2013

Arbovirus Persistence and Selection of Persistent Variants Following Chronic Infection in Aedine Mosquitoes: A Comparative Study Between Ae. Aegypti and Ae. Albopictus 30 Days Post Infection With Sindbis Virus

Zoe Leah Lyski
University of North Florida, zoelyski@lyski.net

ARBOVIRUS PERSISTENCE AND SELECTION OF PERSISTENT VARIANTS FOLLOWING CHRONIC INFECTION IN AEDINE MOSQUITOES: A COMPARATIVE STUDY BETWEEN *AE. AEGYPTI* AND *AE. ALBOPICTUS* 30 DAYS POST INFECTION WITH SINDBIS VIRUS.

By

Zoë Leah Lyski

A thesis submitted to the Department of Biology

in partial fulfillment of the requirements for the degree of

Master of Science in Biology

UNIVERSITY OF NORTH FLORIDA

COLLEGE OF ARTS AND SCIENCES

April, 2013

CERTIFICATE OF APPROVAL

The thesis submitted by Zoe L. Lyski

Approved by the thesis committee:

Date

Signature Deleted

4-3-2013

Dr. Doria F. Bowers
Committee Chair Person

Signature Deleted

4-3-2013

Dr. James J Gelsleichter

Signature Deleted

4-3-2013

Dr. Raquel Hernandez

Accepted for the Department:

Signature Deleted

4 April 2013

Dr. Dan Moon
Chairperson

Accepted for the College:

Signature Deleted

4-8-13

Dr. Barbara Hetrick
Dean

Accepted for the University:

Signature Deleted

5/13/13

Len Roberson
Dean of the Graduate School

Acknowledgements

I would like to thank my mentor Dr. Doria Bowers who supported me, always stood up for me, never gave up on me, and always reined me in when I started getting too far off subject. My committee members Dr. James Gelsleichter and Dr. Raquel Hernandez who were never too busy to answer my questions and my lab mates past and present, thanks for the good times.

I want to thank my parents who have always supported me and helped me out along the way, especially my dad who always pushes me to be the best that I can be.

Most importantly I'd like to thank my family. My husband AJ who is both my biggest critic and my biggest fan, and my three amazing sons, Gavin, Corbin, and Bear, thanks for understanding and never letting me give up, I do this for you guys.

This work was supported by NIH AREA Grant R15 AI060654-01 to Dr. Doria Bowers, tuition waiver awards from the UNF Office of Academic Affairs 2011 and 2012, and a Graduate Scholars award 2011.

Table of Contents

List of Figures and Tables

Abstract

In the present investigation two container breeding mosquitoes, *Aedes aegypti* and *Aedes albopictus* were exposed to Sindbis virus (SINV). Mosquitoes were proffered a viremic bloodmeal using a vertical membrane feeder in an attempt to infect females while preserving virus-gut interactions that are speculated to be responsible for amplification and selective roles encountered in nature. Cohorts of 50 mosquitoes of each species were exposed to SINV strain TR339 or EMEM in a bloodmeal and incubated in insectary conditions for 30 days. Once infected, the mosquito remains persistently infected for life, accumulating mutations in the virus RNA genome. Plaque size variants may indicate changes to the virus genome, therefore in this investigation TR339 persistent disseminated isolates (TR339-PDIs) were isolated from mosquito legs 30 days post infection (p.i.) and assayed for plaque size differences. These TR339-PDIs represent virus selected for by the whole mosquito. TR339-PDIs following a long term infection were used to study virus adaptations selected for during persistent infection of the mosquito host. The two mosquito species exhibited different host responses to SINV; survivorship, dissemination rates, and immunohistochemistry of mosquito midguts and hindguts were assayed at 30 days p.i. *Aedes aegypti* and *Ae. albopictus* along with SINV strain TR339 provide a good model system to study the selective pressures that occur within the whole mosquito. Greater knowledge of the mechanisms of virus persistence in the mosquito host is vital for controlling arthropod-borne disease at the vector level.

Chapter 1: Introduction

Two common container- breeding mosquitoes, *Aedes aegypti*, the Yellow Fever mosquito, and *Aedes albopictus*, the Asian Tiger mosquito are encountered worldwide. Both species are found in the United States and coexist in many urban areas of Florida (Alto et al., 2003). First detected in Harris County, Texas in 1985 (Sprenger & Wuithiranyagool 1986), *Ae. albopictus* now resides in 866 counties in 26 states, and has been dubbed the most invasive mosquito in the world (Andreadis, 2009). This invasiveness correlates to the decline and displacement of *Ae. aegypti* in rural and suburban areas (Alto et al., 2003; Gratz, 2004; O'Meara et al., 1995) therefore, *Ae. albopictus* is speculated to become the principle vector of arthropod-borne viruses (arboviruses) in the United States (Gratz, 2004). *Aedes aegypti* and *Ae. albopictus* are susceptible hosts to numerous arboviruses, including Sindbis virus (SINV), Dengue, Yellow Fever, and Eastern Equine Encephalitis viruses, many of which are emerging and re-emerging global health concerns (Delatte et al., 2010; Moore, 1997).

In nature, a blood meal is directly essential for energy and reproduction, and indirectly responsible for arbovirus transmission (Vo & Bowers, 2006). In the laboratory, blood feeding is necessary for colony maintenance and for arbovirus-host investigations. Intrathoracic inoculation is an effective way to infect insects (Rosen & Gubler 1974), however because this method bypasses potential gut barriers (Kramer et al., 1981), virus dissemination cannot be effectively studied. In the present investigation mosquitoes were infected via the oral route in an attempt to preserve the virus-gut

interactions, speculated to be responsible for amplification and selective roles encountered in nature.

Numerous techniques for blood feeding have been published (Gerberg et al., 1994; Higgs & Beaty, 1996; Jones & Potter, 1972; Lyski et al., 2011; Mourya et al., 2000; Tseng, 2003). *Aedes aegypti* is efficient at artificial membrane feeding in a laboratory setting, coined an "easy feeder" while *Ae. albopictus* has established itself as a "difficult feeder" (Lyski et al., 2011). Recent studies by Lyski and colleagues (2011) varied blood feeding presentation to promote more successful blood feeding for both species. Presenting *Ae. albopictus* with a vertical feeding position increased blood feeding success from ≤ 20% with a traditional membrane feeder (upside-down feeding presentation) to 67%. This increase in blood feeding success should translate to an overall increase of infected individuals available for investigation.

When a mosquito ingests a viremic blood meal, the infectious blood enters the lumen of the midgut where it encounters a simple monolayer of epithelial cells as well as the peritrophic membrane (Clements, 1996). Together they form a construct known as the midgut infection barrier (MIB). The MIB is considered a natural barrier to infection and transmission. Virus strains as well as host genetics are thought to play a role in the success of this barrier (Pierro et al., 2007). Several SINV studies have shown that the genetic determinants associated with midgut infection rates are associated with the E2 glycoprotein (Pierro et al., 2007). A single amino acid change within the E2 glycoprotein can alter host cell entry as well as affect pathogenesis (Klimstra et al., 1998). Once inside the midgut cells, virus replicates, amplifies, and buds from the basal aspect of the gut epithelium. From there it disseminates to secondary tissues via the hemolymph (Houk &

Hardy, 1979; Turell et al., 1984) and eventually multiplies in the salivary glands where it can be transmitted through saliva to a vertebrate host. The gut serves as a potential barrier to virus infection; presumably it also provides important steps in amplification and virus selection. There is a significant difference in midgut infection rates and midgut dissemination rates, when mosquitoes are proffered SINV, indicating the presence of a midgut escape barrier (MEB) (Khoo et al., 2010). MIB prevents SINV from infecting the midgut epithelial cells whereas the MEB prevents SINV from budding from the basal lateral aspect of the midgut, never allowing virus to escape the hemocoel to become a disseminated infection. The molecular nature of the MIB and MEB appear to be dependent on specific virus and mosquito strain combinations that have not been fully characterized or well understood (Khoo et al., 2010).

