virus replication in mosquitoes infected with the miRNA-targeted viruses, compared to their respective controls. When all mosquitoes, including those that did not have detectable virus titer, were included in the analysis, there was a significant difference between MRE3′miRT and MRE3′control titers at all time points in both midgut and carcass (Fig. 2.5A). However, when we compared the midgut and carcass titers only in the mosquitoes that had detectable virus (Fig. 2.5B), there was no statistical difference in titer between the two viruses except at the 72 hr time point in midgut. It should be noted that only a small number of mosquitoes developed disseminated infection with MRE3′miRT, which could affect these statistical analyses. Regardless, the observations that the carcass titers were similar between MRE3′miRT and MRE3′control, and both were substantially higher than the midgut

titers, rule out the possibility that the presence of virus in the carcass was simply due to contamination from the dissected midgut.

These experiments were repeated in mosquitoes blood-fed with MRE-ORFmiRT or MRE-ORFcontrol (Fig. 2.5C-D) and we again found that when all mosquitoes, including those without detectable virus, were included in the analysis there were significant differences between titers in MRE-ORFmiRT and MRE-ORFcontrol-fed mosquitoes (Fig. 2.5C), but when mosquitoes with undetectable levels of virus were removed, there was no significant difference between MRE-ORFmiRT and MRE-ORFcontrol titers in either the midgut or carcass at any timepoint (Fig. 2.5D). Overall, these results indicate that significantly less mosquitoes became infected after exposure to midgut-attenuated viruses than control viruses, but when a mosquito was infected, viral replication occurred at normal levels.

**MRE3′miRT replicates normally in mosquito tissues when the midgut is bypassed**

Since *miR1174* and *miR1175-5p* are preferentially expressed in midgut, we predicted that MRE3′miRT would replicate normally if the midgut was bypassed by injecting virus directly into the hemocoel. To test this, we injected mosquitoes intrathoracically with MRE3′miRT or MRE3′control and titered the carcass samples (Fig. 2.6). There was no difference in the prevalence of infection, with almost all mosquitoes in both groups having detectable infection at all timepoints tested. We also did not find any significant differences between the mean virus titers at any timepoint. From these data we conclude that the reduced percentage of infection in the mosquitoes orally infected with MRE3′miRT was related to decreased replication in the midgut and not due to a reduced ability to replicate in other mosquito tissues.

**No evidence of a minimum threshold midgut titer necessary for dissemination**

Using these data, we also examined whether there was evidence of a threshold titer that needed to be achieved in the midgut for dissemination to occur. We first analyzed the MRE3′control and MRE3′miRT data in mosquitoes that developed infection to determine if a higher midgut titer was correlated with a higher carcass titer and whether midgut titer could be used to predict disseminated infection. We found no significant correlation between midgut and carcass titer (r=0.15) for either virus (Fig. 2.7A). Furthermore, examining the data for both viruses, there was no indication of a threshold midgut titer that predicted disseminated infection. The minimum level of detection in this $TCID_{50}$ assay was $10^2$ PFU/ml, which explains why there were no observed titers between $10^2$ and zero. In addition, we observed mosquitoes with midgut titers that did not have detectable carcass titers, especially at the 48 hr time point (Fig. 2.3), and there were also a small number of mosquitoes infected by either virus that had disseminated infection but did not have detectable midgut titers (Fig. 2.7A). To examine this further, we constructed a Receiver Operating Characteristic (ROC) curve using all of the combined data from both viruses to determine whether midgut titer is a useful predictor of disseminated infection. We found that midgut titer was only a fair predictor of disseminated infection with an area under the curve (AUC) or probability value of 0.775 (Fig. 2.7B). Because there were a higher number of midgut-only infections at 48 hpbm than at the later timepoints (Fig. 2.4), presumably due to the virus not having escaped from the midgut yet in some mosquitoes, we also analyzed the data after removing the 48 hpbm results. However, this resulted in only a small increase in the AUC value, to 0.830.

**Virus populations do not contain significant sequence alterations in the insert region**

A potential explanation for the ~20% of mosquitoes that developed disseminated infection with MRE3′miRT could be selection for virus mutants with alterations in the miRNA target sequence, which allowed them to escape inhibition by miRNAs in the midgut. To examine this possibility, we performed deep sequencing of amplicons derived from this region of the viral genome in the virus populations of individual infected mosquitoes. The virus populations were individually analyzed from the carcasses of 12 mosquitoes orally infected with MRE3′miRT and 10 mosquitoes orally infected with MRE3′control, as well as 10 mosquitoes that were injected with MRE3′miRT. These sequence analyses indicated that only a very small proportion of reads contained alterations among the viral populations in any of these mosquitoes (Fig. 2.8 and Dataset S1). A single mutation hotspot was observed at nucleotide 11563 (corresponding to position 9 in Fig. 2.2C), which was primarily comprised of the transition mutations A to G in MRE3′miRT and G to A in MRE3′control. Position 11563 is the initial nucleotide in the first target sequence for *miR1174* in MRE3′miRT. An alteration in this position was observed in an average of 5-12% of sequence reads from all three groups of infected mosquitoes, including the control virus, implying it does not correlate with selective pressure due to the presence of the miRNA target sequences. While detected at some level in nearly all the mosquitoes, the percentage of reads with a mutation at this hotspot varied significantly by individual, ranging from <1% to 33%. Based on these results, we conclude that disseminated infection in the MRE3′miRT blood fed mosquitoes was not the result of a large proportion of the viral population having alterations in the 3′miRT region. While the significance of the mutation hotspot at position 11563 is not clear, it does not seem to be due to the presence of functional miRNA target sites since the same nucleotide position was altered in MRE3′control.

Additionally, given that the miRNA target sequences are repeated, a mutation at this single position in one target site is unlikely to affect miRNA binding at other target sites.

## Discussion

The goal of this study was to determine if midgut replication is necessary for midgut escape and dissemination to other tissues. We found that SINV constructs targeted by midgut-specific miRNAs had reduced ability to infect mosquitoes via infectious blood meal, indicating that the ability to replicate in midgut is important for establishing infection. However, when mosquitoes did become infected with the targeted viruses, the amount of replication in both midgut and carcass was not significantly different from those of control viruses. Our results did not support the hypothesis that there is a minimum midgut titer necessary for dissemination. We did find that midgut titer has some predictive value in whether the carcass titer will be positive or negative, although the ability to predict disseminated infection was not absolute. Not all mosquitoes that developed midgut infections also developed disseminated infection, and vice versa, but these examples were relatively uncommon. Overall, this study improves upon previous work since we were able to limit viral replication in a tissue-specific manner without altering major mosquito immune processes, which may affect mosquito physiology in ways that are difficult to predict.

If midgut replication was not important for dissemination, we had predicted two potential outcomes: 1) the miRNA-targeted viruses would be able to cause disseminated infection at rates approaching those of control viruses, and/or 2) we would find abundant examples of mosquitoes infected with the miRNA-targeted viruses that had disseminated infection but lacked detectable midgut infection, presumably by an intercellular escape route. Instead, we did not observe either of these outcomes. The prevalence of disseminated infections was reduced to less than 50% in

mosquitoes that were exposed to the miRNA-targeted viruses, and dissemination without detectable midgut titer was observed in only 3 of 31 mosquitoes infected with MRE3’miRT. Similarly, in mosquitoes infected with MRE3'control, only 4 of 136 mosquitoes with disseminated infection had no detectable midgut titer. These results support the hypothesis that midgut replication is important for viral dissemination, consistent with other studies that have found reduced replication in the midgut leads to less dissemination (Kading et al., 2014; Molina-Cruz et al., 2005). This is further supported by comparing our results with MRE3′miRT and MRE-ORFmiRT; MRE3′miRT replication appeared to be more affected than MRE-ORFmiRT by the miRNA mimics in transfected BHK21 cells, and MRE3′miRT also had less ability to establish midgut infection than MRE-ORFmiRT. These latter results, although correlative, are also consistent with the conclusion that the ability of a virus to replicate in midgut cells affects its ability to establish midgut infection, which in turn affects its ability to disseminate.

It appears that infection with the midgut-attenuated viruses was an all-or-nothing phenomenon. If infection was successfully established in the midgut, then the levels of midgut replication were normal, and dissemination almost always occurred. On the other hand, if infection was not established in the midgut, as was the case for the majority of mosquitoes that were exposed to the miRNA-targeted viruses, then dissemination usually also did not occur. One reason for the requirement for midgut replication may be due to a requirement for a virion to encounter a transient breech or tear in the midgut basal lamina or a site of basal lamina remodeling that allows escape. If a virus can replicate and spread to adjacent midgut cells, then it is more likely to be located near a location in the basal lamina that allows a virion to pass through. This idea is supported by studies that have shown structural changes in the basal lamina after blood-meal ingestion, including changes in thickness as well as the appearance of

perforations (Kantor et al., 2018; Reinhardt & Hecker, 1973). Additionally, studies have shown that only a few cells in the midgut get infected initially (Smith et al., 2008; Whitfield et al., 1973), and only a small number of virions escape from the midgut (Forrester et al., 2012). If this is the case and a virus is only able to infect a few cells and then cannot spread to nearby cells, it may remain trapped until it is cleared by the immune system or cell turnover occurs via apoptosis. A similar model has been proposed based on the finding that viral dissemination is enhanced by a second non-infectious blood meal, presumably by giving virions a second chance to encounter a perforation in the basal lamina (Armstrong et al., 2020; Cui et al., 2019). There is also a possibility that viruses use another means of midgut escape such as the trachea, which would also be supported by this model (Romoser et al., 2005; Romoser et al., 2004).

An important question that is raised by our results is why ~20-40% of mosquitoes still developed midgut and disseminated infection with the targeted viruses when they should, in theory, not be able to replicate in the midgut. There are several potential explanations for this observation. First, these miRNAs may not be expressed equally in all midgut cell types or in all regions of the midgut. Liu et al (2014) found that *miRNA1174* was predominantly expressed in the posterior midgut, and since these two miRNAs are expressed from the same miRNA cluster, *miRNA1175-5p* expression would be expected to follow a similar pattern. If a midgut cell is infected that does not express high levels of these miRNAs, the virus may be able to replicate and spread more efficiently. Second, there may be variability between individual mosquitoes in either expression of these miRNAs in the midgut or in the strength or timing of the midgut immune response. In this regard it was interesting that the percentage of mosquitoes with a positive midgut titer decreased over time, but only with the midgut-attenuated viruses. This could indicate that decreased midgut replication coupled with RNAi and other immune responses

were able to clear the midgut infection in some mosquitoes at later timepoints. It also suggests that ability to clear the virus may be temporally variable and might further explain the occurrence of mosquitoes that had disseminated infection but did not have a detectable midgut titer. It has previously been found that viruses can be cleared from the midgut, but generally not at such early time points (Saredy et al., 2020). This idea is also consistent with results of a recent study that found that a chikungunya virus construct with a slower replication rate displayed a reduced midgut escape rate (Merwaiss et al., 2020). These authors suggested that there is a limited time window for the virus to reach a particular level and then escape from the cells.

A small number of mosquitoes infected with the targeted viruses developed infection in either the midgut or the carcass but not both. We propose that in mosquitoes that developed only midgut infection, the virus was unable to access a cell near a transient perforation in the basal lamina in the time window needed. Thus, even though the virus was able to replicate, it remained confined to the midgut. On the other hand, the mosquitoes that developed only carcass infection suggest that an intercellular route might exist in rare cases, reminiscent of the "leaky gut" phenomenon and other studies suggesting that arboviruses may sometimes pass directly through the gut wall (Chandler et al., 1998; Houk & Hardy, 1979; Weaver et al., 1991). However, there are other potential explanations for these rare "carcass-only" infections, such as midgut titers that were below the level of detection by $TCID_{50}$ assay or infections that were cleared from the midgut after dissemination occurred.

We considered the possibility that selection for mutations in the virus populations in the mosquitoes that developed disseminated infection with miRNA-targeted viruses could play a role in these results. However, deep sequencing of the insert region from virus populations in individual mosquitoes indicated that the number of sequence alterations following oral infection

and dissemination was extremely low, and the large majority of viruses contained intact miRNA target sites. It is thus unlikely that selection for alterations in the inserted sequence was responsible for the mosquitoes that developed disseminated infection with the midgut-attenuated viruses.

We also sought to examine the possibility of a midgut threshold titer. It has been previously hypothesized that midgut titer must reach a certain threshold for dissemination to occur (Kramer et al., 1981; Merwaiss et al., 2020; Vazeille et al., 2019), although this idea has been refuted by other studies (Bosio et al., 1998; Dickson et al., 2014). We did not find a significant correlation between midgut and carcass titers, and we observed no evidence of a threshold midgut titer that led to dissemination. Taken together, this indicates that while there may not be a defined threshold titer that must be achieved for disseminated infection to occur, replication in the midgut does appear to greatly increase the chance of midgut escape. This likelihood is probably also affected by which midgut cells the virus initially infects, and perhaps by variation in the immune responses of individual mosquitoes.

In summary, this study used a novel method to demonstrate that replication of SINV in *Ae. aegypti* midgut cells is important for the crucial process of arbovirus dissemination from the midgut, although other factors besides replication are likely also involved. The observation that dengue virus dissemination was reduced by manipulation of the midgut RNAi response (Franz et al., 2006) suggests these results may be generalizable to flaviviruses, but this will require more work to confirm. The reasons underlying the finding that 20-40% of mosquitoes still developed disseminated infection when exposed to a midgut-attenuated virus will be the subject of further study. Our findings also suggest the strategy of using tissue-specific miRNA targeting of viruses in mosquitoes could be used further to study whether replication in other tissues is important for

transmission. Finally, the results of this and other studies support the idea that preventing arbovirus replication in the vector midgut is a viable strategy for preventing the spread of arboviral disease.

## Methods

### Cell lines

BHK21 cells were propagated at 37º C with 5% $CO_2$ and kept in DMEM media (Gibco) plus 10% fetal bovine serum (FBS, Atlanta Biologicals). C6/36 cells were maintained at 27º C in Leibovitz′s media (Gibco) plus 10% FBS.

### Insect rearing

*Ae. aegypti* mosquitoes (Orlando strain (Kuno, 2010), obtained in 2008 from James Becnel, USDA ARS, Gainesville, FL) were reared at 27º C and 80% humidity with a 12-hr light-dark cycle. Eggs were obtained by allowing female mosquitoes to feed on defibrinated sheep blood (Colorado Serum Company) through stretched parafilm using a Hemotek feeding system (Hemotek Ltd). All experiments with mosquitoes were done in a BSL-2 insectary at Kansas State University.

### MRE3′miRT and MRE3′control plasmid generation

The 5’dsMRE16ic infectious clone was obtained from Ken Olson (Colorado State University) and its construction has been described (Foy et al., 2004). Oligos containing alternating repeats of complementary sites to *miR1174* and *miR1175-5p* or a mutated control sequence flanked by *Xho*I and *BamH*I restriction enzyme sites (Fig. 2.2C) were obtained from Integrated DNA Technologies (IDT) and cloned into the pGEM-T vector (Promega). Splicing by overlap extension PCR was used to amplify the 3′ UTR region and part of the structural gene region of 5′dsMRE16ic corresponding to nucleotides 3499-4064 of Genbank entry U90536 (Foy

et al., 2004), with the first nucleotide of the start codon for the structural proteins considered as position 1. *Xho*I and *BamH*I sites were simultaneously inserted between positions 3753 and 3754 in the 3'UTR, and the resulting fragment was cloned into pGEM-T. The *miR1174* and *1175-5p* pGEM-T vector and control pGEM-T vectors were then digested with *Xho*I and *BamH*I and the resulting insert fragments were gel-purified (Qiagen). The 3′ UTR region in pGEM-T was also digested with *Xho*I and *BamH*I and the insert *miR1174* and *1175-5p* fragment or control fragment was inserted. The resulting sequences in pGEM-T were then digested with *Nhe*I and *Asc*I and sub-cloned into 5′dsMRE16ic. The final plasmids (MRE3′miRT and MRE3′control) possessed either four tandem miRNA target sequences or a control sequence as well as the above-mentioned restriction enzyme sites inserted in the 3'UTR between nucleotides 3753 and 3754, with the final sequence of the insert and flanking regions verified by Sanger sequencing (Fig. 2.2A).

**MRE-ORFmiRT and MRE-ORFcontrol plasmid generation**

5′dsMRE16ic was digested with *Not*I and *Avr*II and the resulting fragment (nucleotides -156 to 2132 in relation to the structural gene start codon in Genbank entry U90536) was ligated into pGEM-T (Promega). A gBlocks gene fragment was ordered from IDT which contained the 54 nucleotide FMDV 2A self-cleaving peptide sequence (5′-CAGCTGTTGAATTTTGACCTTCTTAAGCTTGCGGGAGACGTCGAGTCCAACCCTGGG CCC-3′) and part of the PE2 sequence (nucleotides 799-828 in relation to the structural gene start codon in Genbank entry U90536) of MRE16. This was then cloned into the *Not*I-*Avr*II MRE16 genome fragment (above) immediately after the capsid sequence, using exponential megapriming PCR cloning (Ulrich et al., 2012). The resulting product, as well as the previously discussed *miR1174* and *1175-5p* and control pGEM-T vectors, were digested with *Xho*I and

*BamH*I and the resulting miRT and control fragments were ligated into the MRE16 gblock in pGEM-T. These plasmids were then digested with *Not*I and *Avr*II and the miRT and gblock fragment were ligated into 5′dsMRE16ic. The Q5 site-directed mutagenesis kit (New England BioLabs) was then used to add the first 9 nucleotides of PE2 between the insert and the end of the capsid sequence (5′-TCAGCAGCA-3′). This duplication of the first 3 codons of PE2 was necessary to allow proper autocleavage by the capsid protein. The resulting plasmids contained the miRT or control sequence followed by the FMDV 2A sequence, in frame, inserted between the end of the capsid sequence and the beginning of PE2, with the first 9 nucleotides of PE2 duplicated before and after the insert. During translation of this portion of the viral genome, the autoproteolysis of the capsid protein and the ribosomal skipping created by the FMDV 2A sequence cause the translated miRT or control peptides to be released without affecting viral structural protein conformation or function. It should be noted that the control and miRT inserts encode the same amino acid sequence, with all nucleotide changes in the control sequence being in the third codon position. The final sequences of the insert and flanking regions were verified by Sanger sequencing.

**Preparation of viral stocks**

Infectious clone plasmids were *in vitro* transcribed (MEGAscript SP6 transcription kit, Thermo Fisher Scientific) in the presence of cap analog (New England BioLabs). The RNA was then transfected into BHK21 cells using Lipofectamine 3000 (Thermo Fisher Scientific) and the resulting P1 stock was passaged once in C6/36 cells to generate the P2 stock that was used for the experiments described below. The titer of the virus stocks was determined by $TCID_{50}$ assay in BHK21 cells.

**$TCID_{50}$ assay**

BHK21 cells were used to seed 96-well tissue culture plates at a density of $1x10^4$ cells per well. Cells were supplemented with 15 μg/ml of pencillin/streptomycin (Invitrogen). Mosquito samples were homogenized in a 1.5 ml microcentrifuge tube using a disposable pestle. Both mosquito and cell culture samples were centrifuged to remove cellular debris. Samples were stored at -80$^o$ C until being assayed. Assay samples were thawed on ice and serial dilutions of each sample were added to 6 wells of BHK21 cells. The plates were scored for cytopathic effects after 5 days, and the proportion of infected wells was used to calculate $TCID_{50}$/ml values. $TCID_{50}$/ml was converted to PFU/ml by multiplying by a factor of 0.69 (O' Reilly et al, 1994).

**Cell transfection experiments**

BHK21 cells were plated in 6-well plates at a density of $5 \times 10^5$ cells per well in serum-free opti-MEM media (Gibco) and allowed to sit for 2 hrs. Six μl of Lipofectamine 3000 was then mixed with 100 μl opti-MEM and allowed to sit for 5 min. After 5 min, 200 pmol of each miScript miRNA mimic, *miR1174* (5′-UCAGAUCUACUUAAUACCCAU-3′) and *miR1175-5p* (5′-AAGUGGAGUAGUGGUCUCAUCG-3′) (Qiagen) or 400 pmol of All-stars control siRNA (Qiagen) were added and the mixture was incubated at room temperature for 15 min. For the mock transfected samples, no mimic or control siRNA was added. After incubation, 900 μl of opti-MEM was then added to the tube containing the mixture. The cells were rinsed with opti-MEM and the mixture was added to the cells and incubated at 37$^o$ C. After 6 hrs, the mixture was then removed, and cells rinsed twice with DMEM plus 10% FBS. Two ml of DMEM plus 10% FBS were then added to the cells and transfected cells were immediately infected with MRE3′miRT or MRE3′control at an MOI of 0.1 or 0.01. This experiment was repeated with the MRE-ORFmiRT and MRE-ORFcontrol viruses at an MOI of 0.01. At 14, 24, 38 and 48 hrs post

infection (hpi), 200 µl of media from each well was removed and frozen at -80$^{o}$ C. After completion of the experiment, the samples were titered by $TCID_{50}$ assay.

To measure transfection efficiency, BHK21 cells were transfected by the same procedure as above but using control fluorescent mimics. At 24 hrs post transfection, pictures were taken of five fields with and without fluorescence. These images were overlaid using ImageJ software. The number of cells displaying fluorescence were counted and divided by the number of total cells in the field to get a percentage of transfected cells. The average of these five fields was used to determine approximate transfection efficiency.

**RT-qPCR**

At 3 days post-emergence of adult mosquitoes, raisins were removed from the mosquito enclosure and mosquitoes were provided with only water for 2 days. After this starvation period, mosquitoes were allowed to feed on defibrinated sheep blood (Colorado Serum Company) using a Hemotek system for 2 hrs. Fully engorged females were separated from the remaining mosquitoes and provided with raisins and water. At 10, 24, 48- and 72-hrs post blood meal (hpbm) mosquitoes were dissected in phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, 1 L $H_2O$) and midguts removed from the carcasses. The midguts were pooled into groups of 5, with 15 mosquitoes dissected for each time point. Tissues were homogenized using a disposable pestle and total RNA extracted using the miRNeasy mini kit (Qiagen). The RNA was treated with DNase and then reverse transcribed with the miScript II RT kit (Qiagen). The resulting cDNA was diluted 1:10 and used for RT-qPCR using a miScript SYBR Green PCR kit with miScript primer assays specific to *miR1174* and *miR1175-5p* (Qiagen). The housekeeping gene RPS7 was used as an internal control.

**Mosquito infection – blood feeding experiments**

Following emergence, female mosquitoes were permitted to feed on raisins and water *ad libitum.* At 3 days post emergence, raisins were removed and mosquitoes starved for 1 day. The mosquitoes were then separated into smaller containers. Virus stocks were diluted with Leibovitz's media to be the same titer ($2.2 \times 10^8$ PFU/ml). The resulting stocks were then mixed 1:1 with defibrinated sheep blood and mosquitoes were allowed to feed for 1 hr on this mixture using a Hemotek membrane feeder. The mosquitoes were chilled at $4^o$ C and then fully engorged females were separated from non-fed mosquitoes and maintained on raisins and water. At 48, 72 and 120 hpbm the mosquitoes were dissected to separate the carcass from the midgut. The tissues were placed in 200 μl DMEM media plus 10% FBS and homogenized in 1.5 ml microcentrifuge tubes using disposable plastic pestles. The samples were frozen at $-80^o$ C until they were titered by $TCID_{50}$ assay.

**Mosquito infection – intrathoracic injection experiments**

Following emergence, adult mosquitoes were maintained on raisins and water until used for the experiment. 3-4 day old female mosquitoes were cold anesthetized and injected with one pulse of 69 nl of either MRE3′miRT or MRE3′control diluted so that each mosquito received a dose of 10 PFU, using a Nanoject II injector (Drummond Scientific Company). After recovering, the mosquitoes were maintained on raisins and water until dissection. At 24, 48- and 72-hrs post injection the mosquitoes were dissected to remove the midgut from the carcass. The carcasses were placed in DMEM media plus 10% FBS, homogenized in 1.5 ml microcentrifuge tubes using disposable plastic pestles and frozen at $-80^o$ C until they were titered by $TCID_{50}$ assay.

**Illumina sequencing**

Mosquito carcass samples were selected for sequencing from mosquitoes that had positive virus titers. Twelve carcass samples from mosquitoes infected by blood feeding with MRE3′miRT, 10 carcass samples from mosquitoes injected with MRE3′miRT and 10 carcass samples from mosquitoes infected by blood feeding with MRE3′control were used for sequencing. RNA was extracted from the individual samples using TRIzol reagent (Invitrogen). From these, cDNA was generated using the Improm-II reverse transcription system (Promega) using a SINV specific primer (5′-CAGCATTATGCACTGCACTTCC-3′). A volume of 3 µl of the resulting cDNA was used in a PCR reaction with Q5 high fidelity polymerase (New England Biolabs) to amplify the 3′miRT region as well as add Illumina overhang adaptor sequences (forward primer -5′-TCGTCGGCAGCGTCAGA TGTGTATAAGAGACAGCTTTGCTGACAAGCACCCGACGATG-3′, reverse primer - 5′-GTCT CGTGGGCTCGGAGATGTGTATAAGAGACAGTGTACCAGCCCGATGCGTTATGC-3′). Thermocycling conditions were as follows: 95º C for 30 seconds, then 95º C for 30 seconds, 66.5º C for 60 seconds and 68º C for 60 seconds repeated for 30 cycles, then 68° C for 5 min. Following this step, 60 ng of each sample were used for Index PCR. The reaction was set up as follows: 25 µl of 2X KAPA HiFi Hot Start Ready mix (Roche Diagnostics); 10 µl of PCR grade water and 5 µl of each Nextera XT Index 1 and Index 2 primers from Nextera XT Index kit (Illumina). The PCR was performed on a Veriti Thermal Cycler (Thermo Fisher Scientific) using the following program: 95°C for 3 min; 8 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec; 72°C for 5 min; hold at 4°C. Amplicons were purified with Pure magnetic beads (Roche Diagnostics) according to the manufacturer's recommendations. The obtained libraries were

validated on a 7500 DNA Bioanalyzer chip (Agilent), quantified with Qubit dsDNA high sensitivity assay (Thermo Fisher Scientific), pooled into equimolar amounts, and sequenced with 2x150bp Illumina MiSeq run using MiSeq 300 cycles reagent micro kit v.2 (Illumina) at the K-State Integrated Genomics Facility.

To identify potential single nucleotide polymorphisms (SNPs) and insertion/deletion (indel) variation within pools of amplicons, all 150 bp paired end reads per sample were remapped to their respective reference genome using BWA 0.7.17-r1198-dirty under the BWA-MEM algorithm (Li, 2013). The resulting Sequence Alignment/Map (SAM) file was converted to binary format (i.e., BAM), sorted and indexed using SAMtools v1.10 (H. Li et al., 2009). Variant calling by reference position of each sample was conducted with bam-readcount 0.8.0-unstable-7-625eea2 (available from https://github.com/genome/bam-readcount), with filtering and removal of false positive calls having minimum base (-b) and mapping (-q) qualities of less than 30 and 20, respectively. Output from bam-readcount was converted to tab-delimited (TSV) files for presentation as Dataset S1.

**Data availability**

Illumina sequence data have been deposited in the Sequence Read Archive under accession number PRJNA699225.

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## Chapter 2 figures

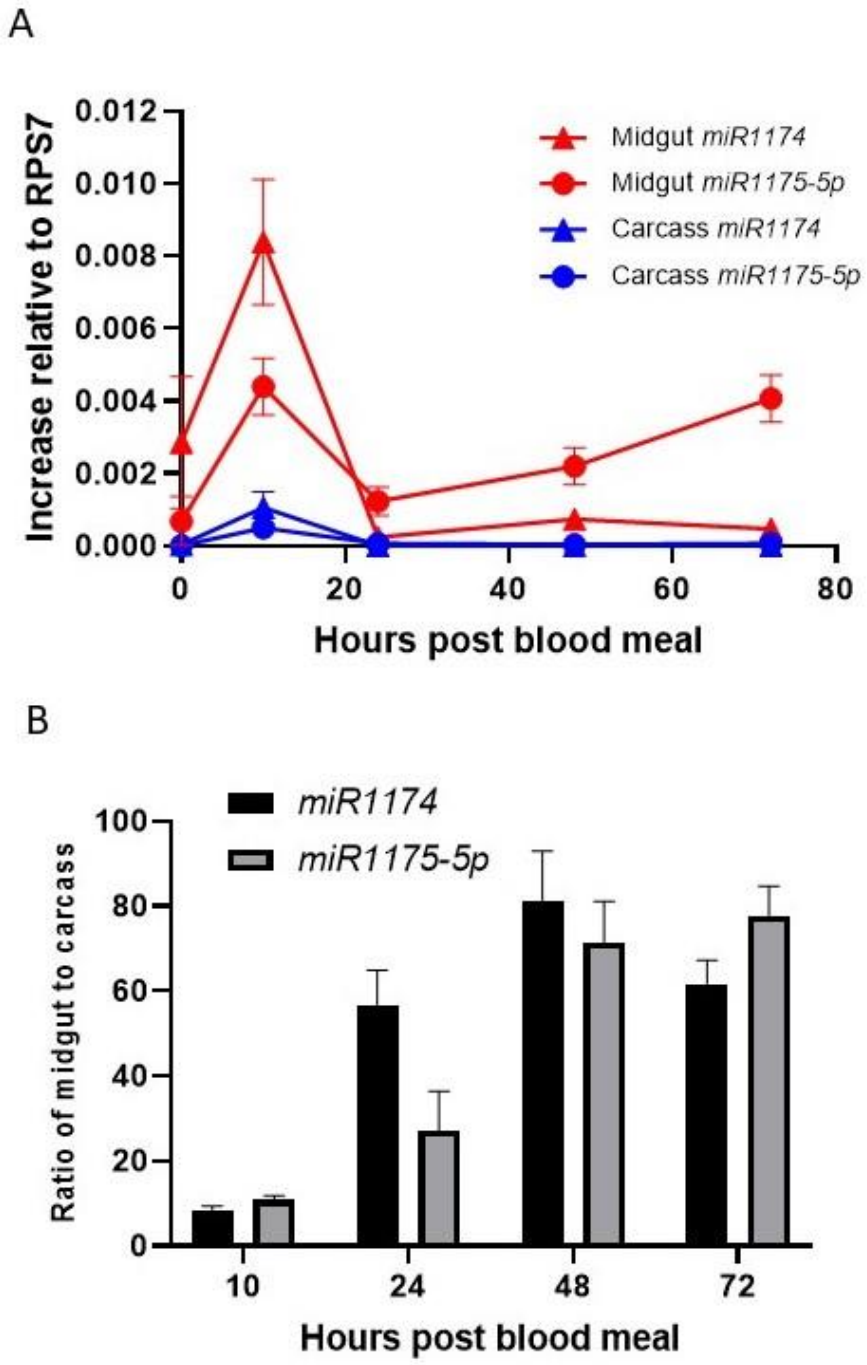
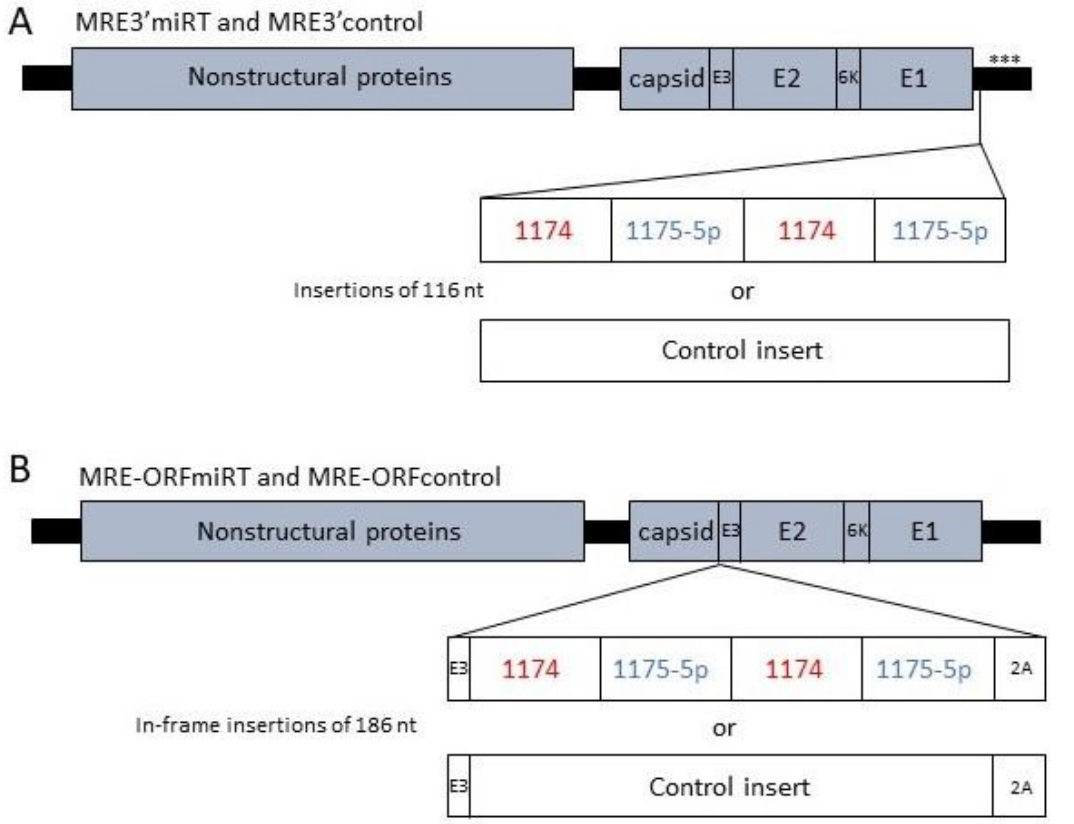



**Figure 2.1 Relative expression of miR1174 and miR1175-5p in midgut compared to carcass**

RNA was isolated from midguts and carcasses of non-blood fed or blood-fed female mosquitoes at 10, 24, 48 and 72 hpbm and miRNA expression was analyzed by RT-qPCR. A) Expression levels of *miR1174* and *miR1175-5p* in midgut and carcass, relative to the housekeeping gene RPS7. B) The ΔΔCt method was used to compare the expression between midgut and carcass using RPS7 as an internal control. The average of 3 experiments is shown with the error bars representing SD.




C

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                 XhoI
miRT         1 CTCGAGAAATGGGTATTAAGTAGATCTGACCGATCGATGAGACCACTACT     50
               ||||||||.||||||.||.||.||.||.||||||||.||||||.||.||.||
Control      1 CTCGAGAAGTGGGTCTTGAGCAGGTCCGACCGATCAATGAGGCCCCTGCT     50