Arboviruses are usually maintained in nature by a complex continuous cycling between insect and vertebrate hosts (Fields, 2001). Arboviruses are unique, in that they are able to replicate and assemble in two unrelated genetic and biochemical environments (Hernandez et al., 2003). The arbovirus SINV belongs to the genus *Alphavirus* and family *Togaviridae*. Viruses belonging to the *Togaviridae* family are responsible for the largest variety of serious diseases worldwide and can infect many vertebrate and invertebrate hosts as well as a plethora of cultured cell lines (Strauss & Strauss, 1994). SINV is a structurally simple, positive sense, single-stranded RNA virus enclosed in an icosahedral capsid with a genome approximately 11.7kb long with a 5' cap and 3' polyadenylated tail. This nucleocapsid is further enveloped with a host-derived membrane bilayer in which the glycoproteins E1 and E2 are incorporated (Strauss & Strauss, 1994). The virion consists of three structural proteins, E1, E2, and capsid. The

structural proteins of SINV E1, E2, capsid, are encoded in a large open reading frame, then proteolytically cleaved to form capsid, PE2, 6K, and E1. They are then translocated to the endoplasmic reticulum where they are glycosylated and transported to the cell surface where PE2 is cleaved into E2 and E3. E3 is discarded in the growth medium (Presley et al., 1991). SINV is a unique hybrid structure, with glycoproteins and genomic RNA the product of viral genetic information, and carbohydrate side chains and a biological membrane that are host cell specific (Hernandez et al., 2003).

The prototype Alphavirus SINV, strain TR339, was used in this investigation. TR339 is generated from a cDNA clone of the consensus sequence of the ancestral strain AR339. The consensus sequence TR339 eliminates cell culture adapted mutations that are present in laboratory strains of AR339 (Klimstra et al., 1998). TR339 exists in a quasispecies population, where the quasispecies represents a balance between changes due to mutation and selection, a grab-bag of potential virus genotypes available to rapidly exploit new niches (Borucki et al., 2001).

In the arbovirus transmission cycle, virus is challenged in the insect vector as well as the vertebrate host. Not only does the virus cycle between vertebrate and invertebrate hosts, but also within the invertebrate host, replicating in different tissue types, such as gut epithelial cells, gut and thoracic muscles, haemocytes (Parikh, et al., 2009), fat bodies, and salivary glands (Bowers et al., 1995). Virus dissemination may begin with midgut associated visceral muscles which are an integral part of virus transmission (Bowers et al., 2003). Virus must enter cells in the midgut, replicate, amplify, and disseminate to secondary organs (Houk & Hardy, 1979; Turell et al., 1984). According to Borucki and others (2001), the majority of changes to the RNA genome occur during

selection in the midgut and these mutants then disseminate to other secondary sites of infection. Bowers and colleagues (1995) noted a widespread presence of virus antigen at 5 days p.i. following intrathoracic inoculation. Of particular interest in that investigation was the presence of virus antigen in the anterior midgut, posterior midgut, and hindgut visceral muscles. Partial virus clearance was observed in the midgut associated muscle 5-10 days p.i. At 18 days p.i. virus was still present in the hindgut. These results were observed following intrathoracic inoculation which bypasses potential gut barriers. Once a mosquito becomes infected it remains persistently infected for life (Bowers et al., 1995, 2003; Theilmann et al., 1984). Studies indicate that mosquitoes can survive for greater than 20 days in the wild (Harrington et al., 2001) and at least 90 days in a controlled laboratory setting (Doria F. Bowers, personal communication). Invertebrate cells infected with SINV display varying degrees of cytopathic effect and remain persistently infected for long periods of time, maintaining virus information. SINV replicates to a high titer (~10^8 PFU/insect) in the mosquito host by two days p.i. and then decreases to a moderate titer (~10^6 PFU/insect) where it persists and is detectable until at least day 18 p.i. following intrathoracic inoculation (Bowers et al., 1995).

The objective of the current investigation was to localize the presence of SINV antigens in the midguts and hindguts of adult mosquitoes 30 days after exposure to SINV via the oral route. This will provide further insight into the effects of an arbovirus on mosquito midgut and hindgut following the development of a persistent infection. Various tissues in the whole mosquito present unique biochemical environments and therefore different selective pressures on the virus lifecycle. SINV replication and assembly have been extensively studied in vertebrates, but much remains unknown about

these mechanisms in invertebrates. Greater knowledge of the mechanisms of virus persistence in the mosquito host is vital for controlling arthropod-borne disease at the vector level (Mudiganti et al., 2006).

The longer virus persists in the whole mosquito mutations accumulate in the virus genome (Holland et al., 1979) and greater chances are for transmission to a susceptible host. A good indication of change to the virus genome is plaque size variance. Plaque assays are conducted using sterile cell culture and are the gold standard infectious assay used to determine virus titer. A plaque is defined as the formation of a localized clearing in the cell monolayer. Each plaque is the result of one infectious virus unit. An infectious unit enters a susceptible cell, replicates, and buds or lyses, this allows progeny virus to infect nearby cells. The plaque formation is restricted to a localized clearing by an agarose overlay. Plaque assays conducted with TR339 result in uniform plaque formations, but it is expected that virus isolated from the legs of mosquitoes 30 days (D30) p.i. with SINV will exhibit varied plaque morphology. In the current investigation persistent disseminated virus was isolated from mosquito legs, D30 p.i. and assayed for plaque size differences. These samples represent TR339 persistent disseminated isolates (TR339-PDI) selected for by the whole mosquito. Original infecting virus strain may acquire mutations during the establishment of persistence. Persistent variants isolated following a long term infection will be used to study virus adaptations selected for during chronic infection in the mosquito host.

The host response is expected to differ between the two mosquito species in response to SINV. Host response will be investigated by the following; survivorship at D30 p.i., dissemination rates, and immune-labeling of mosquito midguts and hindguts to

localize SINV antigens. Differences in host response to SINV will help to further determine differences between the two mosquito species. If differences in blood feeding preference are due to anatomical or physiological differences than it is likely that host response to SINV will differ as well.

Aedes aegypti and *Ae. albopictus* along with TR339 provides a good model system to study the genetic determinants and selective pressures that occur within the whole mosquito. This invertebrate/vertebrate cycling contributes to the global spread of arboviruses in nature. When challenged with the same virus, it is expected that both mosquitoes will respond differently in terms of survivorship, viral replication, post translational processing, and potential deleterious effects on the host organism; tissue tropism.

Chapter 2: Materials and Methods:

Hatching and Rearing of Mosquitoes

Aedes aegypti (Orlando) and *Ae. albopictus* (Skuse) were hatched, reared and maintained in the University of North Florida insectary under standard conditions, 25.5± 0.5^{o}C, 70-80% relative humidity, and 16:8 (light: dark) photoperiod as previously described (Bowers et al., 1995). Mosquito eggs were hatched in 1.0% nutrient broth. First instar larvae were distributed approximately 300 per pan in 1.5L tap water and fed a 2% liver powder suspension three times a week. Following pupation, adults were housed in white bucket cages, no more than 300 per cage, topped with mosquito netting, supplied *ad lib* honey soaked cellucotton (carbohydrate source) and water saturated cotton (hydration source). Males and females were allowed to cohabitate in cages for 5-7 days post-emergence to allow adequate time for mating.

Artificial Blood Feeding

Fifty females, 5-7 days post emergence were used in all experiments. Females were separated from males 48 hours prior to blood feeding and deprived of carbohydrate source 24 hours prior to blood feeding. A vertical membrane feeder, as previously described by Lyski and colleagues (2011), was used to administer blood meals, with fresh collagen sausage casing (22 mm) as the membrane source (The Sausage Maker Inc., Buffalo, New York). Sausage casings were filled with 20mL warmed (37^{o}C) defibrinated bovine blood (Colorado Serum Company, Denver, Colorado), clamped taunt with dialysis clamps and suspended vertically in mosquito cage. Mosquitoes were proffered blood, and allowed to feed for one hour. After an hour blood was removed, and females

were aspirated and cold anesthetized for observation under dissection microscope. Engorged females were counted and returned to cages equipped with a fresh carbohydrate supply and water for hydration. Eggs were collected on oviposition sites and stored in humidity chambers under insectary conditions to ensure proper embryonation (Clements, 1992).

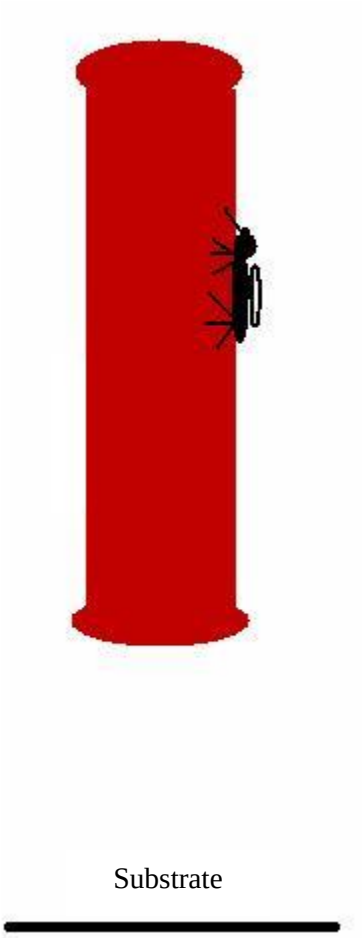

Figure 1. Model of vertical blood feeding apparatus as previously described by Lyski et al., 2011.

Virus Infection of Mosquitoes

Mosquitoes were exposed to SINV through viremic blood meals, utilizing the natural (oral) route of infection. Female mosquitoes, 5-7 days post-emergence were used for all experiments. Mosquitoes were starved of carbohydrate source 24 hours prior to blood feeding. Eighteen (18) mL warmed (37^{o}C) defibrinated bovine blood (Colorado Serum Company, Denver, Colorado) and 2mL SINV strain TR339 at a titer of 10^{8} PFU/mL were added to a 22mm collagen sausage casing (The Sausage Maker Inc., Buffalo, New York) methods previously described by Lyski and colleagues (2011). Control mosquitoes were fed mock infected blood meals containing 18 mL defibrinated bovine blood and 2 mL of Eagle's minimum essential medium (EMEM). Mosquitoes were allowed to feed for 1 hour, aspirated from cages, cold anesthetized and inspected for engorgement. Engorged females were returned to cages and supplied fresh carbohydrate and water sources as described above.

Cell Culture

Vertebrate cell culture

Cultured BHK-21 cells were grown at 37º C, 5% CO_2 in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 10% tryptose phosphate broth, 20ug/ml Gentamycin (MEM-C; Renz & Brown, 1976). Cells were lifted with 0.25% Trypsin and split every 96 hours. Cultured cells were maintained in 25 cm^2 flasks until needed.

Invertebrate cell culture

Cultured C7-10 (*Ae. albopictus*) cells were grown at 28º C, 5% CO_2 in EMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 10% tryptose phosphate broth, 20ug/ml Gentamycin. Cells were split every 96 hours and maintained in 25 cm2 flasks until needed.

Leg Assay and Measure of Cytopathic Effect (CPE)

At D30 p.i. legs were removed from surviving females, placed in sterile cryovials and stored at -80^{o}C. BHK-21 cells were cultured at 37º C in EMEM, supplemented with 20ug/ml Gentamycin and 2.5 ug/ml Amphotericin-B and plated in 6-well plates. Pre-confluent BHK-21 cells were challenged with aliquots of virus samples (positive control) or a single leg removed from an individual mosquito. The absence or presence of viable virus in the sample or leg was scored directly by the absence or presence of CPE at 72-hour p.i. of BHK-21 cells. Cells were observed through a Leica microscope at 10X objective. CPE is characterized by the presence of rounded cells, loss of adhesion to substrate, along with gaps in the cytoplasm and is an indication of cell lysis. Detection of CPE in a leg assay is due to the presence of virus in the hemolymph and indicates a disseminated infection in the adult mosquito. In wells where CPE was present media was removed and stored in -80^{o}C for further analysis.

Clonal Selection and Plaque Assays

Plaque assays were used to determine titer, in plaque-forming units (PFU) per leg isolated from mosquitoes D30 p.i. In this assay, virus from a single infected cell infects nearby cells, but the infection remains localized to the original site of infection by an

agarose overlay. A plaque is identifiable by a localized clearing or plaque that consists of lysed cells (Hernandez et al., 2005). Pre-confluent BHK-21 cells were challenged with 200 µL of a known dilution of persistent disseminated isolates (TR339-PDI), virus isolated from CPE leg assays. Individual plaques were observed, counted, photographed, measured, and identified as small (<3mm), medium (3-6mm), or large (>6mm). Viral titer was calculated and plaques were picked under sterile conditions. This was done using a sterile Pasteur pipette to pick a plug from the center of the plaque. The plaque plug was placed in viral growth medium (VGM), 97% PBS/3% fetal calf serum, and stored at 4^{o} C for 72 hours, to allow virus to diffuse from plaque plug. Following incubation TR339-PDIs were amplified in BHK-21 and C7-10 cells. The virus isolated from the individual plaques was frozen and kept for further analysis.

Amplification of TR339 Persistent Disseminated Isolates (TR339-PDI)

TR339-PDIs were amplified in both vertebrate (BHK-21) and invertebrate (C7-10) cells. TR339-PDI samples were diluted in VGM, applied to pre-confluent cells in $150cm^3$ flask and allowed to adsorb at room temperature on a platform rocker at low speed, for 1 hour. Following incubation EMEM was added to the flask to bring the total volume up to 3 mL. Cells were returned to incubators, 37^{o}C for BHK-21 and 28^{o}C for C7-10 cells, and monitored for signs of CPE beginning at 48 hours p.i. Once CPE was observed virus was harvested by removing the supernatant containing virus particles. This was centrifuged at 10,000 rpm for 10 minutes, and supernatant, free of cell debris, was aliquoted and stored at -80^{o}C. Plaque assays were run on amplified TR339-PDIs, titer and plaque morphology were determined.

TR339 → Whole mosquito → CPE leg assay (BHK-21 cells) → Plaque assay (BHK-21 cells) → Amplify plaques (BHK-21 and C7-10 cells) → Plaque assay (BHK-21 cells)

Figure 2. Flow chart depicting isolation of disseminated persistent isolates.