miRT        51 CCACTTACCGGTATGGGTATTAAGTAGATCTGACCGATCGATGAGACCAC    100
               .||.||||||||.||||||.||.||.||.||.||||||||.||||||.||.|
Control     51 TCATTTACCGGTGTGGGTCTTGAGCAGGTCCGACCGATCAATGAGGCCCC    100
                                 BamHI
miRT       101 TACTCCACTTGGATCC     116
               |.||.||.||||||||
Control    101 TGCTTCATTTGGATCC     116
```

**Figure 2.2 Schematic representations of the viral constructs used in this study**

(A) The MRE3′miRT and MRE3′control viruses were constructed with an insertion of alternating repeats of sequences complementary to *miR1174* or *miR1175-5p*, or a control sequence of the same length, in the 3′ UTR region of the MRE-16 genome. Asterisks represent the position of three repeated sequence elements in the 3′ UTR (Garneau et al., 2008). (B) In

MRE-ORFmiRT and MRE-ORFcontrol, the alternating complementary target sequence or control sequence were inserted in frame into the structural ORF, between the sequences encoding capsid and E3. In addition, the first 9 nucleotides of E3 were duplicated to retain the autoproteolytic activity of the capsid protein, and the FMDV 2A peptide sequence was included so that the peptides encoded by the inserted sequences would be removed during translation of the structural proteins. (C) The inserted miRNA target (miRT) and control sequences are aligned. Sequences complementary to *miR1174* and *miR1175-5p* are underlined and in red and blue, respectively, while restriction sites are underlined in black.

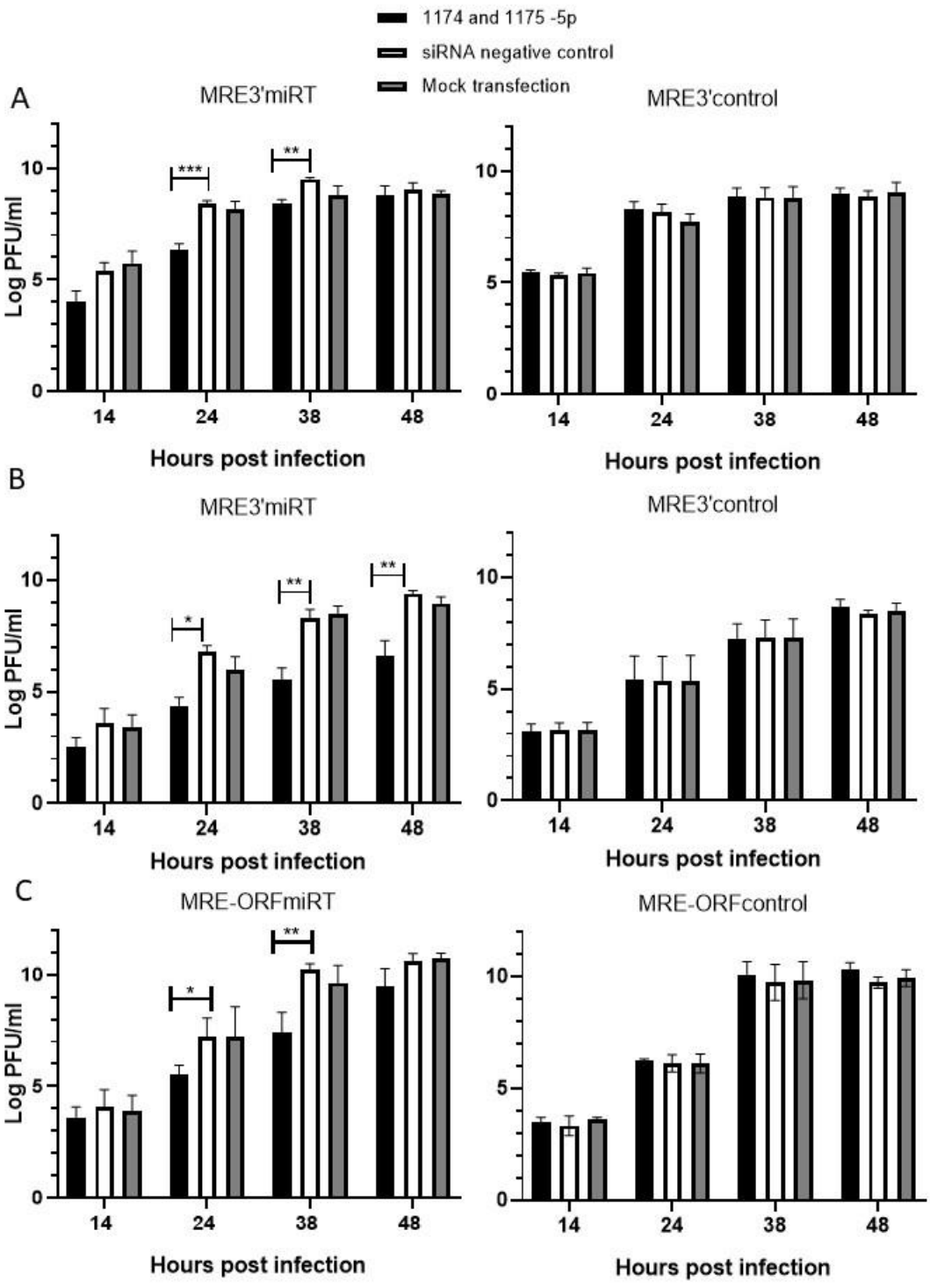


**Figure 2.3 Effect of miRNA mimics on replication of the targeted or control viruses**

(A) BHK21 cells were transfected with miRNA mimics 1174 and 1175-5p or negative control siRNA, or were mock transfected. They were then infected with MRE3′miRT or MRE3′control at an MOI of 0.1. Media was sampled at the time points indicated and titered by $TCID_{50}$. (B) BHK21 cells were transfected and infected as in (A) but at an MOI of 0.01. (C) BHK21 cells were transfected as in (A) but were infected with MRE-ORFmiRT or MRE-ORFcontrol at an MOI of 0.01. Means of the log values are graphed and error bars indicate SD. Titers were compared using Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A lack of brackets indicates non-significant differences.

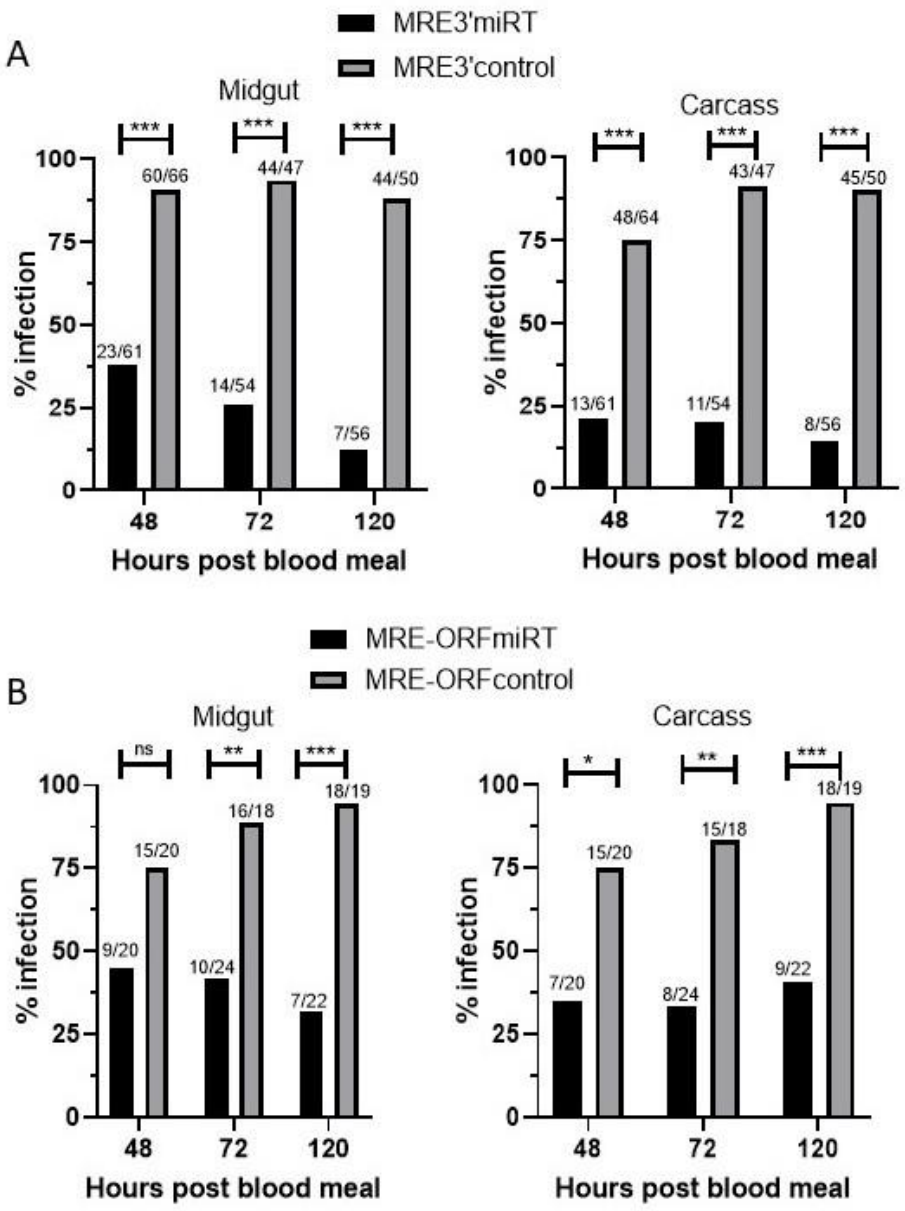


**Figure 2.4 Percentage of mosquitoes with detectable infection in the midgut or carcass when blood-fed with targeted or control viruses**

Mosquitoes were allowed to feed on blood containing (A) MRE3′miRT or MRE3′control or (B) MRE-ORFmiRT or MRE-ORFcontrol, and midguts and carcasses were titered at the indicated timepoints. Results were compared using Fisher′s exact test. Ns, not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

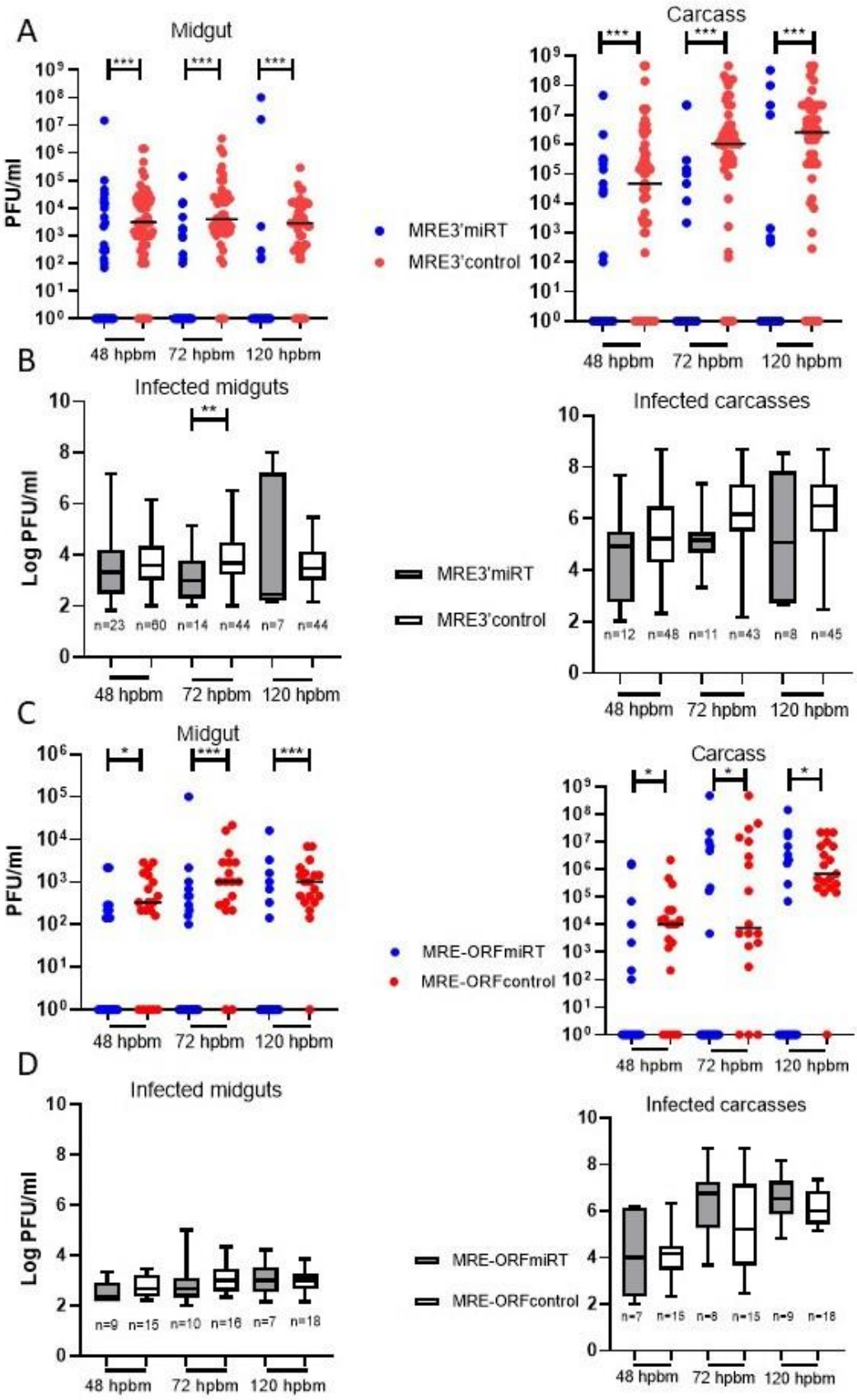
A
Midgut
PFU/ml
48 hpbm
72 hpbm
120 hpbm
MRE3'miRT
MRE3'control
Carcass
B
Infected midguts
Log PFU/ml
n=23 n=60 n=14 n=44 n=7 n=44
MRE3'miRT
MRE3'control
Infected carcasses
n=12 n=48 n=11 n=43 n=8 n=45
C
Midgut
MRE-ORFmiRT
MRE-ORFcontrol
Carcass
D
Infected midguts
n=9 n=15 n=10 n=16 n=7 n=18
Infected carcasses
n=7 n=15 n=8 n=15 n=9 n=18

**Figure 2.5 Titers of midguts and carcasses from mosquitoes blood-fed with targeted or control viruses**

(A) Midgut and carcass titers in mosquitoes given an infectious blood meal containing either MRE3′miRT or MRE3′control. (B) The same titer values as in panel A but with negative values removed and remaining values log transformed. (C) Midgut and carcass titers in mosquitoes given an infectious blood meal containing either MRE-ORFmiRT or MRE-ORF control. (D) The same titer values as in panel C but with negative values removed and remaining values log transformed. The groups in panels A and C were compared by Mann-Whitney test, while those in panels B and D were compared by Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

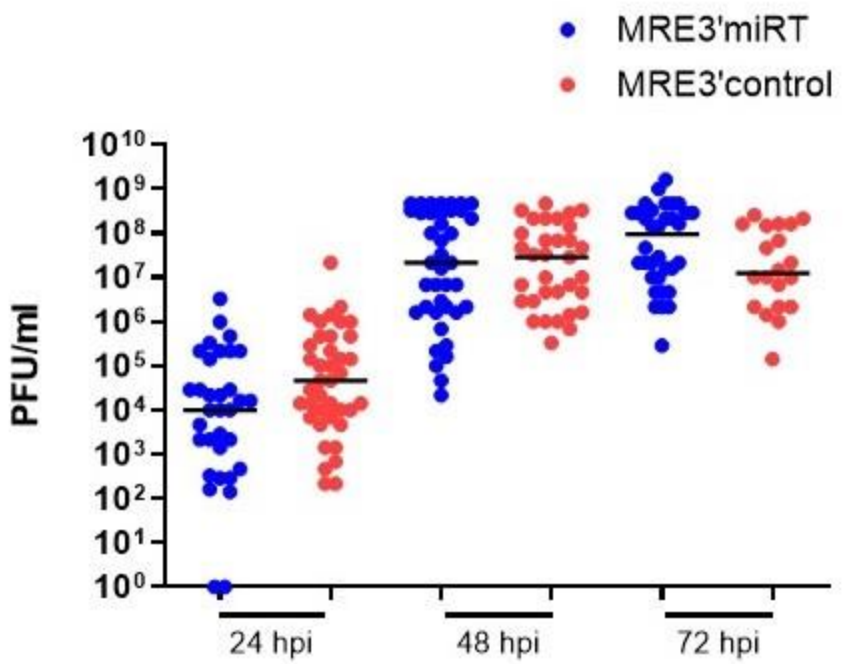


**Figure 2.6 Titers of mosquito carcasses after injection with MRE3′miRT or MRE3′control**

Mosquitoes were injected with 10 PFU of virus and dissected at the timepoints indicated. Carcass titers were compared by Student's *t*-test. No significant differences were found. Median values are shown by a horizontal line.

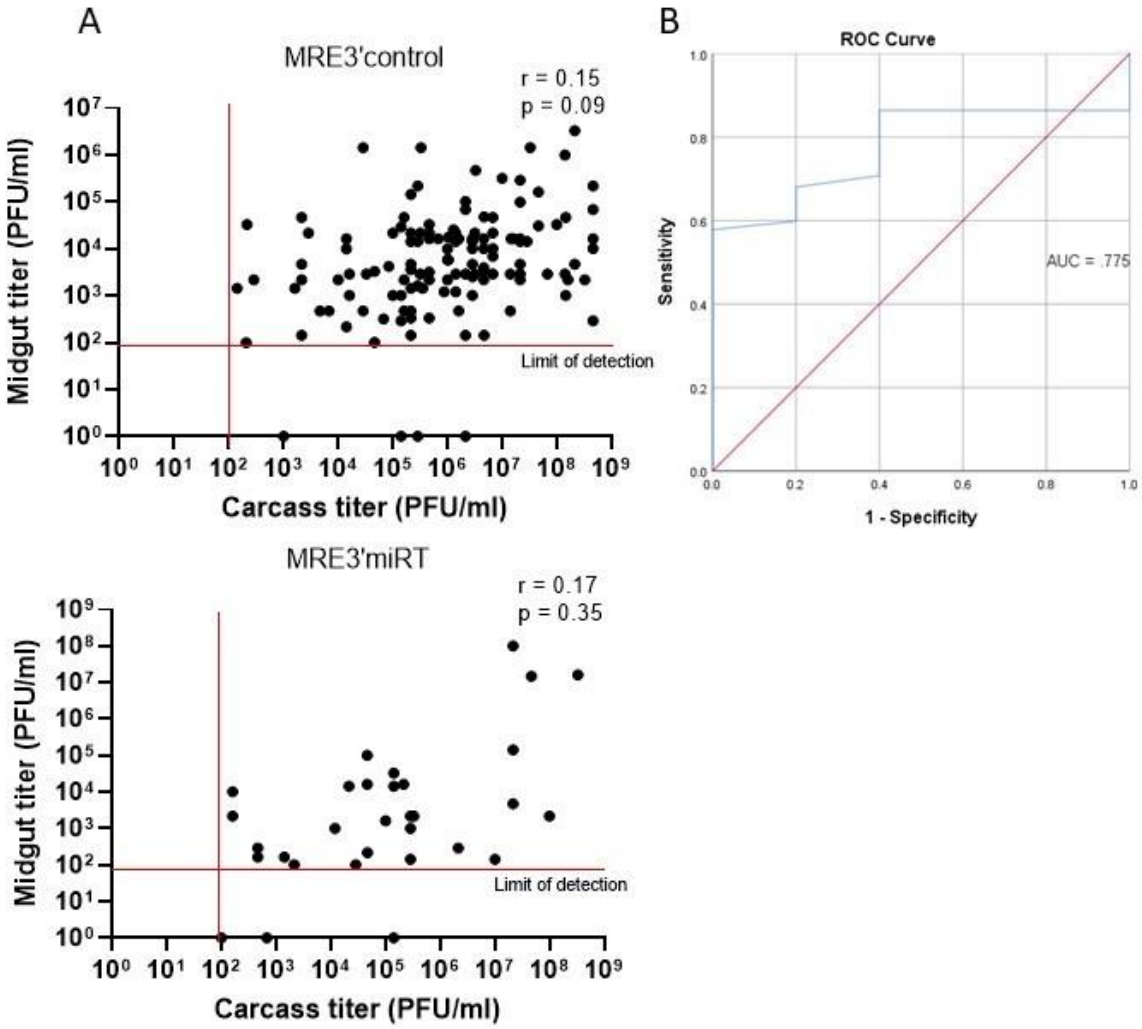


**Figure 2.7 Correlation analysis comparing midgut titers and carcass titers**

(A) Pearson correlation analysis comparing midgut titers and carcass titers in the MRE3′control-infected (n=136) and MRE3′miRT-infected (n=31) mosquitoes that developed disseminated infection. There was no significant correlation for either virus. Red lines indicate the limit of detection for the assay. (B) ROC curve estimating the ability of midgut titer to predict if the carcass titer will be positive or negative. This analysis was done using all MRE3′control and MRE3′miRT data combined. The area under the curve was 0.775 which indicates that midgut titer is only a fair predictor of whether or not the carcass titer will be positive. Data were analyzed using the SPSS software package.

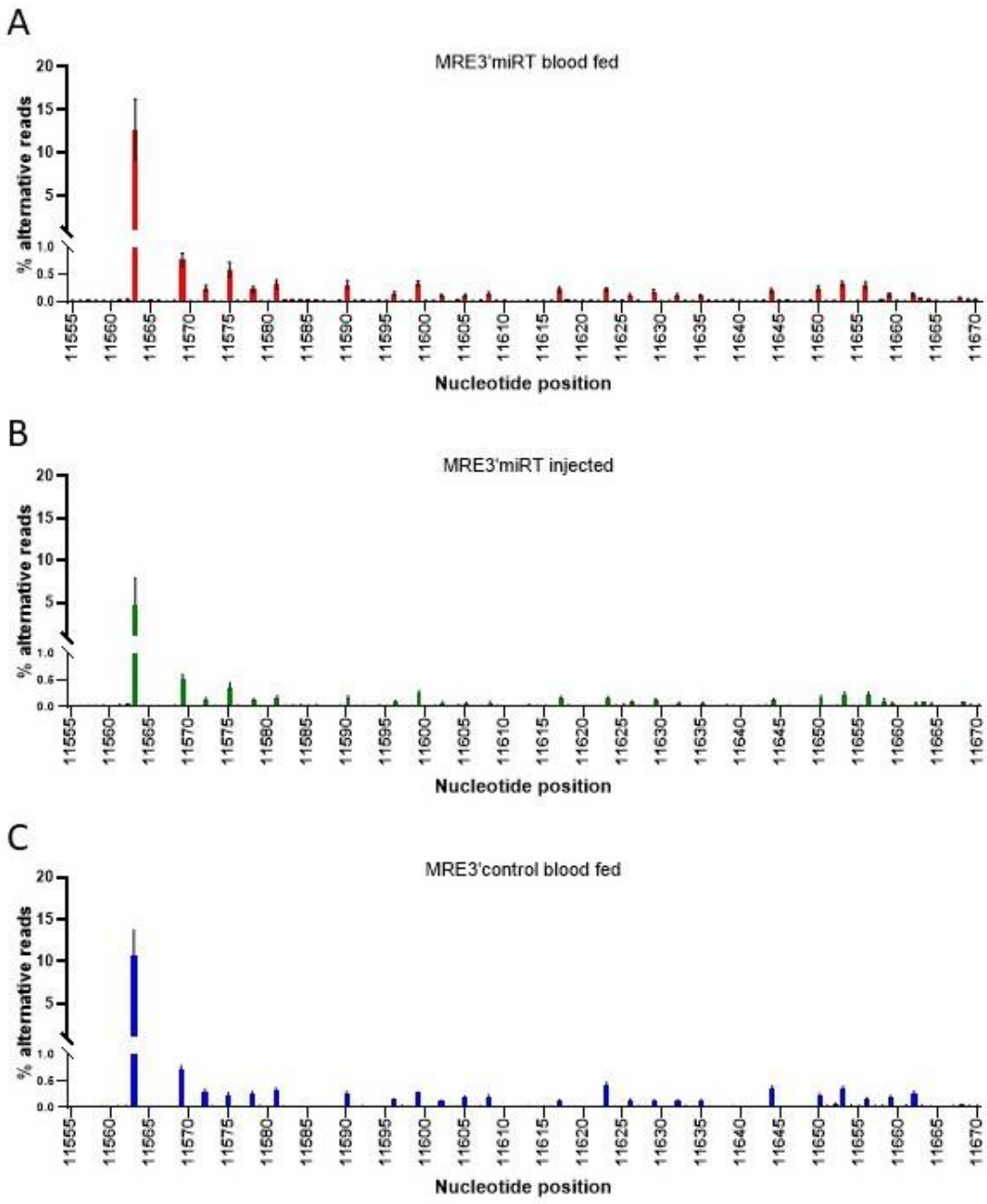


**Figure 2.8 Percentage of alternative reads at each nucleotide position in the inserted sequence in viruses isolated from MRE3′miRT blood-fed, MRE3′control blood-fed and MRE3′miRT-injected mosquitoes**

The percentage of reads that differ from the reference sequence were averaged for each group of mosquitoes at each base pair. Error bars represent SEM.

# Chapter 3 Expressing the pro-apoptotic Reaper protein via insertion into the structural open reading frame of Sindbis virus reduces the ability to infect *Aedes aegypti* mosquitoes

## Abstract

Arboviruses continue to threaten a significant portion of the human population, and a better understanding is needed of the determinants of successful arbovirus infection of arthropod vectors. Avoiding apoptosis has been shown to be one such determinant. Previous work showed that a Sindbis virus (SINV) construct called MRE/rpr that expresses the pro-apoptotic protein Reaper via a duplicated subgenomic promoter had a reduced ability to orally infect *Aedes aegypti* mosquitoes at 3 days post-blood meal (PBM), but this difference diminished over time as virus variants containing deletions in the inserted *reaper* gene rapidly predominated. The goal of this study was to generate a SINV construct that more stably expressed Reaper, to further clarify the effect of midgut apoptosis on disseminated infection in *Ae. aegypti*. We did this by inserting *reaper* as an in-frame fusion into the structural open reading frame (ORF) of SINV. This construct, MRE/rprORF, successfully expressed Reaper, replicated similarly to MRE/rpr in cell lines, and induced apoptosis in cultured cells and in mosquito midgut tissue. Mosquitoes that fed on blood containing MRE/rprORF developed less midgut and disseminated infection when compared to MRE/rpr or a control virus up to at least 7 days PBM, when less than 50% of mosquitoes that ingested MRE/rprORF had detectable disseminated infection, compared with around 80% or more of mosquitoes fed with MRE/rpr or control virus. However, virus titer in mosquitoes infected with MRE/rprORF was not significantly different from control virus, suggesting that induction of apoptosis by expression of Reaper by this method can reduce infection prevalence, but if infection is established then apoptosis induced by this method has limited ability to continue to suppress replication.

# Introduction

Recent decades have seen the emergence and reemergence of a number of significant arboviral diseases such as dengue, Zika, West Nile, yellow fever and chikungunya (Morrison, 2014; Murray et al., 2015; Pan American Health Organization / World Health Organization, 2018). Arboviruses, which are transmitted through the bite of an infected arthropod vector such as a tick or mosquito, are expected to become more significant in the future, and it is predicted that climate change will increase incidence of disease by impacting vector geographical range, feeding behavior and survival (Bouchard et al., 2019; Colón-González et al., 2021; Liu-Helmersson et al., 2016). New ways of protection from these diseases are needed as vaccines are not available for many of these diseases and there is increasing insecticide resistance in some vectors (Deming et al., 2016; Moyes et al., 2017; Weill et al., 2003).

An alternative to traditional means of controlling arboviral diseases is to prevent productive infection of the vector. Tissue barriers in the vector such as the midgut present an obstacle for viruses to overcome and inhibiting escape from midgut tissue would prevent disseminated infection and thus the spread of these viruses (Franz et al., 2015). Several pathways and processes may be considered which when altered could prevent disseminated infection, and improved knowledge of these pathways could lead to new strategies of vector infection control. One cellular process which shows potential promise in preventing disseminated infection is apoptosis. Apoptosis, a specific type of programmed cell death, has been shown to be an important antiviral pathway in insects (Blair & Olson, 2014; Clarke & Clem, 2003). The insect apoptosis pathway has been best studied in *Drosophila melanogaster*, but a similar pathway has been demonstrated in the disease vector *Aedes aegypti* (Bryant et al., 2008; Hoffmann, 2003; Liu & Clem, 2011; Wang & Clem, 2011). During apoptosis, activated initiator caspases cleave and

activate effector caspases, which are responsible for cleaving target proteins in the cell, leading to death. Activation of initiator caspases is prevented by inhibitor of apoptosis (IAP) proteins, the action of which can be overcome by IAP antagonists such as Reaper. The result is a carefully controlled mechanism which prevents unnecessary cell death but promotes cell death in response to activating stimuli such as viral infection.

The role of apoptosis in protecting insects against viral infections brings up the question of what role it plays in vector competence for arboviruses. Several studies have implicated apoptosis as a significant factor in preventing viral escape from the midgut. For example, enhanced midgut apoptosis has been associated with a *Culex pipiens pipiens* strain of mosquitoes that were refractory to West Nile virus infection (Vaidyanathan et al., 2006). Additionally, a SINV construct that expressed the *Drosophila* IAP antagonist Reaper was shown to less effectively infect and disseminate from the *Ae. aegypti* midgut, although viruses with deletions in the *reaper* insert rapidly predominated (O'Neill et al., 2015). Consistent with these results, one study showed that some pro-apoptotic genes were more highly expressed in a refractory strain compared to a susceptible strain of *Ae. aegypti* (Ocampo et al., 2013), while another study found increased rapid induction of apoptosis in mosquitoes that were less susceptible to dengue virus serotype 2 (DENV-2) compared to a more susceptible strain (Ayers et al., 2021). However, it has also been suggested that apoptosis may weaken the barrier that the midgut provides and thus allow viruses to pass through more easily. One study found that knocking down AeIAP1 expression in *Ae. aegypti* and then feeding them with SINV led to increased midgut infection and virus dissemination (Wang et al., 2012). Due to a high rate of mosquito mortality in these AeIAP1 knockdown mosquitoes, it was hypothesized that the level of apoptosis was drastically increased, greatly reducing the structural integrity of the midgut. It is possible that some level of

apoptosis is critical to preventing virus passage through tissues, but if apoptosis levels are too high, viral spread is promoted through gaps in the structure.

A previous series of studies by our group aimed to determine how inserting the *Drosophila* pro-apoptotic gene *reaper* into SINV and then infecting *Ae. aegypti* would affect rates of disseminated infection (O'Neill et al., 2015; Wang et al., 2008). The construct used in these earlier studies, called MRE/rpr, expressed Reaper via a duplicated subgenomic promoter located between the nonstructural and structural genes. The MRE/rpr virus strongly induced apoptosis and had decreased virus yield compared to control viruses in cultured mosquito cells (Wang et al., 2008). When mosquitoes were fed MRE/rpr, it was found that there was less disseminated infection compared to control virus at early timepoints, but by 7 days post-blood meal (PBM) there was no significant difference between MRE/rpr and control. The reason for this was found to be that a significant proportion of the MRE/rpr population by 7 days PBM had deletions in the *reaper* insert, rendering it non-functional (O'Neill et al., 2015). This result indicated strong negative selection against Reaper expression. Indeed, even at early timepoints over half of the mosquitoes developed disseminated infection, which may have been due to the strong selective advantage of mutants lacking functional *reaper*.

To provide more stable expression of Reaper protein, we decided to insert the *reaper* gene into the structural ORF of SINV, a method which has been previously shown to allow more stable insertions into the SINV genome (Carpenter et al., 2021; Thomas et al., 2003). By inserting the sequence into the structural ORF between the capsid gene and PE2, as opposed to the duplicated subgenomic promoter region, we hoped to increase selective pressure for retaining the insert because deletions in *reaper* would more likely negatively impact the correct expression of critical viral structural proteins. To allow proper synthesis of the viral structural proteins, we