SINV Infected Mechanical Mosquito: Virus Viability without Amplification

To mimic a SINV infection in the mosquito host and determine viability of SINV without amplification in gut and secondary tissues, a mechanical mosquito was devised. Samples of SINV consensus sequence strain TR339 and heat resistant strain SVHR were diluted 1:100 in either synthetic insect hemolymph or sterile VGM. Diluted samples were placed in cryovials (1 mL each), wrapped in parafilm, placed on rocker and incubated under insectary conditions, 25.5± 0.5^{o}C, 70-80% relative humidity, and 16:8 (light: dark) photoperiod, with constant movement for 30 days. Following 30 day incubation, titers were determined using plaque assay techniques previously described and compared to initial titer of stock virus.

Figure 3. Image of mechanical mosquito.

Localization of SINV Antigens in the Midgut of *Ae. aegypti* and *Ae. albopictus*

At 30 days p.i. mosquito midguts and hindguts were dissected out of cold-anesthetized mosquitoes, both TR339 virus exposed and mock exposed individuals. Guts were dissected directly onto microscope slides, allowed to air dry for several minutes, fixed in ice cold 100% acetone solution for 3 minutes and stored in -80^{o}C until further processing. At time of labeling slides were rehydrated in 1X PBS for 10 minutes, then blocked in 10% BLOTTO (Johnson et al., 1984) for 1 hour. After several rinses in 1X PBS, rabbit polyclonal Anti-SINV 1:100 dilution in 10% BLOTTO was added to each slide (200 µl per slide) and allowed to incubate overnight. Following incubation slides were rinsed in 1X PBS then, secondary antibody, FITC-conjugated goat anti-rabbit IgG at a 1:40 dilution in 10% BLOTTO was added to each slide (200 µL per slide) and allowed to incubate at room temperature in the dark for 1 hour. Following incubation the tissue was washed in 1X PBS and slides were mounted with Vecta Shield and observed using an Olympus BX60 epifluorescence microscope with a digital SPOT program following protocols previously described by Bowers and others (1995, 2003).

Chapter 3: Results and Discussion

Survivorship of Mosquitoes at D30 Post Infection

Survivorship of female *Ae. aegypti* and *Ae. albopictus* mosquitoes were investigated at 30 days following exposure to either a viremic bloodmeal containing TR339 or a mock infected bloodmeal containing EMEM (negative control). Survivorship was calculated based on the number of mosquitoes alive at D30 compared to the number of mosquitoes that bloodfed at the time of blood feeding (Table 1). Results indicate that mock exposed mosquitoes had reduced survivorship when compared to TR339 exposed mosquitoes. *Aedes aegypti* exhibited higher rates of survivorship in both groups when compared to *Ae. albopictus*. In the mock exposed group, *Ae. albopictus* exhibited a higher survivorship through the first 20 days, however at D30 *Ae. aegypti* had a higher survivorship rate (Fig. 4). For the first 13 days, TR339 exposed *Ae. aegypti* and *Ae. albopictus* both exhibited 100% survivorship. At D30 *Ae. aegypti* had a higher survivorship 94% when compared to *Ae. albopictus*, which had a 63% survival rate at D30 (Fig. 5).

Table 1. Survivorship at D30 following exposure to TR339 or EMEM in a bloodmeal.

	Survival (%)
Ae. aegypti	
TR339 exposed	93 (39/42)
Mock exposed	74 (35/47)
Ae. albopictus	
TR339 exposed	63 (10/16)
Mock exposed	40 (6/15)

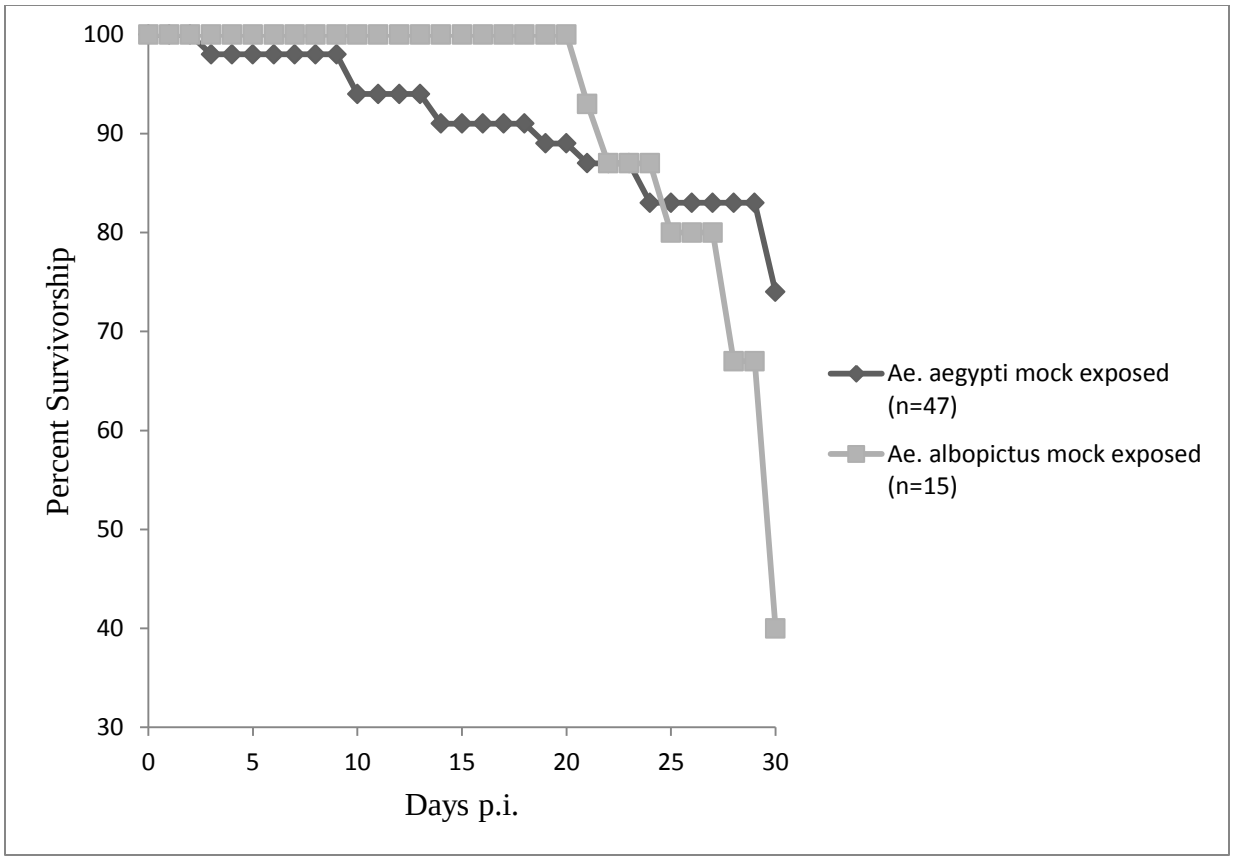

Figure 4. Survivorship of mock exposed (control) *Ae. aegypti* compared to *Ae. albopictus* D0 (time of infection) until D30.

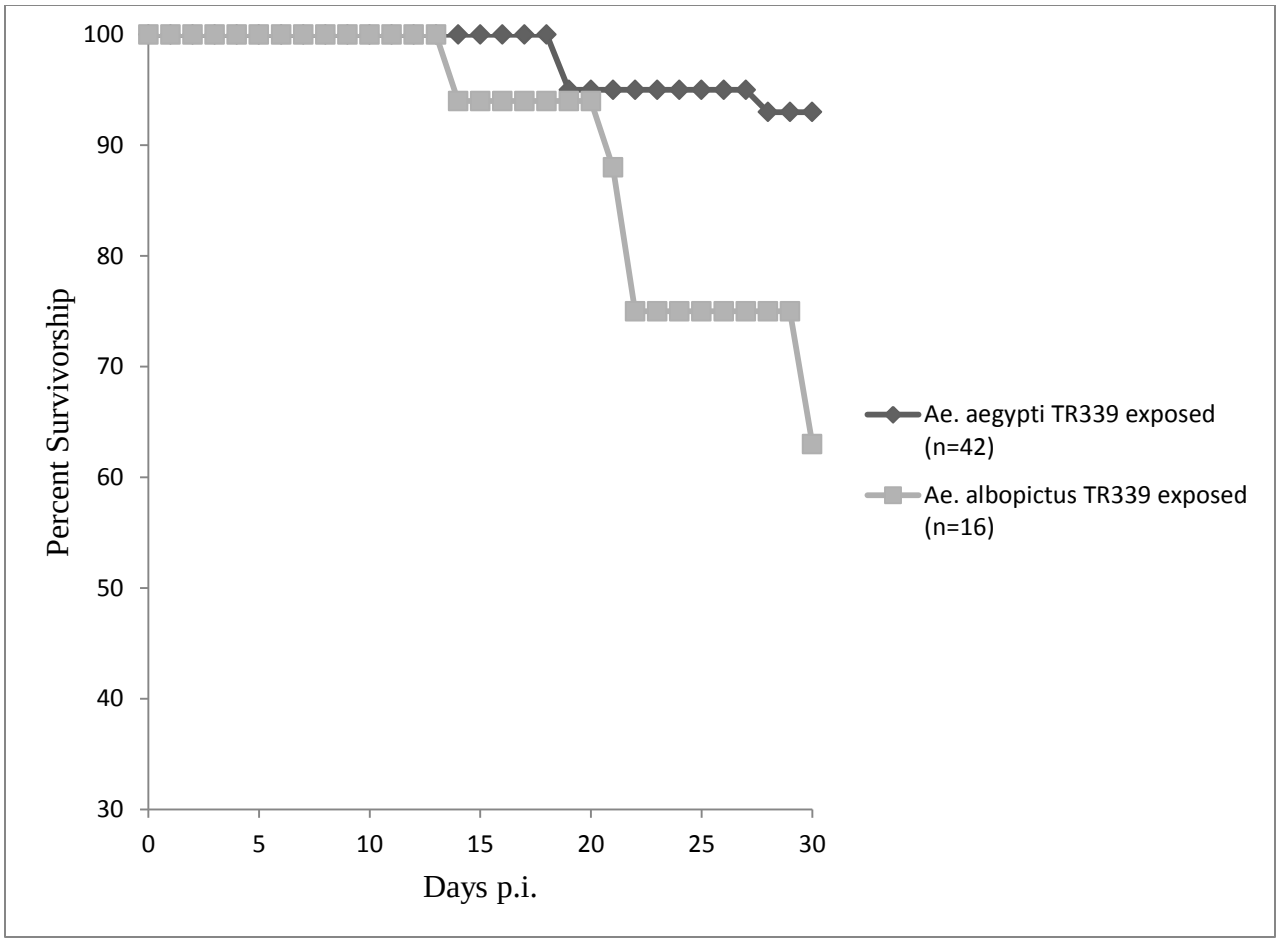

Figure 5. Survivorship of SINV exposed *Ae. aegypti* compared to *Ae. albopictus* D0 (time of infection) until D30.

Dissemination of TR339 at D30 Post Infection

Virus dissemination at D30 following exposure to an infectious bloodmeal was analyzed. A disseminated infection was determined by the presence of infectious virus particles in the hemolymph. This was detected using a CPE leg assay (Turrell, et al., 1984) at D30 p.i. and percent dissemination was calculated (Table 2). Mock infected individuals never exhibited CPE. It was determined that, *Ae. aegypti* displayed a lower dissemination rate than *Ae. albopictus,* at D30 p.i.

Table 2. Dissemination rates of TR339 in *Ae. aegypti* and *Ae. albopictus* at D30 p.i..

	Bloodmeal titer	Dissemination (%)
*Ae. aegypti**	7.5×10^7	15 (7/47)
*Ae. albopictus**	7.5×10^7	20 (3/15)

*Dissemination in mock exposed not detected

Isolation of TR339 Persistent Disseminated Isolates (TR339-PDIs)

TR339-PDIs are defined as; Persistent Disseminated Isolates of TR339 virions that have been selected for by the whole mosquito. During 30 days in the mosquito, TR339-PDI virions were exposed to all permissive tissues. TR339-PDIs isolated from a single mosquito leg contained multiple virus phenotypes as depicted in Figure 6A which shows two different plaque morphologies, a large and medium plaque. Once plaques were sterilely picked and amplified the resulting plaques were of uniform size and shape (Fig. 6B), indicating a single virus phenotype. Additionally stock TR339 exhibited uniform plaque morphology, after incubating in the mechanical mosquito for 30 days, in the absence of cellular amplification (Table 3).

Table 3. Plaque morphology and titer of TR339-PDIs isolated after 30 days in whole mosquito compared with characteristics of stock TR339.

Virus	Initial titer on BHK cells (pfu/mL)	Plaque size Diameter (mm)	Titer on BHK after amplification in BHK cells (pfu/mL)
TR339 (Stock)	7.5 X 10^8	Medium 5.0	NA*
TR339-PDI Ae 20**	6.0 X 10^5	Large 8.0	4.0 X 10^5
		Large 7.8	2.0 X 10^5
		Medium 4.8	1.0 X 10^5
		Medium 3.5	3.0 X 10^5
TR339-PDI Al 13***	4.4 X 10^5	Medium 4.8	2.2 X 10^5
		Medium 4.5	1.8X 10^5
		Small 1.2	NA*
		Small 1.0	NA*

*NA – Not assayed

** - PDI isolate from *Ae. aegypti*

*** - PDI isolate from *Ae. albopictus*

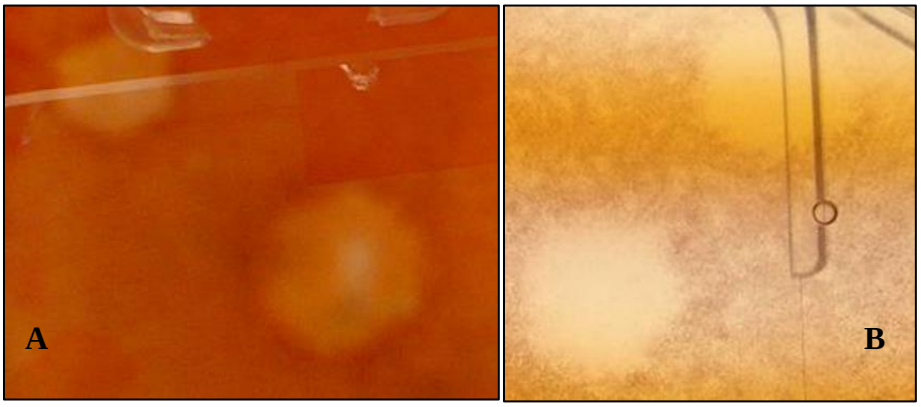

Figure 6. (A) Large and medium plaque size variants isolated from *Ae. aegypti* leg D30 p.i. with TR339, exhibiting plaque size variance an indication of different virus phenotypes. (B) Large plaques sterilely picked from (A), after amplification of TR339-PDI in BHK-cells, single plaque morphology indicates consistent virus phenotype.