utilized the autoproteolytic function of the SINV capsid protein and the ribosomal skipping function of foot and mouth disease virus (FMDV) 2A (Donnelly et al., 2001) to cotranslationally release the Reaper protein. Additionally, we employed a ubiquitin fusion strategy, which has been used successfully to generate proteins with precise N-terminal sequences (Baker, 1996; Hunter et al., 2003). This ensured that we would not impact the N terminus of Reaper, which has been shown to be critical for binding IAPs (Holley et al., 2002).

Using this SINV construct that more stably expressed Reaper, we then re-examined the effect of Reaper expression on establishment of midgut infection and dissemination from the midgut. Our results provide deeper insights into the effects of apoptosis on SINV infection in *Ae. aegypti*.

## Results

### MRE/rprORF virus construction and Reaper expression

To generate a SINV construct with enhanced stability of the pro-apoptotic gene *reaper*, we inserted this gene into the structural ORF of the 5'dsMRE16ic infectious clone (Fig. 3.1A) (Foy et al., 2004). The resulting construct, which we named MRE/rprORF, has *reaper* and flanking sequences inserted immediately after the capsid gene (Fig 3.1B). To ensure proper processing of both Reaper and the SINV capsid, and E2 proteins, the first sequence inserted after the capsid gene was the first three codons of PE3, which are required for an intact capsid autoproteolytic cleavage site. However, this created a problem because these three amino acids would be fused to the Reaper protein, and the N terminus of Reaper has been shown to be critical for its function in binding IAPs (Holley et al., 2002). Following normal translation of cellular Reaper, methionyl peptidases remove the N-terminal methionine, revealing an alanine that is required for binding to IAPs (Yan et al., 2004). To avoid this problem, we used a ubiquitin-

Reaper fusion strategy. After the three PE3 codons, we inserted a *ubiquitin* gene immediately followed by the *reaper* gene (lacking the N-terminal methionine) with a hemagglutinin (HA) epitope tag at its C terminus to facilitate detection of Reaper expression. Cellular proteases cleave ubiquitin fusion proteins immediately following the ubiquitin sequence (Hunter et al., 2003). Thus, the insertion of ubiquitin would result in expression of Reaper with an amino-terminal alanine. Following *reaper*, we inserted the FMDV 2A sequence, which causes ribosomal skipping (Donnelly et al., 2001), and thus should free the Reaper protein from the rest of the polypeptide chain. The result was a predicted Reaper-HA fusion protein expressed from the structural ORF that allows for intact viral structural protein processing. As a control virus we constructed MRE/control, which contained the same elements described above but lacked the *reaper* sequence (Fig. 3.1B). The other constructs used in this study were MRE/rpr (Fig. 3.1C), which has been previously described and expresses Reaper via a duplicated subgenomic promoter, and 5'dsMRE16ic, which also has been previously described and did not contain any of the inserted sequences other than the duplicated subgenomic promoter present in all the constructs (Foy et al., 2004; O'Neill et al., 2015; Wang et al., 2008).

After these viruses were constructed, we first confirmed expression of the Reaper protein from MRE/rprORF by immunoblotting (Fig. 3.2). C6/36 cells were infected with MRE/rprORF, 5'dsMRE16ic (labeled MRE16 in the figure), or MRE/control, or they were mock-infected, and at 24 or 48 hrs post-infection (hpi), protein was isolated and HA-tagged proteins were detected by immunoblotting. At 24 hpi we detected an HA-reactive protein in MRE/rprORF-infected cells of about 18 kDa. This likely represents uncleaved ubiquitin-Reaper fusion protein, which is predicted to be around this size. At 48 hpi, lysates from cells infected with MRE/rprORF still contained the 18 kDa protein but in addition had another protein of around 9 kDa, which likely

corresponds to the HA-tagged Reaper protein after cleavage from ubiquitin. No proteins of similar size were detected in any of the other treatments. We also observed an HA-immunoreactive band of about 57 kDa at both time points in MRE/control-infected cells, as well as some faster migrating bands at 48 hpi (Supplementary Fig. 3.1). Because in this control construct the HA tag is located immediately downstream of ubiquitin, we speculate that these bands may correspond to ubiquitin that was still fused to the HA tag and had been conjugated to unknown protein(s) by ubiquitin ligases. We did not observe any replication deficit in these constructs (see Fig. 3.4), suggesting that the expression of ubiquitin did not significantly affect virus replication. A protein of about 72 kDa that was observed in all treatments (Supplementary Fig. 3.1) was assumed to be of cellular origin and detected as the result of antibody cross-reactivity.

**MRE/rprORF causes increased apoptosis in C6/36 cells**

To confirm that the Reaper protein expressed from MRE/rprORF was functional, we infected C6/36 cells with this virus and then tested for markers of apoptosis, as was previously done for MRE/rpr (Wang et al., 2008). We first demonstrated stimulation of apoptosis by MRE/rprORF infection using a chromatin fragmentation assay (Fig. 3.3A). Endonucleolytic chromatin fragmentation (or nucleosomal laddering), which is characterized by the appearance of a ladder-like pattern on gel electrophoresis, has long been known to be a hallmark of apoptotic cells (Skalka et al., 1976; Williams et al., 1974; Wyllie et al., 1980). A nucleosomal ladder in multiples of approximately 140 bp was evident at 48 hpi in cells that were infected with MRE/rprORF, while DNA from cells that were infected with MRE/control or 5'dsMRE16ic (abbreviated as MRE16) did not display this pattern. We also demonstrated increased apoptosis in these cells using a caspase assay, which measures effector caspase activity by detecting

increased cleavage of a fluorogenic substrate, Ac-DEVD-AFC (Fig. 3.3B). We did not find differences in caspase activity between cells infected with MRE/rprORF, MRE/control or MRE/rpr when they were collected at 24 hpi. However, at 48 hpi, cells infected with MRE/rprORF or MRE/rpr had increased effector caspase activity when compared to MRE/control. This caspase activity increase between 24 and 48 hpi corresponds to the levels of Reaper expression in MRE/rprORF-infected cells as determined by immunoblotting (Fig. 3.2).

**MRE/rprORF and MRE/rpr show replication differences compared to control SINV in BHK-21 and C6/36 cells**

To determine how Reaper expression would affect SINV replication and whether the insertion site of *reaper* in the viral genome would influence this effect, we infected BHK-21 and C6/36 cells with MRE/rprORF, MRE/rpr or MRE/control and sampled the cell culture media by median tissue culture infectious dose ($TCID_{50}$) assay at several timepoints to construct replication curves (Fig. 3.4). In BHK-21 cells we found that MRE/rprORF and MRE/rpr had reduced titers compared to MRE/control at most timepoints, with the largest difference being at 4 days post-infection (dpi) (Fig. 3.4A). There was no significant difference between the MRE/rprORF and MRE/rpr replication curves. In C6/36 cells, we constructed a cumulative replication curve, as well as a non-cumulative growth curve in which the cells were rinsed after sampling at each timepoint (Fig. 3.4B). In the cumulative replication curve, both MRE/rprORF and MRE/rpr had lower titers than MRE/control at most timepoints and the differences were found to increase at later timepoints from about 10-fold at 2 dpi to around 100-fold at days 3 and 4. Differences between MRE/rprORF and MRE/rpr were not found to be significant. In the non-cumulative replication curve, MRE/rprORF and MRE/rpr titers were similar to MRE/control at early timepoints but the Reaper-expressing viruses diverged from MRE/control at the later

timepoints of 4 and 5 dpi. Titers of MRE/control remained near $10^8$ PFU $ml^{-1}$ after 3 dpi, while MRE/rprORF and MRE/rpr titers reached between $10^7$ and $10^8$ PFU $ml^{-1}$ at 3 dpi and then declined at 4 and 5 dpi. This decrease was expected since the Reaper-expressing viruses were causing apoptosis by these later time points. The replication curve of MRE/rprORF was found to be significantly different from MRE/rpr, with MRE/rprORF showing a more drastic decline after peaking at 3 dpi.

**Mosquitoes that ingest blood containing MRE/rprORF show increased caspase activity in midgut**

We next wanted to find out if there was evidence of apoptosis in mosquitoes after they fed on blood containing the MRE/rprORF virus. In this experiment, we allowed mosquitoes to feed on blood with MRE/rprORF, MRE/rpr or MRE/control. After three days, the mosquitoes were dissected and pools of 8 midguts were tested for effector caspase activity. We found that there were significant differences in effector caspase activity between the three treatments using two-way ANOVA (Fig. 3.5). Pooled midguts from the mosquitoes that fed on blood containing MRE/rprORF had the highest effector caspase activity as indicated by the increase in fluorescence over time, suggesting that mosquitoes in this group had the most apoptosis occurring in their midguts. Caspase activity in MRE/rpr-exposed midguts was only marginally higher than in midguts exposed to MRE/control at this time point. Nonetheless, the results confirmed that MRE/rprORF infection induced apoptosis in mosquito midguts.

**MRE/rprORF is less able to infect mosquitoes, but replicates normally if infection is established**

We then allowed mosquitoes to feed on blood containing MRE/rprORF, MRE/rpr or MRE/control to compare how many mosquitoes got infected and whether the infected

mosquitoes showed any differences in viral titer. To do this each mosquito midgut and carcass (defined as all remaining tissues of the mosquito other than the midgut), was titered by $TCID_{50}$. In the midgut, we found that at all timepoints tested, the mosquitoes fed with MRE/rprORF were less likely to have detectable infection compared to MRE/rpr and MRE/control (Fig. 3.6A). This percentage increased over time in the MRE/rprORF-fed mosquitoes, from 25% of midguts showing infection at 3 days PBM to 49% at 7 days PBM. In contrast, the percentage of midgut infection in the MRE/rpr-fed mosquitoes was not significantly different compared to mosquitoes that fed on MRE/control, although it was lower at 3 days PBM (65% versus 81%). MRE/rpr infection also increased over time from 65% at 3 days PBM to 87% at 7 days PBM. Midgut infection percentage in the MRE/control-fed mosquitoes reached 81% by 3 days PBM and remained high over time.

Similar trends were observed in the percentage of mosquitoes with detectable carcass infections, which indicated dissemination of the virus from the midgut (Fig. 3.6B). The prevalence of mosquitoes with carcass infection in the MRE/rprORF-fed group was significantly lower compared to MRE/rpr and MRE/control at all timepoints tested. The percentage of infection prevalence also increased over time and ranged from 10% at 3 days PBM to 41% at 7 days PBM. The prevalence of carcass infection in MRE/rpr-fed mosquitoes was not significantly different from MRE/control and ranged from about 65% at 3 days PBM to 87% at 7 days PBM. The percentage of carcass infection in MRE/control-fed mosquitoes was 74% at 3 days PBM with an increase to 84% at 5 days PBM and then a slight decrease to 79% at 7 days PBM. MRE/rprORF-infected mosquitoes also had lower dissemination rates than MRE/rpr and MRE/control at all timepoints tested (Table 1).

To compare virus replication in the midgut and carcass between the three groups, we focused on only those mosquitoes that had detectable titers (Fig. 3.7). MRE/rprORF titers did not significantly differ from MRE/control at any of the timepoints tested in the midgut or carcass, while MRE/rpr titers were found to be higher than MRE/rprORF and MRE/control at 3 and 7 days PBM in the midgut and at 3 days PBM in the carcass. While statistical analysis of some of the titer data may be unreliable due to small numbers of infected mosquitoes, especially in the case of MRE/rprORF at the earlier time points; overall, there did not appear to be any large differences in titer between the three viruses.

## Discussion

The goal of this study was to generate a SINV construct that would more stably express the pro-apoptotic Reaper protein than the virus used in our previous studies, followed by characterization of this virus in cell culture and mosquitoes. Previous work using a SINV construct that expressed Reaper from a duplicated subgenomic promoter (MRE/rpr) found that fewer mosquitoes developed disseminated infection than a control virus at early timepoints, but this difference disappeared over time due to deletions that accumulated in the inserted *reaper* sequence, resulting in *reaper* defective viruses (O'Neill et al., 2015). Therefore, we were interested in learning what affect apoptosis would have on SINV infection if the Reaper protein was expressed more stably.

The results of our study showed that expressing the Reaper protein from the structural ORF of SINV using a ubiquitin fusion strategy to proteolytically release Reaper was successful. We were able to detect Reaper protein in MRE/rprORF-infected cells by immunoblotting and measured an increase in apoptotic markers in both infected cells and mosquito midgut. We detected a protein of approximately 9 kDa, which is likely free Reaper protein at 48 hpi. We also