SINV Infection of Mechanical Mosquito

SINV consensus sequence strain TR339 and heat resistant strain SVHR were used to investigate the viability of virus under insectary conditions, without cellular amplification for 30 days. Results indicate that Sindbis strain TR339 survives and maintains titer for 30 days at 25^{o} C (Table 4), however with SVHR samples, the virus titer decreased by 2 logs. The different solutions, haemolymph or VGM, appeared to have little effect on the survivability of both virus strains.

Table 4. Virus titers on BHK cells, after 30 days in mechanical mosquito, under standard Insectary conditions with constant motion.

Virus	Starting titer	Titer D30 hemolymph	Titer D30 VGM
TR339	3.3 X10^{5}	1.7 X10^{5}	2.5 X10^{5}
SVHR	4.5 X 10^{8}	7.0 X10^{6}	3.0 X 10^{6}

SINV Localization in TR339 Exposed Mosquitoes Exhibiting a Disseminated Infection

Localization of TR339 infection was detected by immunofluorescence labeling of SINV antigen on whole-mount tissue preparations of both *Ae. aegypti* and *Ae. albopictus* midguts and hindguts at D30 p.i. (Figs. 7-11). TR339 was detected in the midgut epithelium as well as peristaltic muscle bundles of orally infected *Ae. aegypti* at D30 p.i. (Fig. 7). All *Ae. aegypti* mosquitoes with positive CPE leg assays had extensive SINV labeling in both horizontal and longitudinal muscle bundles (Fig. 8). In *Ae. albopictus* mosquitoes exhibiting a positive CPE leg assay, SINV antigen was localized in isolated

patches of epithelium at 30 days p.i. (Fig. 9). Virus antigen was also observed in the gut associated peristaltic muscles (Fig. 10), however not as prominent as that seen in *Ae. aegypti*.. Virus antigen was not detected in tissues from the control mock-infected group (Fig. 11). Differences in SINV localization between species is described in Table 5.

Table 5. Gut tropism in adult female mosquitoes at D30 p.i. following a disseminated infection with TR339. (+) indicates the presence of SINV antigens, (-) indicates the absence of SINV antigens.

Ae aegypti	**Epithelium**	**Muscle bundles**
Ae 1	+	++
Ae 4	+	++
Ae 13	+	++
Ae 60	-	++
Mock	-	-
Ae albopictus		
Al 1	+ patchy	-
Al 12	+	+
Mock	-	-

++ Extensive muscle banding

+ Less prominent muscle banding

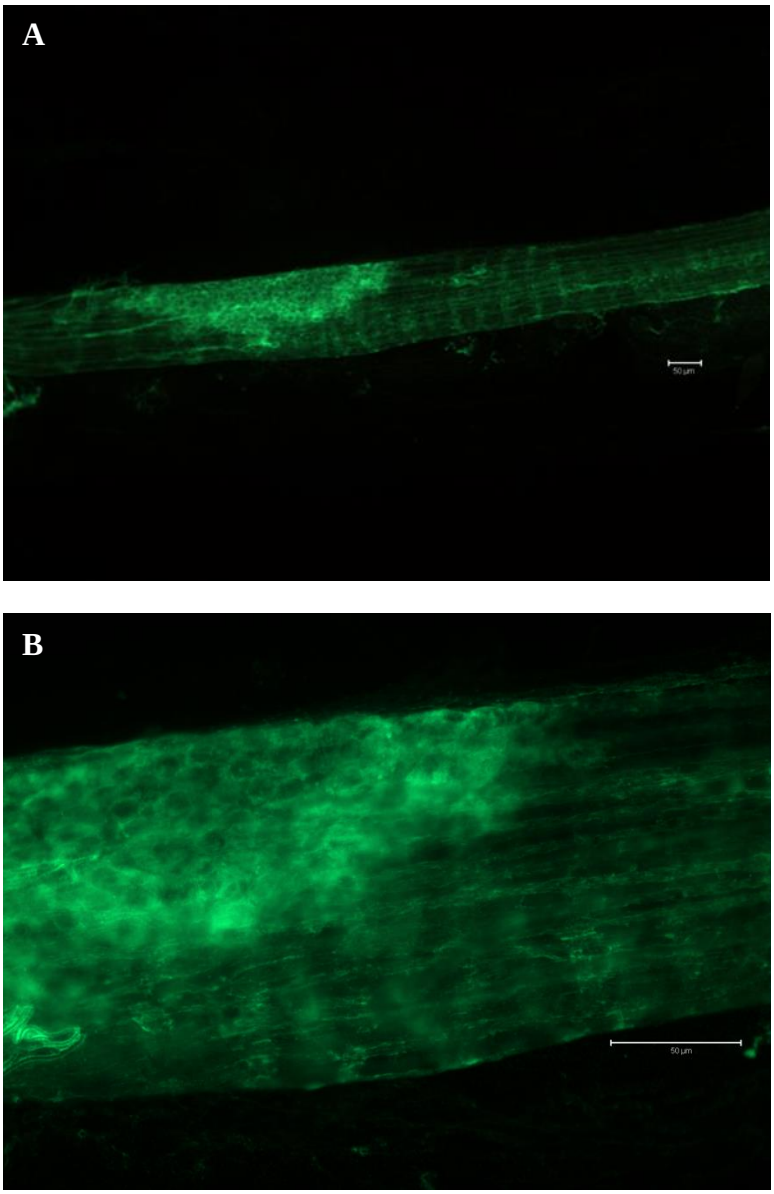

Figure 7. Immune-labeling of *Ae. aegypti* midgut dissected at D30 p.i. with TR339. (A) Note staining of epithelial patches and muscle banding. (B) Higher magnification of the same epithelial patch. Magnification bar 50µm.

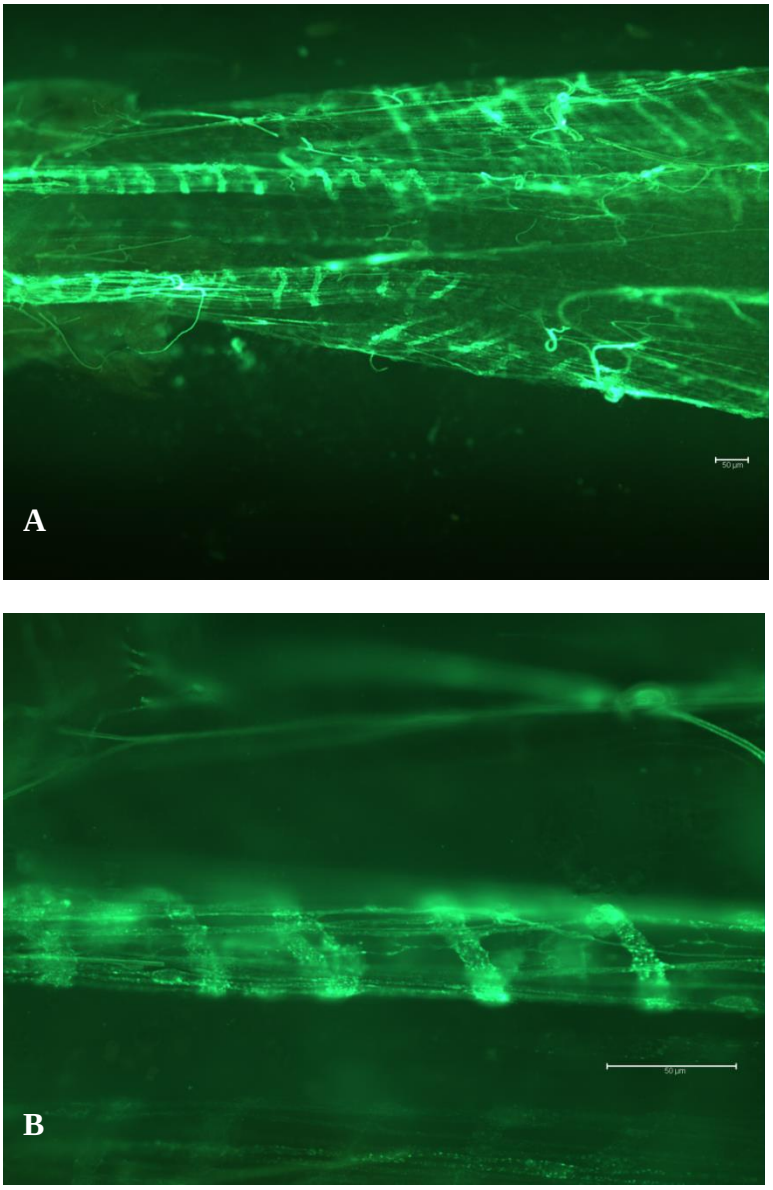

Figure 8. A&B Immune-labeling of *Ae. aegypti* midgut dissected at D30 p.i. with TR339. Note staining of horizontal and longitudinal muscle bands. Magnification bar 50µm.

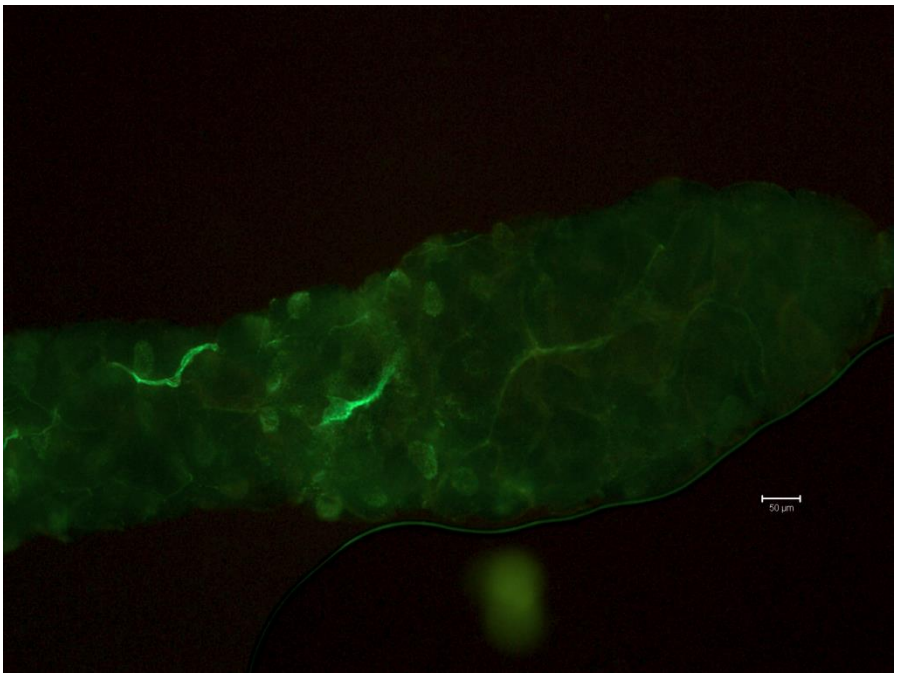

Figure 9. Immune-labeling of *Ae. albopictus* midgut dissected at D30 p.i. with TR339. Note staining of epithelial patches throughout the midgut. Magnification bar 50µm.

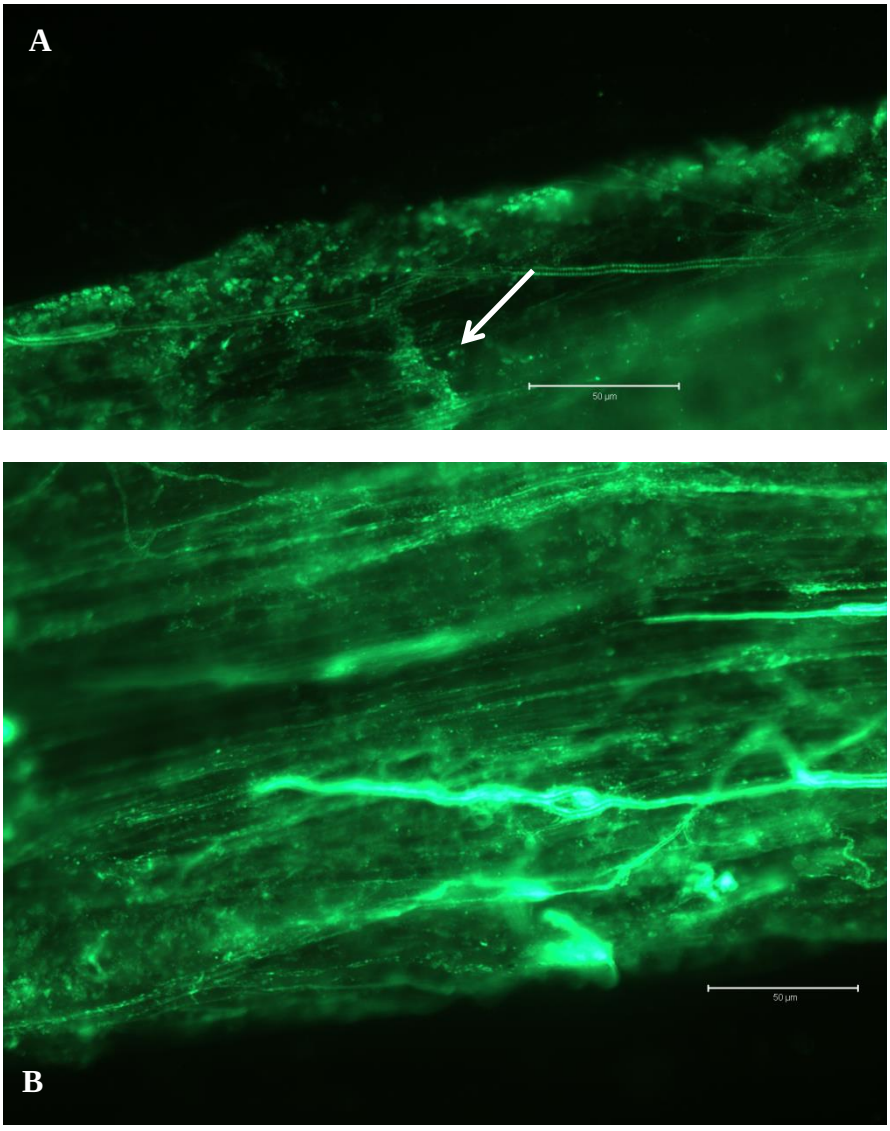

Figure 10. Immune-labeling of *Ae. albopictus* midgut dissected at D30 p.i. with TR339. Note staining of gut peristaltic muscles throughout the midgut. (A) Arrow indicates horizontal muscle band. (B) Note longitudinal muscle fibers labeling for the presence of SINV antigens. Magnification bar 50µm.