likely detected uncleaved ubiquitin-Reaper fusion protein at both 24 hpi and 48 hpi. Thus, it appears that cleavage of ubiquitin fusion proteins in this system is relatively inefficient. We also detected a larger band of unknown origin in the MRE/control virus, which suggests that the ubiquitin expressed from these constructs may be utilized as a substrate by cellular ubiquitin ligases. We cannot conclude with certainty whether expression of ubiquitin has any effect on virus structure or replication. However, the results from the replication curve experiments indicated that the ubiquitin fusion strategy did not result in any significant replication defects under the conditions tested. In BHK-21 cells, MRE/control replicated to the highest level, with MRE/rprORF and MRE/rpr replicating to modestly lower levels before decreasing after 3 dpi. Although SINV naturally causes lytic cell death in BHK-21 cells, it is not surprising that Reaper expression would increase cell death to some degree (Tait et al., 2004), leading to lower titers. In the cumulative replication curve in C6/36 cells, the differences between MRE/control and the Reaper-expressing viruses were more pronounced. This was expected since SINV does not naturally cause a considerable amount of death in these cells (Wang et al., 2008). In this experiment, the titers of MRE/rprORF and MRE/rpr closely matched each other, suggesting that the location of the *reaper* insert did not obviously affect replication. In the C6/36 cells noncumulative replication curves, the titers of MRE/control, MRE/rprORF and MRE/rpr closely matched each other until 3 dpi, after which it appears that enough Reaper protein had accumulated to cause significant cell death and a decrease in titer was seen. MRE/rprORF titers decreased after 3 dpi to a greater extent than MRE/rpr, and these two replication curves were found to be significantly different by Tukey's multiple comparisons test. The reason for this is not clear but it could be due to greater instability of the *reaper* insert in MRE/rpr, which may have decreased its effectiveness in causing cell death. It is important to note that C6/36 cells

have an impaired RNAi response and, therefore, results in these cells may not accurately reflect an in vivo situation (Brackney et al., 2010).

In mosquitoes, MRE/rprORF had the decreased ability to establish both midgut and carcass infection compared to MRE/rpr. At all timepoints tested, MRE/rprORF had a lower infection prevalence compared to both MRE/control and MRE/rpr, while the prevalence of MRE/rpr infection did not significantly differ from MRE/control at any of the timepoints. This result differed from our previous study, which showed that the percentage of mosquitoes infected with MRE/rpr was lower than the control virus used in that study at early timepoints in both the midgut and the carcass (O'Neill et al., 2015). Although we observed that MRE/rpr infection prevalence was lower than MRE/control at 3 dpi (65% versus 81%), this difference did not reach statistical significance. Perhaps additional replicates would accentuate this difference. It is also possible that this difference could be due to different control viruses being used in the two studies. Regardless, the most important result in this study was that MRE/rprORF showed decreased ability to establish infection in the midgut and disseminate compared to MRE/rpr. We conclude that this is likely due to the increased selective pressure to retain the insert in the structural ORF, since deletions within the *reaper* insert would have a 2 out of 3 chance of altering the reading frame and eliminating expression of the downstream essential envelope proteins, resulting in defective viruses. In contrast, any MRE/rpr virus with a deletion within the *reaper* insert would remain viable.

The results of our study also differed from previous results (O'Neill et al., 2015) when we examined the virus titers in mosquitoes that were infected. We found that when MRE/rprORF was able to establish infection in the midgut, the titers were similar to those of MRE/control, and this pattern held true in the carcass. One possible reason for this is that in the sub-population of

mosquitoes that developed infection with MRE/rprORF, the *reaper* gene became non-functional due to mutations, despite the greater stability of the insert than in MRE/rpr. This possibility is supported by the increase in the percentage of mosquitoes that developed infection with MRE/rprORF over time, which suggests that as the virus replicated and was exposed to selective pressures, mutations may have accumulated. Another possibility is that if the virus is able to accumulate to a certain level in midgut cells of some mosquitoes, apoptosis may no longer be able to effectively limit viral replication and spread. When we examined the titers of mosquitoes that became successfully infected with MRE/rpr, we found that titers were higher compared to MRE/rprORF and MRE/control in the midguts at 3 and 7 days PBM and at 3 days PBM in the carcass. It is possible that in many of these MRE/rpr-infected mosquitoes the frequency of viruses with mutated *reaper* was higher, allowing MRE/rpr to replicate to higher levels compared to MRE/rprORF. However, it is unclear why the titers would be higher than MRE/control. In any case, none of the three viruses appeared to have a large replication advantage over the others in infected mosquitoes.

One conclusion that can be drawn from this study is that apoptosis negatively affects viral midgut infection and dissemination. This is supported by several studies in which similar conclusions have been reached (Ayers et al., 2021; O'Neill et al., 2015; Ocampo et al., 2013; Vaidyanathan et al., 2006). However, there have been studies which suggest a seemingly opposite conclusion. In one study where expression of AeIAP1, which inhibits apoptosis, was knocked down in mosquitoes, both midgut infection and dissemination increased (Wang et al., 2012). However, AeIAP1 knockdown caused a high mortality rate and extensive pathology of the midgut tissue was observed. The systemic induction of apoptosis thus likely greatly decreased the structural integrity of the midgut, which may have allowed viral passage through

the tissue. In the present study as well as in O'Neill et al (2015), the pro-apoptotic gene was inserted into the viral genome, ensuring that apoptosis was stimulated in infected cells only. Additionally, two studies have shown that knockdown of the caspase *Aedronc* reduces infection with SINV or DENV-2 (Brackney et al., 2010; Eng et al., 2016; Wang et al., 2012). If apoptosis was strictly inhibitory for viral infection and dissemination, the opposite result would be expected. One possible explanation for this result is that caspases are involved in remodeling of midgut basal lamina (Means & Passarelli, 2010), which appears to be necessary for viral midgut escape (Dong et al., 2017). Thus either a low basal level of midgut caspase activity or extensive, widespread stimulation of apoptosis may be advantageous for a virus, while stimulation of apoptosis specifically in infected cells has a negative effect.

There are several limitations in our study that could be explored and improved upon in future studies. In this study, we only looked at the effect of Reaper expression on SINV in *Ae. aegypti*. This should be explored in other virus/vector combinations to determine if the results of this study are generalizable to other situations. Additionally, while less than 50% of mosquitoes exposed to MRE/rprORF developed disseminated infection by 7 days PBM, we did see this percentage increase from 3 to 7 days PBM. It is possible that at further timepoints the percentage of infection of MRE/rprORF would become equivalent to that of control viruses. The durability of this effect should be further explored in the future.

Overall, this study provides additional evidence that expression of the pro-apoptotic protein Reaper has a negative effect on the ability of SINV to establish infection in the midgut and disseminate to the carcass. Expressing Reaper by inserting the gene into the structural ORF caused a more robust reduction in infection prevalence than expression via the duplicated subgenomic promoter, with the differences in both midgut and carcass infection prevalence

between MRE/rprORF and MRE/control being significant at all timepoints tested. These results provide additional evidence that the apoptotic pathway is antiviral in mosquitoes and possibly could be exploited to prevent transmission of arboviruses. Additionally, this study is consistent with previous findings that inserting genes into a viral ORF is a more successful strategy for durable expression of genes that are subject to negative selection.

## Methods

### Cells

BHK-21 cells were maintained at 37$^{o}$C with 5% $CO_2$ in Dulbecco modified Eagle medium (DMEM, Gibco) plus 10% fetal bovine serum (FBS, Atlanta Biologicals). C6/36 cells were maintained at 27$^{o}$C in Liebovitz's medium (Gibco) plus 10% FBS.

### Insect rearing

Orlando strain *Ae. aegypti* mosquitoes (obtained in 2008 from James Becnel, USDA ARS, Gainesville, FL) were reared in a 27$^{o}$C incubator with 80% humidity and a 12-hr light-dark cycle. To obtain eggs used in experiments, females were allowed to feed on defibrinated sheep's blood (Colorado Serum Company) using a Hemotek feeding system (Hemotek Ltd.). Adult mosquitoes were maintained on raisins and water.

### Plasmid design and construction

A previously described plasmid containing a fragment of 5'dsMRE16ic extending from the NotI site to the AvrII site as well as microRNA target sites and a 2A self-cleaving peptide sequence ligated into a pGEM-T backbone was used as the starting plasmid (Carpenter et al., 2021). This plasmid was digested with BstEII and AfIII and the intervening sequence containing the miRNA target sites was replaced with the following sequence (synthesized by Genewiz) containing ubiquitin (human K48R), the *Drosophila reaper* sequence (lacking the initiator

methionine) and an HA tag:

(TGGAATAGCAAGGGAAAGACCATCAAGACGACGCCCGAAGGGACAGAGGAATGGT
CAGCAGCACTCGAGATGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACT
CTAGAAGTTGAACCATCCGATACCATCGAAAACGTTAAGGCTAAAATTCAAGACAA
GGAAGGCATTCCACCTGATCAACAAAGATTGATCTTTGCCGGTAGGCAGCTTGAGG
ACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCCACCCTGCACCTGGTCC
TCCGTCTCAGAGGTGGTGCAGTGGCATTCTACATACCCGATCAGGCGACTCTGTTGC
GGGAGGCGGAGCAGAAGGAGCAGCAGATCCTTCGCTTGCGGGAGTCACAGTGGAG
ATTCCTGGCCACCGTCGTCCTGGAAACCCTGCGCCAGTACACTTCATGTCATCCGAA
GACCGGAAGAAAGTCCGGCAAATATCGCAAGCCATCGCAATACCCATACGATGTTC
CAGATTACGCTGGATCCCAGCTGTTGAATTTTGACCTT). A control plasmid was generated in the same way, but the intervening sequence was replaced with a synthesized sequence not containing *Drosophila reaper* but still containing ubiquitin and an HA tag:

(TGGAATAGCAAGGGAAAGACCATCAAGACGACGCCCGAAGGGACAGAGGAATGGT
CAGCAGCACTCGAGATGCAGATCTTCGTCAAGACGTTAACCGGTAAAACCATAACT
CTAGAAGTTGAACCATCCGATACCATCGAAAACGTTAAGGCTAAAATTCAAGACAA
GGAAGGCATTCCACCTGATCAACAAAGATTGATCTTTGCCGGTAGGCAGCTTGAGG
ACGGTAGAACGCTGTCTGATTACAACATTCAGAAGGAGTCCACCCTGCACCTGGTCC
TCCGTCTCAGAGGTGGTTATCCATACGATGTTCCAGATTACGCTGGATCCCAGCTGT
TGAATTTTGACCTT). Both plasmids were then digested with NotI and AvrII and the purified fragments were ligated into 5'dsMRE16ic which had been digested with the same restriction enzymes. Proper insertion was verified by Sanger sequencing. The generation of MRE/rpr has previously been described (Wang et al., 2008).

**Virus production**

Infectious clone plasmids were linearized using AscI (New England Biolabs) and then in vitro transcribed using the MEGAscript SP6 transcription kit (Thermo Fisher Scientific) with added cap analog (New England Biolabs). Following transcription, RNA was transfected into BHK-21 cells using Lipofectamine 3000 (Thermo Fisher Scientific). After two days, the media was removed and used to infect a T75 flask of C6/36 cells. After 5 days the resulting P2 virus stock was frozen in aliquots and titer was determined using $TCID_{50}$ assay.

**$TCID_{50}$ assay**

BHK-21 cells were plated at a density of $1x10^4$ cells per well in a 96-well tissue culture plate in 100 µl of DMEM plus 10% FBS and supplemented with 15 µg per ml of penicillin/streptomycin (Invitrogen). Mosquito and cell samples were removed from -80$^o$ C and thawed on ice. Samples were then spun to remove debris and DMEM was used to make serial dilutions of each sample. Each dilution was transferred to five wells containing BHK-21 cells. After 5 days, each well was scored for cytopathic effects. The number of wells of each dilution scored as positive was used to determine $TCID_{50}$ $ml^{-1}$ and this was converted to PFU $ml^{-1}$ by multiplying by 0.69 (O' Reilly et al., 1994).

**Replication curves**

For replication curves in C6/36 cells, the cells were plated at a density of $1x10^6$ cells per well in a 6-well plate in 2 ml of Leibovitz's medium containing 10% FBS. For replication curves in BHK-21 cells, the cells were plated at a density of $5x10^5$ cells per well in a 6-well plate in 2 ml DMEM containing 10% FBS. Cells were allowed to recover for 2 hrs and then were infected with MRE/rprORF, MRE/rpr or MRE/control at a multiplicity of infection (MOI) of 0.1. The cells were placed on a rocker and the virus was allowed to adsorb for 1 hr. The media was then

removed, and the cells were rinsed before replacing the media. For the cumulative replication curves, 100 μl of media were sampled for analysis at 1, 2, 3 and 4 dpi. For the non-cumulative replication curves, 100 μl of media were sampled at each time point for analysis and then the remaining media was removed, the cells were rinsed twice and then 2 ml of media were added. All samples were frozen and stored at -80°C until analysis by $TCID_{50}$ assay.

**DNA fragmentation assay**

C6/36 cells were plated at a density of 2 x $10^6$ cells per well in a 6-well plate in 2 ml Leibovitz's medium containing 10% FBS. Cells were allowed to recover for 2 hrs and then were infected with MRE/rprORF, MRE/control, or 5'dsMRE16ic at an MOI of 1. After 48 hrs cells were removed from the 6-well plates and pelleted at 500 x g. They were washed twice with phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$) and then resuspended in 200 μl lysis buffer (0.1 M SDS, 0.1 M Tris pH 8.0, 0.05 M EDTA pH 8.0, 200 mg $ml^{-1}$ Proteinase K) and incubated at room temperature for 1 hr. The samples were then extracted twice with phenol/chloroform and then ethanol precipitated. The pelleted DNA was resuspended in 100 μl Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA) containing 100 μg $ml^{-1}$ RNase A (Thermo Fisher Scientific) and incubated at room temperature for 5 min. Twenty μl of each sample was loaded into a 1.2% agarose gel containing 0.5 μg $ml^{-1}$ ethidium bromide. The gel was visualized using an AlphaImager gel imaging system. Sizes of the bands were compared to a Versaladder DNA ladder (Gold Bio).

**Mosquito infection for $TCID_{50}$ and caspase assay**

Prior to blood feeding, mosquitoes were placed in cups containing 20-30 females and 10% males and were only provided water for 24 hrs. MRE/rprORF, MRE/rpr and MRE/control stocks were diluted to 1.45 x $10^7$ PFU $ml^{-1}$ with Liebovitz's medium. The diluted virus stocks

were then mixed 1:1 with defibrinated sheep blood and the mosquitoes were then allowed to feed on one of the virus blood mixtures using the Hemotek feeding system for 90 min. Fully engorged females were separated from unfed and partially fed females and males and were given water and raisins to feed *ad libitum*. At 3, 5, and 7 days PBM, the mosquitoes were cold anesthetized, and the midguts were dissected from the rest of the mosquito (the carcass). The midguts and carcasses used for $TCID_{50}$ assays were placed in 1.5 ml tubes containing 200 µl DMEM media containing 10% FBS and homogenized using disposable pestles. The samples were then frozen at -80$^{o}$ C. The midguts used for caspase assays were collected in 30 µl of caspase reaction buffer (20 mM Hepes-KOH, pH 7.5, 50 mM KCl, 1.5 mMMgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT), homogenized with disposable pestles and stored at -80$^{o}$C.

**Immunoblotting**

C6/36 cells were plated at a density of $2x10^{6}$ cells per well in a 6-well plate containing 2 ml Leibovitz's media plus 10% FBS. They were then infected with MRE/rprORF, MRE/control or 5'dsMRE16ic at an MOI of 10. After 24 or 48 hrs, the cells were rinsed three times with cold PBS. The plate was placed on ice and 100 ul of cold Laemmli sample buffer (Bio-Rad) was added to each well. The wells were scraped, and the lysate was collected in a 1.5 ml tube. The samples were then heated to 100$^{o}$C for 5 min, centrifuged at 4$^{o}$C and the supernatant was transferred to a new tube. SDS-PAGE was performed using 30 µl of sample with 4-20% Bis-Tris gels (Genscript) in Tris-MOPS-SDS running buffer (Genscript), and proteins were transferred to PVDF membrane. After blocking for 1 hr in 5% dried skim milk in TBST, a 1:1000 dilution of the anti-HA (Biolegend) or anti-β-actin (Santa Cruz Biotechnology) primary antibody was incubated with the membrane overnight at 4$^{o}$C with constant agitation. After incubation, the membrane was washed three times with TBST. A 1:15000 dilution of goat anti-mouse IgG-

horseradish peroxidase secondary antibody (Thermo Fisher Scientific) was added and was incubated for 1 hr at room temperature with agitation. Bands were detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized using a LI-COR Western Blot Imager.

**Caspase assay**

In the cell experiments, C6/36 cells were plated at a density of $2x10^6$ cells per well in a 6-well plate in 2 ml Leibovitz's media plus 10% FBS. The cells were infected with MRE/rprORF, MRE/rpr or MRE/control at an MOI of 1. At 24 and 48 hpi the cells were rinsed with PBS and then collected in 400 µl of caspase reaction buffer. Mosquito midgut samples were collected in pools of 8 in caspase reaction buffer and the tissues were disrupted by sonication. The tubes were centrifuged, and the supernatant was moved to a new tube. All samples were frozen at -80$^o$C until testing. Bradford protein assay (Bio-Rad) was used to determine protein concentration, and cell and mosquito samples were diluted to 100 µg $ml^{-1}$. 50 µl of each sample were added to white 96-well plates (Costar) and incubated at 37$^o$C for 15 min. Then 10 µl of Ac-DEVD-AFC (ApexBio) was added to each well at a final concentration of 20 µM. Cleavage of this fluorogenic substrate was monitored at an excitation wavelength of 405 nm and an emission wavelength of 535 nm using a Victor3 1420 Multilabel Counter (Perkins-Elmer). Readings were taken at 15 min intervals for a period of 1 hr.

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# Chapter 3 figures

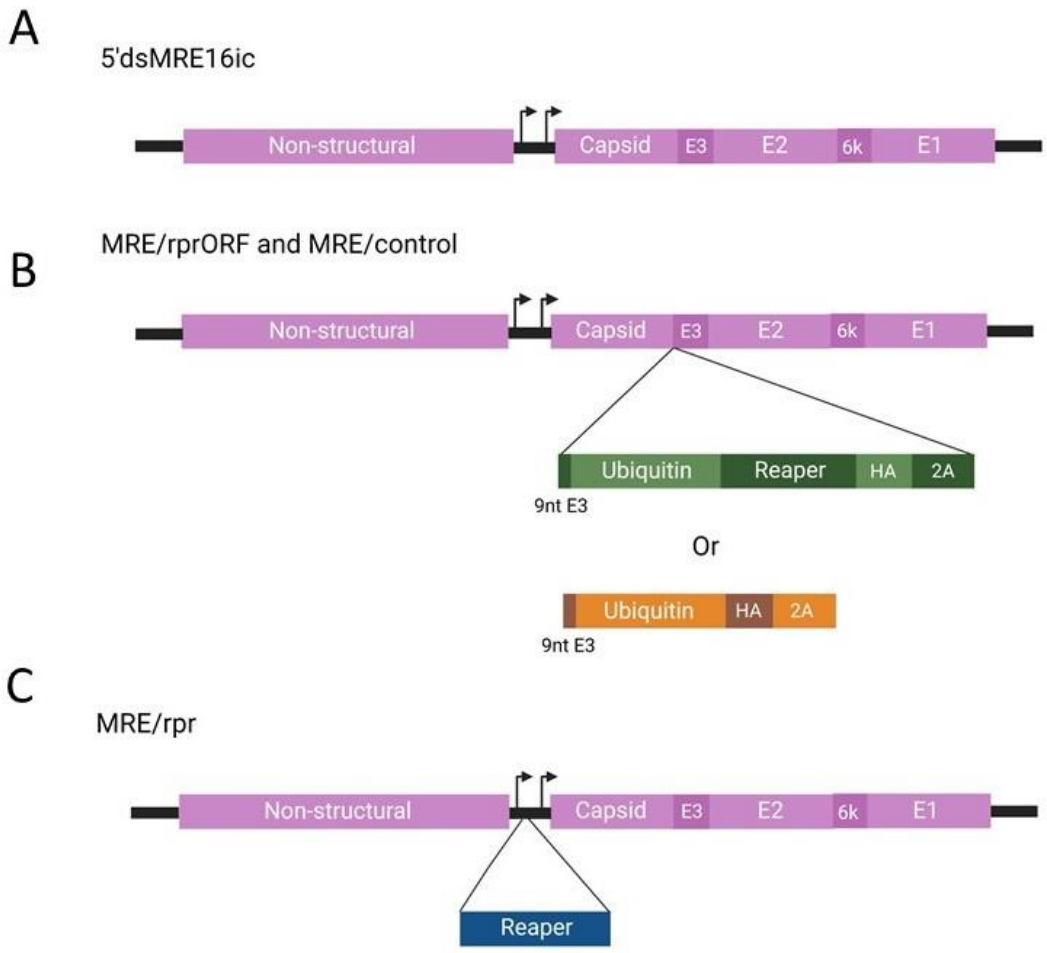



**Figure 3.1 Diagrams of virus constructs used in this study**

(A) 5'dsMRE16ic was used as the starting backbone for the other viruses and does not contain any inserted sequences in the duplicated subgenomic promotor region or the structural ORF. Arrows indicate subgenomic promoter transcription start sites. (B) MRE/rprORF and MRE/control contain sequences inserted after the final codon of the capsid sequence in the structural ORF. In both constructs, the first 9 nucleotides of PE3 are duplicated following the capsid to allow autoproteolytic activity of the capsid. Immediately following the PE3 insertion, MRE/rprORF contains a ubiquitin-*reaper* fusion with an HA tag. MRE/control contains

ubiquitin and HA but does not contain *reaper*. The inserted sequence of both constructs ends with FDMV 2A which will release peptides as they are translated. (C) MRE/rpr contains the *reaper* gene inserted in the duplicated subgenomic promoter region. The figure was created with Biorender.com.

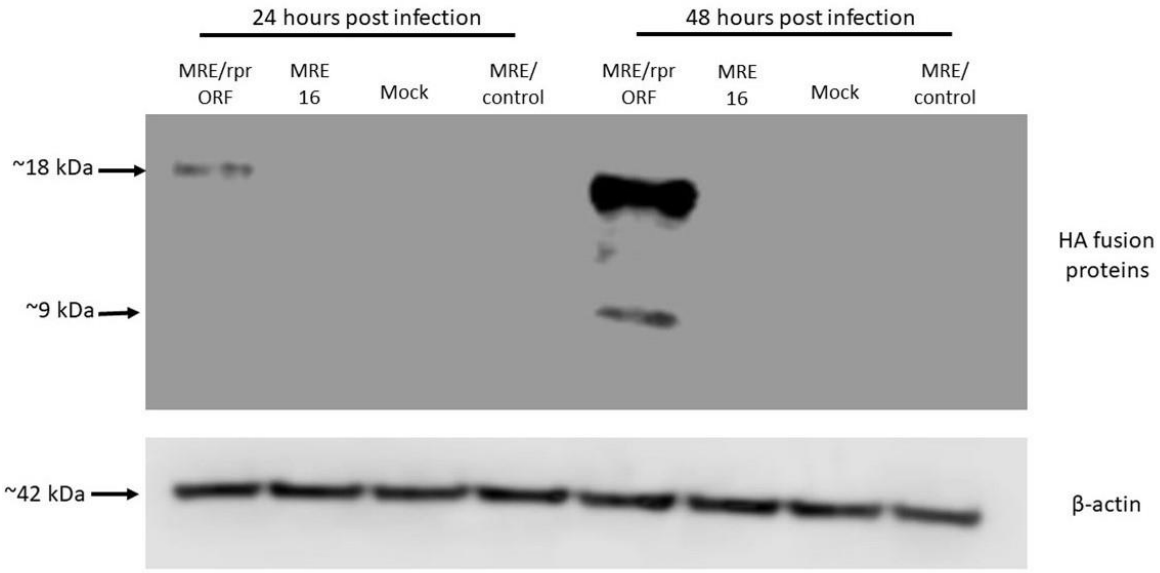



**Figure 3.2 MRE/rprORF expresses the Reaper protein in infected cells**

C6/36 cells were infected with MRE/rprORF, 5'dsMRE16ic (labeled MRE16), or MRE/control or were mock infected and protein was extracted at 24 and 48 hpi. Immunoblotting was done using antibodies against HA (upper panel) or β-actin as a loading control (lower panel).

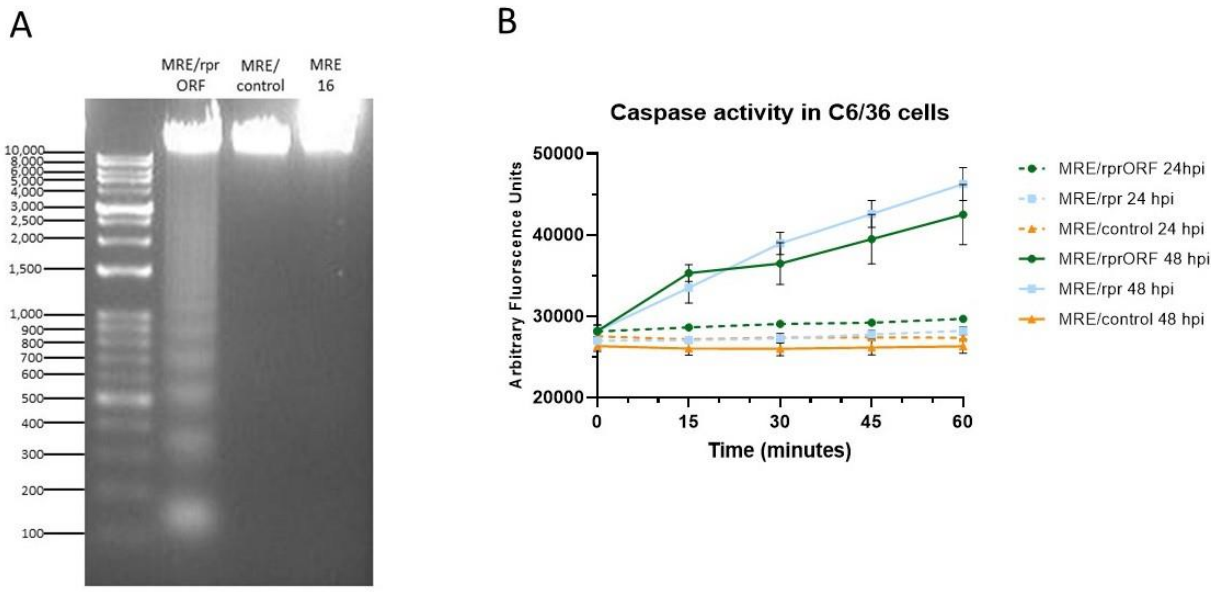



**Figure 3.3 MRE/rprORF causes apoptosis in C6/36 cells**

(A) C6/36 cells were infected with MRE/rprORF, MRE/control or 5'dsMRE16ic (labeled MRE16). After 48 hrs the cells were collected, DNA was extracted and run on an agarose gel containing ethidium bromide. (B) Cells infected with MRE/rprORF, MRE/rpr or MRE/control were collected and lysed at 24 and 48 hpi. Caspase activity from these cell lysates was measured by examining their ability to cleave the fluorogenic substrate Ac-DEVD-AFC. Fluorescence measurements were taken every 15 min for 1 hr. Three biological replicates were performed. Error bars indicate SEM.

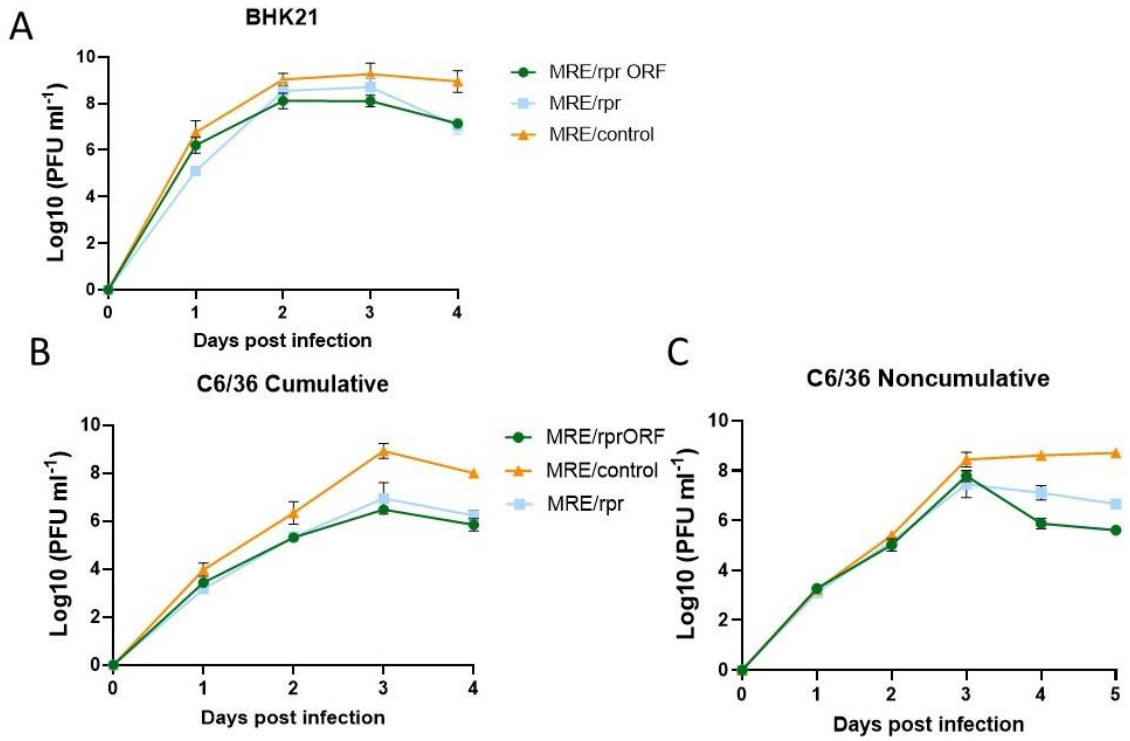



**Figure 3.4 Virus growth curves in BHK-21 and C6/36 cells**

(A-B) BHK-21 or C6/36 cells were infected with MRE/rprORF, MRE/rpr or MRE/control at an MOI of 0.1 and a sample of supernatant was removed and titered by $TCID_{50}$ assay at each of the indicated timepoints. (A) In BHK-21 cells, MRE/rprORF ($p$=0.003) and MRE/rpr ($p$=0.0002) were significantly different compared to MRE/control. MRE/rpr and MRE/rprORF were not significantly different ($p$=0.9879). (B) In C6/36 cells, MRE/rprORF ($p$<0.0001) and MRE/rpr ($p$<0.0001) were significantly different compared to MRE/control. MRE/rprORF and MRE/rpr were not significantly different ($p$=0.7702). (C) Non-cumulative growth curve. C6/36 cells were infected with MRE/rprORF, MRE/rpr or MRE/control at an MOI of 0.1. At each indicated timepoint, a sample of supernatant was removed for $TCID_{50}$ assay. The remaining cell culture medium was then removed from each well, the cells were rinsed, and the media was replaced. MRE/rprORF ($p$<0.0001) and MRE/rpr ($p$<0.0001) were significantly different compared to

MRE/control. MRE/rprORF and MRE/rpr were also significantly different ($p$=0.0291). In A-C, three independent biological replicates were performed. Titers were log transformed and compared with 2-way ANOVA and Tukey's multiple comparison test. Error bars represent SEM.

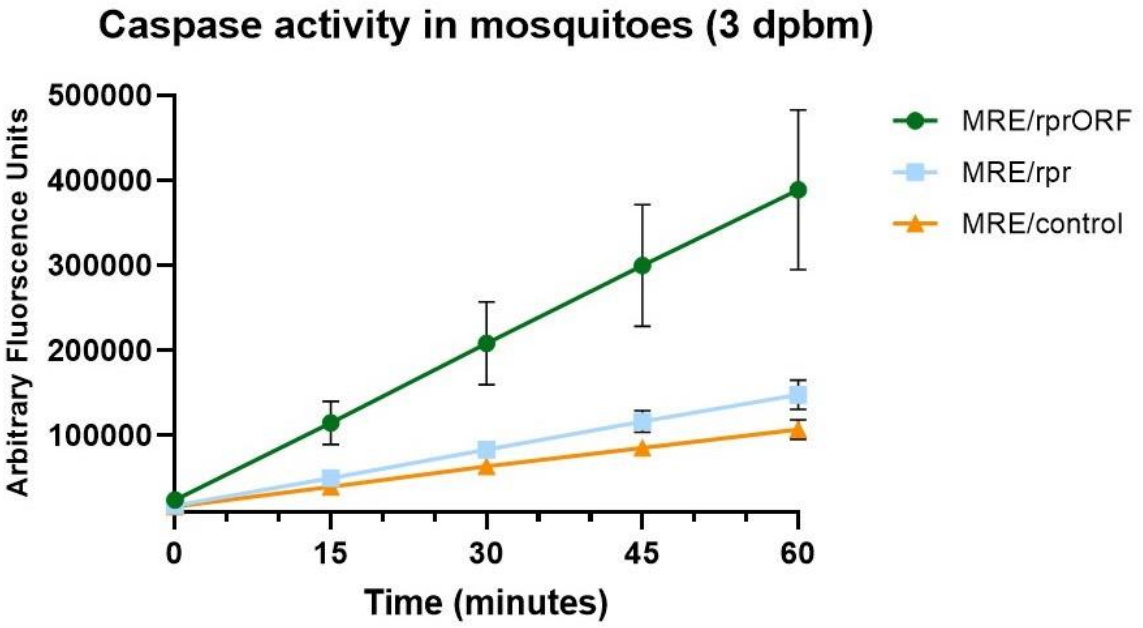



**Figure 3.5 MRE/rprORF causes apoptosis in Ae. aeygpti midgut**

Mosquitoes were fed blood containing MRE/rprORF, MRE/rpr or MRE/control and midguts were dissected after 3 days. Midguts were pooled in groups of 8 with 3 pools/treatment. Each pool thus represents an independent biological replicate. Cells were lysed by sonication and the supernatant was used for caspase assay. Cleavage of the Ac-DEVD-AFC substrate was monitored every 15 min for 1 hr. Viral treatment was judged to significantly contribute to variation using 2-way ANOVA ($p$=0.0055).

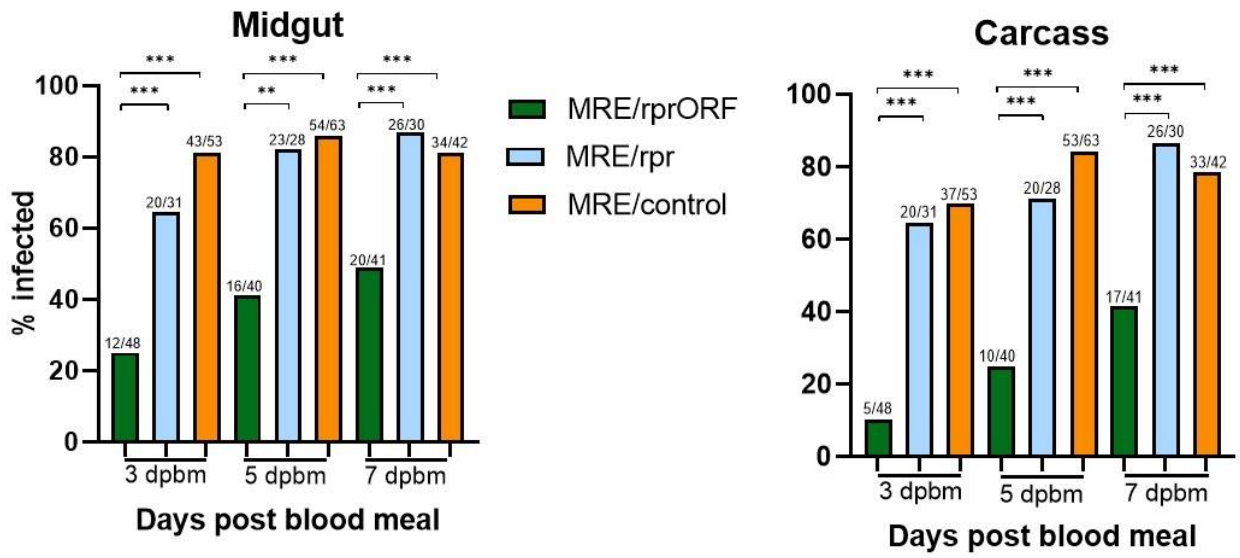



**Figure 3.6 MRE/rprORF is less able to establish mosquito infection than MRE/rpr and MRE/control**

Mosquitoes were fed blood containing MRE/rprORF, MRE/rpr or MRE/control and dissected at 3, 5 or 7 days PBM**.** Midguts (left) and carcasses (right) were titered by $TCID_{50}$. Mosquitoes that did not have detectable titer were considered to be negative while any positive titer by $TCID_{50}$ was considered to be positive. Treatments were compared using Fisher's exact test (*** $p<0.001$, ** $p<0.01$). Statistical comparisons (brackets) are shown for all statistically significant differences.

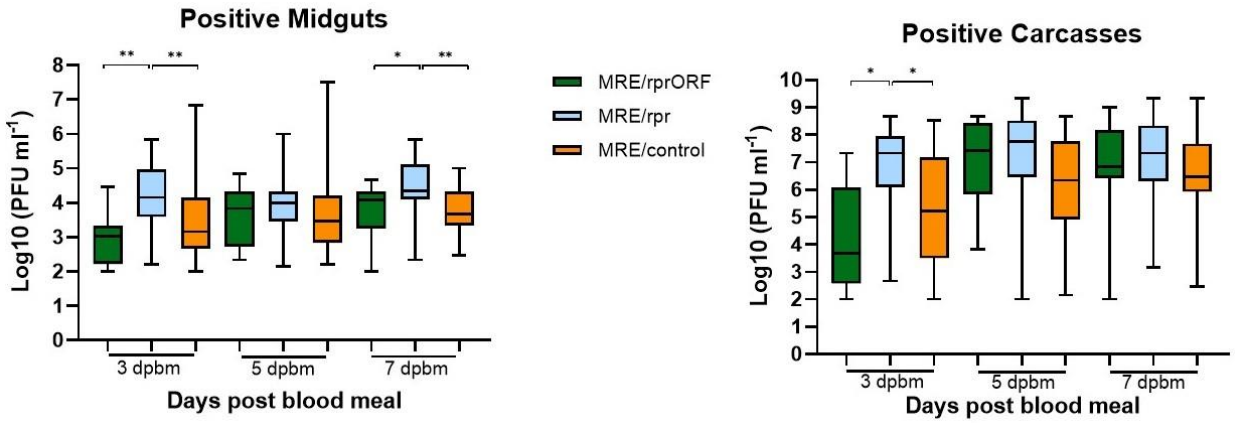


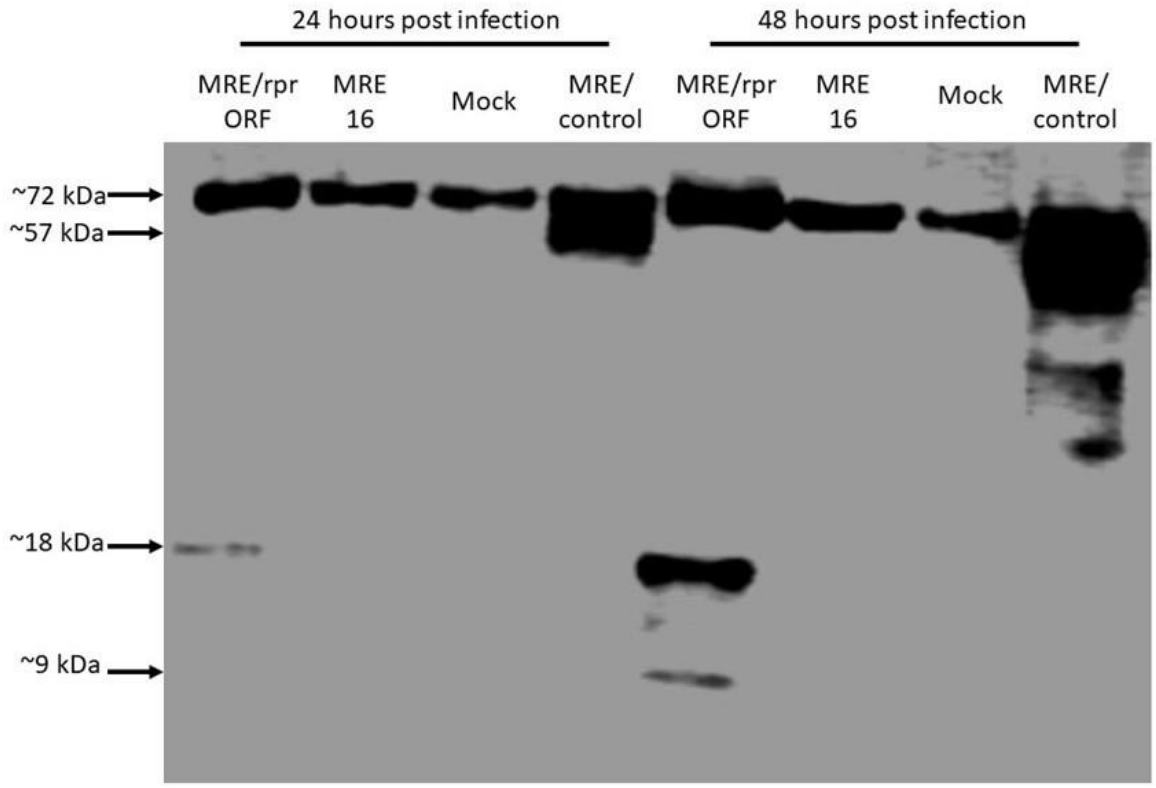

**Figure 3.7 Mosquitoes infected with MRE/rprORF have similar titers compared to MRE/control**

The titers of the infected midgut (left) and carcass (right) samples from Fig. 6 are shown. Titer values were log transformed and compared by 1-way ANOVA and Tukey's multiple comparisons test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical comparisons (brackets) are shown for all statistically significant differences.

**Table 3.1 Dissemination rates at different days PBM**

| Virus | 3 days PBM | 5 days PBM | 7 days PBM |
|---|---|---|---|
| MRE/rprORF | 41.7[a] | 62.5 | 85.0 |
| MRE/rpr | 100.0 | 87.0 | 100.0 |
| MRE/control | 86.1 | 98.2 | 97.1 |

[a]Percentage of infected mosquitoes having disseminated infection, as calculated by the number of positive carcass infections divided by the number of positive midgut infections.




**Supplementary Figure 3.1**

# Chapter 4 Conclusions

The destructive impacts of arboviral diseases are not likely to abate in the future without new advances in prevention and treatment, and therefore improved understanding of virus-vector interactions will be vital to progress in controlling these diseases. The constantly fluctuating nature of these viruses, their hosts, and the environment necessitates ongoing research into these complex interactions. One important piece of this immense and shifting puzzle is why some mosquito species are such efficient vectors of certain arboviruses when the vast majority are not, and how the midgut may contribute to this enduring mystery. In the studies described in the previous two chapters we sought to improve the understanding of how midgut replication and apoptosis contribute to the processes of midgut infection and escape; yet there remains much to explore regarding these factors and the many other factors that contribute to this important barrier.

The purpose of the experiments described in Chapter 2 was to gain a better understanding of how viral replication in midgut cells contributes to the ability of a virus to escape from the midgut. While there has been significant published research that suggests that viral replication can be an important contributor to midgut escape (Franz et al., 2006; Khoo et al., 2010; Romoser et al., 2004), there have also been several findings, such as red blood cells from a blood meal found in the mosquito hemocoel, that suggests that a midgut escape route via intercellular means may exist (Weaver et al., 1991). We inserted target sites for midgut-specific miRNAs into the SINV genome to cause a tissue-specific reduction in replication. Our primary finding was that midgut replication significantly improves the chances that a mosquito will develop disseminated viral infection. However, the infrequent occurrence of mosquitos with carcass infection that were not found to have midgut infection does not completely exclude the possibility that an intercellular route may exist in rare cases. Both midgut and carcass infections rarely occurred in

mosquitoes that were fed with the miRNA-targeted viruses. Nevertheless, when midgut and disseminated infections were established, virus levels were mostly equivalent to control virus levels. The reasons why midgut replication occurred in the small percentage of mosquitoes that did develop infection with the miRNA targeted viruses was not related to the loss of the inserted sequence and will require more study in the future.

In the next studies described in Chapter 3, we aimed to clarify the role of apoptosis in midgut escape. Evidence for the negative impacts of apoptosis on midgut escape has been reported in several studies (Ayers et al., 2021; O'Neill et al., 2015; Ocampo et al., 2013; Vaidyanathan & Scott, 2006). However, the results of other studies suggests that there may be a level of apoptosis in which the structural integrity of the midgut is damaged, which may lead to increased dissemination rates (Wang et al., 2012). A previous study showed that when the pro-apoptotic gene *reaper* was inserted into the SINV genome and the resulting virus was fed to mosquitoes in a blood meal, the percentage of mosquitoes with disseminated infection was initially lower compared to control virus-fed mosquitoes, although not strikingly so, but this difference dissipated at later timepoints due to loss of *reaper* by negative selection (O'Neill et al., 2015). We aimed to improve upon this study by inserting the *reaper* gene into the structural ORF of SINV, which allowed us to gain an improved understanding of the impact of apoptosis on midgut escape. Our primary finding was that fewer mosquitoes blood fed with the MRE/rprORF virus developed disseminated infection compared to MRE/rpr and control fed mosquitoes. This showed that Reaper had a greater negative effect on the ability of SINV to establish infection than that shown by the previous study, which used a less stable Reaper-expressing virus.

Taken together, these two studies have added to our understanding of how these two factors affect the ability of an arbovirus to cause disseminated infection and could lead to a greater understanding of virus-vector specificity. Both reduced midgut replication and apoptosis appear to have a negative effect on the ability of a virus to establish infection and escape from the midgut. Studies have shown that disruptions in the basal lamina may occur after a blood meal and it has been speculated that this may allow virions to pass through (Dong et al., 2017; Kantor et al., 2018). These breeches may not occur extensively throughout the basal lamina but may instead be associated with particular areas (Kantor et al., 2018). The virions may need to replicate and spread to nearby cells to increase the likelihood of infecting a cell that is near one of these breeches. A similar model was suggested by Armstrong et al., who proposed that a mosquito taking a second blood meal could give a virus another chance to access a transient disruption in the basal lamina (2020). We feel that this could explain the results that we observed in our first study; when midgut replication was limited, the virus was less able to spread to nearby cells and access an escape portal. The results of our second study could also fit in with this model. Apoptosis has been shown to limit proliferation of some viruses in the *Aedes aegypti* midgut by rapidly inducing apoptosis (Ayers et al., 2021). This potentially rapid cell death could stop viral replication early and prevent viral spread in the midgut and therefore access to an exit through the basal lamina. However, it is unclear how quickly apoptosis was induced by Reaper expression in our study, as the level of apoptosis in mosquito midguts was not assessed until three days post blood meal. Even though much evidence points to apoptosis as a limiting factor in viral dissemination, disruptions in the basal lamina near apoptotic cells have been observed (Weaver et al., 1988), which could increase the access of a virion to an escape portal. Nonetheless, it is not clear if this happens regularly in compatible virus-vector combinations, or

if this disruption might occur too late for virions that are still in the midgut lumen to access it. In any case, limited midgut replication and apoptosis may ultimately prevent disseminated infection in much the same way; by preventing cell-to-cell spread which eliminates access to rare or transient disruptions in the basal lamina.

This knowledge may be applicable to disease prevention in a few different ways. First, there may be some predictive value to midgut replication and apoptosis. When assessing a new mosquito species or population for their ability to transmit a certain virus, observations of extensive apoptosis or limited midgut replication could be an early indication of a poor virus dissemination rate. Also, the knowledge that these pathways negatively affect dissemination could make them attractive targets for prevention of mosquito infection. RNAi technology may be one way to prevent viral replication and already has had successful applications in other insects. For example, a dsRNA developed against Israeli acute paralysis virus has been shown to be protective against this virus in honeybees when they are fed with this dsRNA (Hunter et al., 2010). Genetic engineering of mosquitoes to be refractory to viruses could be another route of disease control. If mosquitoes could be engineered to have increased midgut apoptosis in infected cells, this may reduce the number of mosquitoes that are able to transmit the virus.

The strategies that we used to engineer SINV in both of our studies may also have practical applications. Inserting miRNA target sites into a viral genome has been developed for a variety of applications, including in vaccines, oncolytic viral therapy, and research safety (Barnes et al., 2008; Kelly et al., 2008; Langlois et al., 2013; Ylosmaki et al., 2013). To our knowledge we were the first group to use this technique to study tissue tropism in mosquitoes. Our success in using this technique may open the door to finding other mosquito tissues in which replication is critical for transmission. Furthermore, we observed greater reduction in

dissemination when these target sites were inserted into the 3' UTR as opposed to the structural ORF and did not find significant mutations in our inserted sequence in mosquitoes after the virus replicated. This knowledge could be valuable in future studies.

In our second study we inserted the *reaper* gene into the structural ORF of SINV as a strategy to prevent loss of gene function, as observed in a previous study (O'Neill et al., 2015). We showed that Reaper was successfully expressed and induced apoptosis using this strategy, although we did note that cleavage of Reaper from ubiquitin appeared to be relatively inefficient in C6/36 cells. However, this virus did not appear to have any replication defects because of this insertion. We propose that this strategy may be a more reliable option for inserting genes that may be subject to negative selection into SINV, which may be useful in future studies.

There are several ways that these studies could be expanded upon to better understand the impacts of apoptosis and viral midgut replication on midgut escape. First, it would be useful to understand if there are circumstances which could make these factors less effective at preventing dissemination. For example, mosquitoes taking multiple blood meals has been shown to increase dissemination rates (Armstrong et al., 2020). It would be important to find out if multiple blood meals or other differences in mosquito behavior could decrease the need for midgut replication. We would also like to better understand why some mosquitoes develop infection with the MRE3'miRT virus. Since the midgut contains several cell types, this could be related to which cells are initially infected by the virus. It is possible that the midgut-specific miRNAs that we used are not expressed at a high enough level in some midgut cell types to block virus replication. Future studies could use fluorescent in situ hybridization to examine where these miRNAs are expressed and where the virus is replicating. Should this avenue of research not explain the results that we observed, another potential strategy could be to use RNA sequencing

to look at differences in gene expression between mosquitoes that do develop disseminated infection with the MRE3’miRT virus and those that do not.

We also think that there are still questions to be answered regarding the role of apoptosis in the midgut escape barrier. While most evidence seems to suggest that apoptosis negatively affects midgut escape when it occurs in virally infected cells, we would like to further explore how apoptosis occurring before viral infection could affect dissemination rates. One way to approach this idea would be to orally infect mosquitoes with the Reaper-expressing virus, MRE/rprORF, and then subsequently feed the mosquitoes with the MRE3'miRT virus to see if apoptosis occurring prior to MRE3'miRT feeding would increase the rates of dissemination by disrupting the midgut barrier.

To achieve a future in which arboviral diseases are better controlled or even eradiated, we need to invest in our understanding of arboviral-vector interactions. If we learn more about the midgut it may, perhaps, be one gateway to transmission that could be shut. In this work, we explored viral midgut replication and apoptosis but hope that there will be continued research to understand the nuances of how these and many other factors contribute to disseminated infection in mosquitoes.

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# Appendix A: Effects of an additional noninfectious blood meal and previous infection with an apoptosis-inducing virus on dissemination of a midgut replication attenuated virus in *Aedes aegypti*

# Introduction

Worldwide public health continues to be threatened by the emergence and reemergence of mosquito-borne arboviruses. One way to prevent transmission of arboviruses could be to block the virus from escaping from the mosquito midgut, which is the initial site of infection. If midgut escape is blocked, then viral dissemination and transmission cannot occur. Our previous work has shown that poor midgut replication negatively impacts the ability of a virus to establish midgut and disseminated infection (Carpenter et al., 2021 and Chapter 2 of this dissertation). Reducing midgut replication may therefore be a viable means of preventing disseminated infection and thus disease transmission. However, before midgut replication can be targeted as a means of disease control, we need to know if there are factors that may decrease the need for midgut replication. If an outside factor makes it so that midgut replication is no longer needed for dissemination, then replication in this tissue might not be a useful target. Under natural conditions, mosquitoes often take multiple blood meals, even after oogenesis has started, and giving infected mosquitoes a subsequent blood meal has been shown to increase viral dissemination (Armstrong et al., 2020; Scott & Takken, 2012). In addition, prior work showed that inducing apoptosis in *Aedes aegypti* by knocking down expression of the anti-apoptotic protein AeIAP1 resulted in higher rates of infection and dissemination of Sindbis virus (Wang et al., 2012). In this study, we wanted to examine how these two different factors may impact dissemination of a midgut replication-attenuated virus.

## Results

**A noninfectious blood meal increases rates of midgut and carcass infection in mosquitoes previously fed with MRE3'miRT and rates of carcass infection in mosquitoes previously fed with MRE3'control**

We fed mosquitoes a blood meal containing MRE3′miRT or MRE3′control and then fed them a noninfectious blood meal 5 days later. Two days after the noninfectious blood meal, we dissected the midguts from the carcasses and used TCID50 to determine the viral titer in each midgut and carcass. We then compared titers and infection rates of the mosquitoes that received a subsequent noninfectious blood meal to mosquitoes that only received the initial infectious blood meal (Figure A1).

In mosquitoes that received a blood meal containing MRE3′miRT but that did not receive a subsequent noninfectious blood meal, which we called the not refed or NRF group, the midgut infection rate was less than 20% while the mosquitoes that received a subsequent noninfectious blood meal, called the refed group, had a midgut infection rate of about 46%. This difference was determined to be significant by Fisher's exact test. In the MRE3′control infected mosquitoes the midgut infection rate was about 60% in NRF mosquitoes and 72% in refed mosquitoes, however this difference did not reach statistical significance.

The carcass infection rates were significantly different between the refed and NRF mosquitoes in both the MRE3′miRT fed mosquitoes and the MRE3′control fed mosquitoes. In the MRE3′miRT mosquitoes the subsequent noninfectious blood meal raised the carcass infection rate from 17% to 41% while in the MRE3′control mosquitoes it raised the rate from 50% to 69%.

**A second noninfectious blood meal does not impact the titer of MRE3'miRT or MRE3'control in infected mosquitoes**

We were also interested to test whether mosquitoes that were successfully infected in the refed group would have a titer that was significantly different from those that were infected in the NRF group (Figure A2). For this analysis we only examined mosquitoes that had a detectable titer, thus all negative values were removed. We did not find a significant difference in titer between the refed and NRF groups with either of the viruses or in either of the tissues.

**Previous blood feeding with MRE/rprORF does not appear to impact MRE3'miRT infection rates or titer**

We also wanted to examine whether initial infection with an apoptosis-inducing virus (MRE/rprORF) would allow increased midgut escape of a virus with limited midgut replication (MRE3′miRT). To achieve this, we fed mosquitoes a blood meal containing MRE/rprORF or the non-apoptosis-inducing MRE/control. After five days the mosquitoes received a second blood meal containing MRE3′miRT and 72 hours after that the mosquitoes were dissected, separating the midguts from the carcasses. Because these mosquitoes were co-infected with two viruses, and TCID50 assay would not distinguish between them, the percentage of infection with each virus as well as the viral titer was determined by qRT-PCR using virus-specific probes (Figure A3).

The percentage of midgut infection with MRE3′miRT was 40% regardless of whether the mosquito initially received a blood meal containing MRE/rprORF or MRE/control (Figure A3, upper left). Meanwhile, the percentage of infection in the carcass with MRE3′miRT was 10% in the mosquitoes that first received MRE/control and 0% in the mosquitoes that received MRE/rprORF. This difference did not reach statistical significance. The percentage of infection

in the midgut was 80% with MRE/rprORF and 100% with MRE/control (Figure A3, upper right), which was not judged to be significant. The infection rate was significantly different between MRE/rprORF and MRE/control in the carcass with 50% and 80% infection rates, respectively. This is consistent with our previous results which have suggested that apoptosis plays a negative role in midgut escape (Carpenter & Clem, 2022; O'Neill et al., 2015).

## Discussion

Our previous work has shown that poor midgut replication negatively impacts the ability of a virus to establish midgut and disseminated infection (Carpenter et al., 2021 and Chapter 2 of this dissertation). We wanted to know if mosquitoes ingested a second blood meal that was noninfectious or previous infection with an apoptosis inducing virus would impact dissemination of a virus with limited midgut replication. Our results show that a subsequent noninfectious blood meal does appear to increase rates of dissemination of a virus with limited midgut replication while previous infection with an apoptosis inducing virus does not. These factors should be considered if any treatment that works to block transmission via impacting midgut replication of a virus is developed in the future.

A previous study showed that when mosquitoes imbibe a noninfectious blood meal after ingesting a virus containing blood meal, dissemination rates are enhanced (Armstrong et al., 2020). We were therefore interested in investigating if this effect would also be seen with a virus that had reduced replication in the midgut. We had previously generated a virus that has reduced replication specifically in the midgut (MRE3′miRT) and demonstrated that this virus has a reduced dissemination rate (Chapter 2 and Carpenter et al., 2021). If feeding mosquitoes a noninfectious blood meal after feeding them with a blood meal containing MRE3′miRT

improved the dissemination rate, then this would suggest that the need for midgut replication can be overcome.

We found that mosquitoes that were fed with MRE3′miRT and then refed with a noninfectious blood meal had higher midgut and carcass infection rates compared to mosquitoes that were not refed. One reason that the carcass infection rate may be higher in the refed group is because a second blood meal may give the virus a second chance to encounter a transient tear or breach in the basal lamina. If a virus has reduced midgut replication, then it may not be able to spread from cell to cell to access a breach in the basal lamina. However, when a second blood meal is ingested, this will result in more disruptions in the basal lamina that may allow a second chance to escape from the midgut. A similar idea was proposed in Armstrong et al. (2020). This increased dissemination rate was also seen in the control virus-fed mosquitoes.

The reasons why the midgut infection rate was also enhanced in in refed MRE3′miRT mosquitoes compared to those that were not refed is unclear. It is possible that in many MRE3′miRT fed mosquito midguts the level of virus is too low to be detected by TCID50 before a second blood meal is ingested. When a blood meal is ingested there are significant changes in gene expression in many pathways including nutrient uptake, metabolism and stress responses (Sanders et al., 2003). These changes may enhance viral replication and allow detection of the virus by TCID50. Other possible explanations may exist, and further study may clarify this result.

We were also interested in seeing if prior infection with an apoptosis-inducing virus would enhance dissemination of MRE3′miRT from the mosquito midgut. Previous studies using viruses that express the pro-apoptotic reaper gene have shown that apoptosis that occurs simultaneously with virus infection has a negative effect on dissemination (Chapter 3 and

Carpenter & Clem, 2022; O'Neill et al., 2015). However, in a study which induced apoptosis in mosquitoes prior to viral infection by knocking down the anti-apoptotic gene Aeiap1, the apoptosis was significant and widespread which led to high mosquito mortality rates and which likely impacted midgut structure, coincident with increased viral dissemination (Wang et al., 2012). We wanted to see if prior infection with an apoptosis-inducing virus, which would be expected to cause less dramatic and more limited apoptosis, would increase the dissemination rates of MRE3′miRT, which has a low dissemination rate. We did not find this to be the case as mosquitoes that were first fed with MRE/rprORF and then fed with MRE3′miRT did not have increased rates of infection with MRE3′miRT or increased titers of MRE3′miRT. Therefore, it appears that widespread apoptosis induced prior to infection increases the dissemination rate, but more limited apoptosis occurring only in cells infected with the Reaper-expressing virus does not have any significant effect. Previous research has shown that only a few cells in the midgut initially get infected with a virus (Smith et al., 2008; Whitfield et al., 1973). Therefore, it is likely that in our case there was not significant overlap between the MRE/rprORF infected cells and the MRE3'miRT infected cells which could explain why we did not find a significant effect on midgut escape.

In conclusion, if replication of a virus in the mosquito midgut is to be used as a target for preventing dissemination and transmission of arboviruses, we need to understand what circumstances or conditions could potentially decrease its utility. We found that when a mosquito receives a noninfectious blood meal after a virus containing blood meal the dissemination rate increases even with a virus that has limited midgut replication. This will need to be considered as a potential limitation of using midgut replication to prevent transmission. However, prior infection with an apoptosis-inducing virus had no effect on dissemination of a virus with limited

midgut replication so it is unlikely to affect usefulness of midgut replication as a target. The more that we understand about the conditions that affect midgut escape, the better equipped we will be to deal with arbovirus outbreaks in the future.

## Methods

### Mosquito rearing

Orlando strain Aedes aegypti mosquitoes (obtained in 2008 from James Becnel, USDA ARS, Gainesville, FL) were reared in a 27°C incubator with 80% humidity and a 12-hr light-dark cycle. To obtain eggs used in experiments, females were allowed to feed on defibrinated sheep's blood (Colorado Serum Company) using a Hemotek feeding system (Hemotek Ltd.). Adult mosquitoes were maintained on raisins and water.

### Virus generation

MRE3′miRT and MRE3′control viruses were used for these experiments. Their design and construction has been described in a previous study (Chapter 2 and Carpenter et al., 2021). Also MRE/control and MRE/rprORF

### Blood feeding – refeeding experiment

Mosquitoes that were 3 days post-emergence were placed into feeding cups containing around 30 females and 3 males. The mosquitoes were only given water for 24 hours prior to blood feeding. MRE3′miRT or MRE3′control virus stocks were diluted to 2.2x108 pfu/ml and then were mixed with sheep's blood in a 1:1 ratio. The mosquitoes were then allowed to feed on one of the blood-virus mixtures for 90 minutes using a Hemotek feeding system. After 90 minutes, engorged females were separated from females that did not feed and males. They were returned to cups and were allowed to feed on water and raisins ad libitum. Four days after receiving the infectious blood meal, raisins were removed for 24 hours. After 24 hours, the

refeeding group were given a noninfectious blood meal using the Hemotek system while the non-refeeding group were not. Mosquitoes in the group provided with a non-infectious blood meal were allowed to feed for 90 minutes, after which engorged females were again separated from females that did not feed. The refed and non-refed mosquitoes were then provided with water and raisins to feed from ad libitum. After 48 hours, mosquitoes from the MRE3′miRT refed, MRE3′miRT not refed, MRE3′control refed and MRE3′control not refed groups were dissected. The midguts were dissected from the carcasses and the tissues were stored in 200µl of DMEM (Gibco) containing 10% fetal bovine serum (FBS, Atlanta Biologicals). The tissues were ground with a disposable pestle and stored at -80° C until titering by $TCID_{50}$.

**$TCID_{50}$**

BHK-21 cells were plated at a density of $1x10^4$ cells per well in a 96-well tissue culture plate in 100 µl of DMEM plus 10% fetal bovine serum and supplemented with 15 µg per ml of penicillin/streptomycin (Invitrogen). Mosquito and cell samples were removed from -80$^o$ C and thawed on ice. Samples were then spun in a microcentrifuge to remove debris and DMEM was used to make serial dilutions of each sample. Each dilution was transferred to five wells containing BHK-21 cells. After 5 days, each well was scored for cytopathic effects. The number of wells of each dilution scored as positive was used to determine $TCID_{50}$ $ml^{-1}$ and this was converted to PFU $ml^{-1}$ by multiplying by 0.69 (O' Reilly et al., 1994).

**Blood feeding – MRE/rprORF or MRE/control and MRE3′mirt**

Mosquitoes that were 3 days post emergence were placed into feeding cups containing around 30 females and 3 males. The mosquitoes were given only water for 24 hours prior to blood feeding. MRE/rprORF and MRE/control stocks were diluted to approximately the same concentration of $1.3x10^8$ pfu/ml and then mixed 1:1 with sheep's blood. Mosquitoes were

allowed to feed on one of the mixtures using a Hemotek feeding system for 90 minutes. Engorged females were then separated from females that did not completely feed. After blood feeding mosquitoes were provided with water and raisins to feed on *ad libitum*. After 4 days, the raisins were removed. One day after the raisins were removed the mosquitoes received a second blood meal containing MRE3′miRT stock that was diluted to $1.3x10^8$ pfu/ml. The mosquitoes were allowed to feed for 90 minutes after which the engorged females were separated from females that did not refeed and were provided with water and raisins to feed on *ad libitum*. After 72 hours the mosquitoes were dissected. Midguts were separated from the carcasses and tissues were homogenized using disposable pestles. Tissues were stored in 0.5 ml TRIzol reagent (Invitrogen) at -80° C until RNA extraction.

**RNA extraction and cDNA generation**

Samples stored in TRIzol were thawed on ice and then 0.1 ml of chloroform was added to each tube. The tubes were then shaken for 15 seconds and incubated at room temperature for 2 minutes. The samples were spun at 12,000xg for 15 minutes at 4° C. The aqueous phase was then moved to a new tube. RNA was precipitated by adding 0.25 ml of isopropyl alcohol and incubating at room temperature for 10 minutes. After incubation, samples were spun at 12,000xg for 10 minutes. The supernatant was then discarded, and the RNA pellet was washed with 75% ethanol twice. After washing, the RNA was resuspended in 30µl of nuclease-free $H_2O$. Samples were treated with DNase and stored at -80° C until cDNA generation. cDNA samples were generated using the ImProm-II Reverse Transcription System (Promega) using oligo dT as a primer and were diluted 1:5 before use in RT-PCR.

**qRT-PCR**

qRT-PCR was used to detect either MRE/rprORF or MRE/control and MRE3′miRT in each midgut and carcass sample. Detection was achieved using Taqman probes (Applied Biosystems). MRE3′miRT was amplified using the following primers: F-5′CTCTCGAGAAATGGGTATTAAGTAGA3′ and R-5′GTTCCTCGGTAGTACGTC3′. MRE3′miRT was detected using a custom Taqman probe that bound to part of the inserted miRT sequence (5′ACCGATCGATGAGACCACTACTCCA3′) with FAM as the fluorescent dye and MGB-NFQ as a quencher. MRE/rprORF and MRE/control were amplified with the following primers: F-5′CAGAGGAATGGTCAGCAGCAC3′ and R-5′TAATCAGACAGCGTTCTACCGTCC3′. MRE/rprORF and MRE/control were detected with a custom Taqman probe bound to part of the ubiquitin region of the inserted sequence (5′AGAAGTTGAACCATCCGATACCATCGA3′) with VIC as the fluorescent dye and MGB-NFQ as the quencher. qRT-PCR was done using an ABI 7500 instrument (Applied Biosystems). Samples and reagents were loaded into 96-well plates (Applied Biosystems) using a total reaction volume of 20µl. Taqman 2x Universal PCR master mix (Applied Biosystems) was used as the master mix. Each reaction had a primer concentration of 750nm and probe concentration of 250nm. 7µl of each 1:5 diluted sample was added to each reaction. Three technical replicates were used for each sample. Plasmids for each construct were used to generate standard curves by creating 1:10 serial dilutions. Each standard curve sample was run in duplicate. To determine the final concentration of each sample, the average of the technical replicates was plotted on the standard curve.

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# Appendix A figures

Figure A1

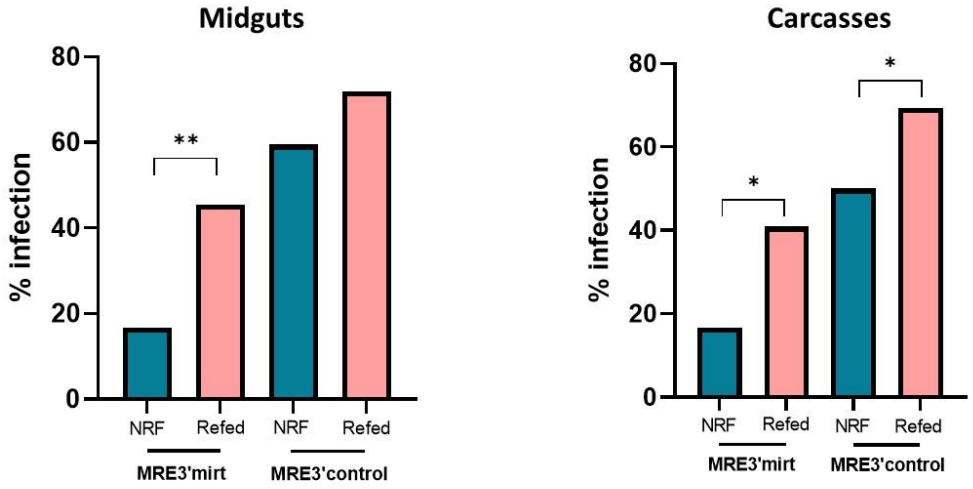


**Figure A.1 infection prevalence in refed and not refed mosquitoes with MRE3'miRT and MRE3'control**

Mosquitoes were fed with MRE3'miRT or MRE3'control. The mosquitoes then received a noninfectious blood meal (Refed) or did not (NRF). Forty-eight hours after the noninfectious blood meal, mosquitoes from all groups were dissected and the midguts were separated from the carcasses. $TCID_{50}$ was used to determine if infection was present. Fisher's exact test was used to compare infection rates between the refed and NRF groups with each virus and in both tissues. *$p<0.05$, **$p<0.01$.

Figure A2

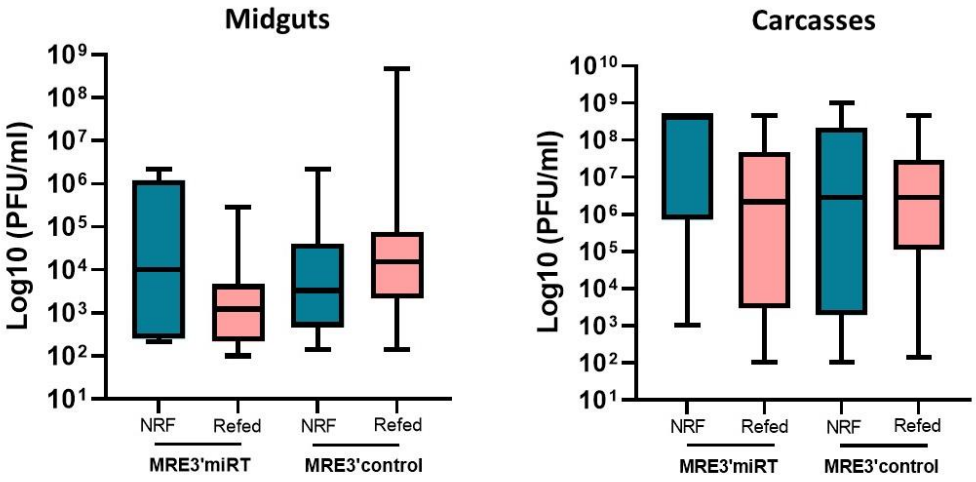


**Figure A.2 titers of MRE3'miRT and MRE3'control in infected mosquitoes that were refed or not refed with a noninfectious blood meal**

$TCID_{50}$ was used to determine the titer of virus in each group. Figure above shows titers only in mosquitoes that had a positive titer in the midgut (left) or carcass (right). Refed and NRF groups in each virus were compared using the Mann-Whitney test. No bracket indicates that a significant difference was not found.

Figure A3

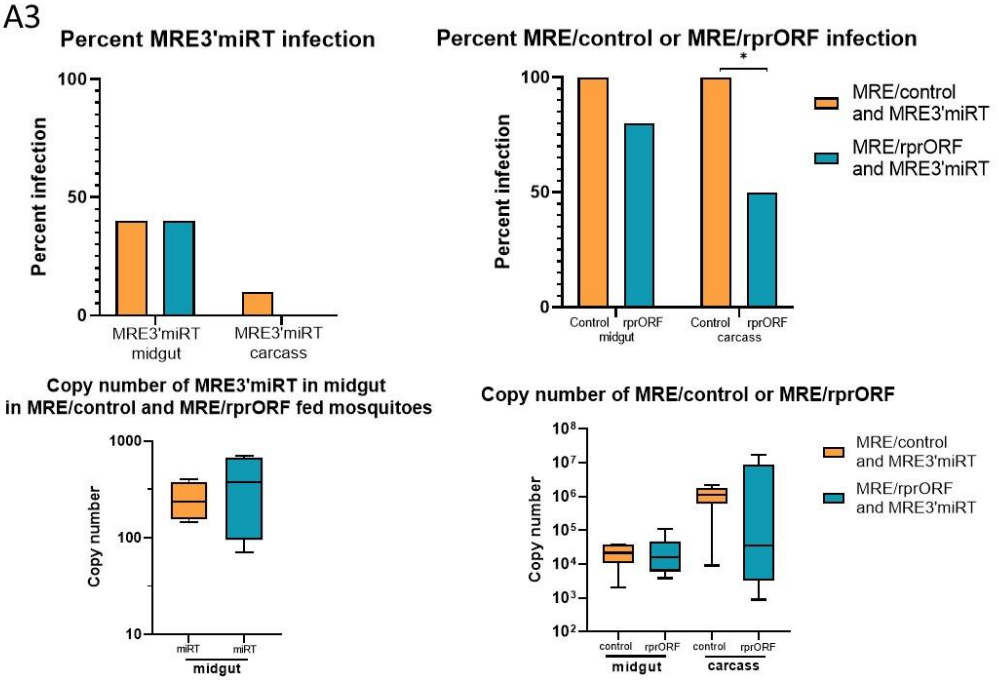


**Figure A.3 Infection prevalence and copy number in mosquitoes fed with MRE/rprORF or MRE/control and subsequently fed with MRE3'miRT**

Mosquitoes were fed with MRE/rprORF or MRE/control and then were subsequently fed with MRE3'miRT. qRT-PCR was used to determine infection prevalence (top) as well as copy number (bottom) of each virus. Infection prevalence was compared with Fisher's exact test and copy number was compared with the Mann-Whitney test. *$p<0.05$, no bracket indicates that a significant difference was not found.