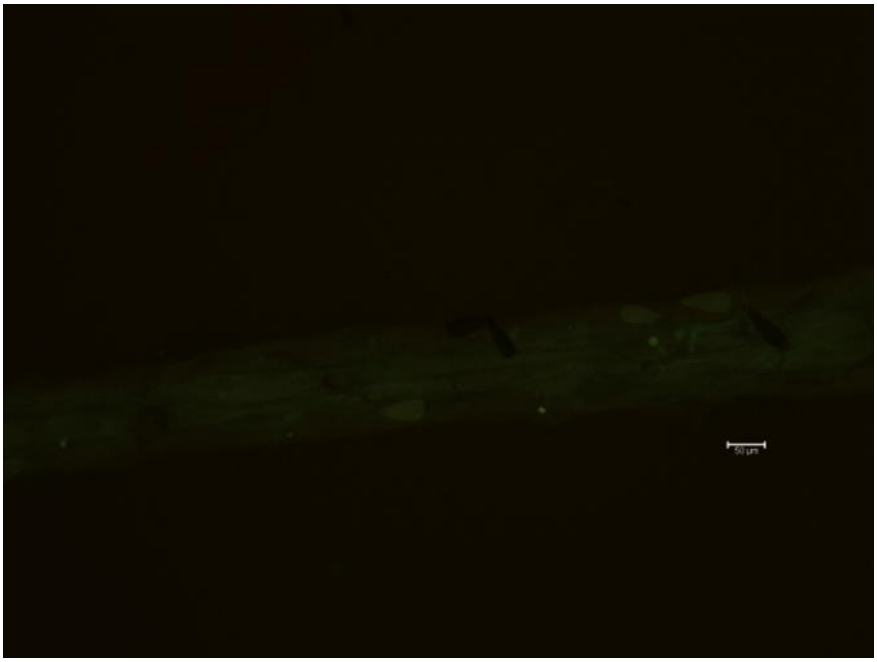

Figure 11. Control *Ae. aegypti* midgut dissected D30 following mock infection with EMEM. Note an absence of SINV antigens. Magnification bar 50µm.

SINV Localization in TR339 Exposed Mosquitoes Exhibiting a Non-Disseminated Infection

Some *Ae. aegypti* and *Ae. albopictus* mosquitoes exposed to TR339 through a viremic bloodmeal, had negative CPE leg assays at D30 p.i. This indicates either an uninfected mosquito or mosquito with a non-disseminated infection. In some cases, the mosquito had a negative leg assay; however SINV antigens were identified trapped inside cells within the hindgut (Fig. 12). Figure 13 depicts an unknown globular structure and stippling where SINV antigens were localized within a non-disseminated *Ae. albopictus* mosquito. This phenomenon was not observed in mock infected mosquitoes. Figure 14 shows a TR339 exposed, but uninfected mosquito midgut. There was no sign of SINV antigens in the midgut or hindgut.

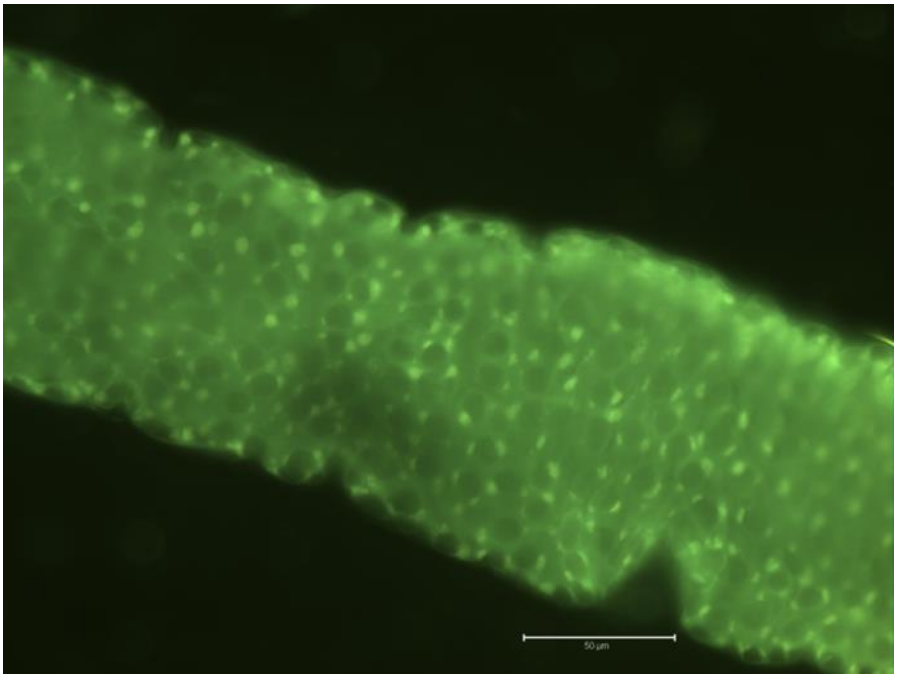

Figure 12. Immune-labeling of *Ae. aegypti* midgut dissected at D30 p.i. with TR339. Note staining of SINV antigens localized within cells in the hindgut of a non-disseminated mosquito. Magnification bar 50µm.

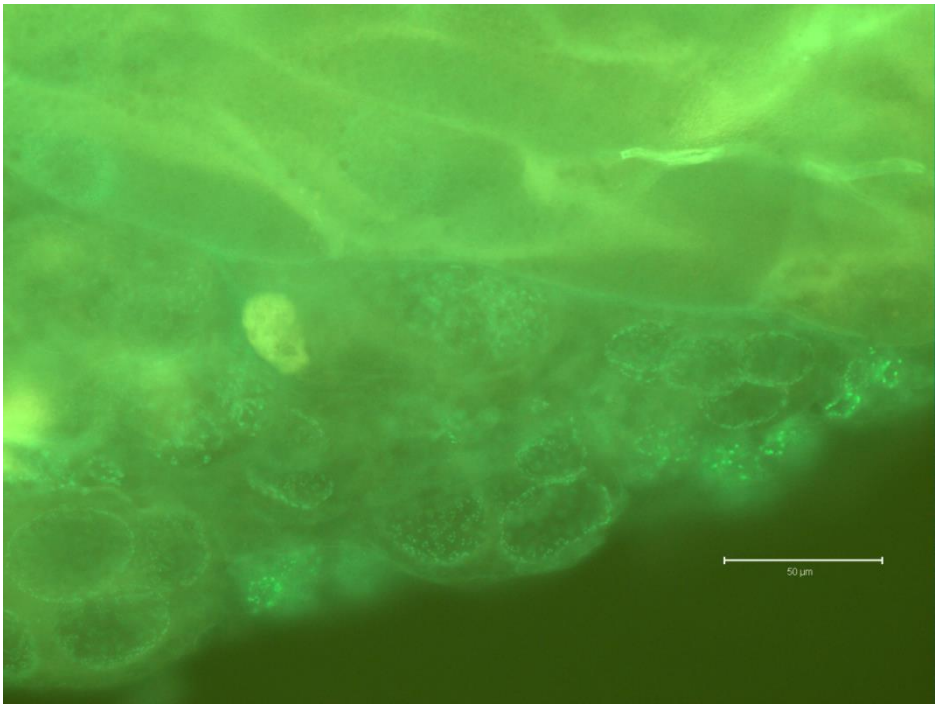

Figure 13. Immune-labeling of *Ae. albopictus* midgut dissected at D30 p.i. with TR339. Note staining of SINV antigens localized in an unknown structure. Magnification bar 50µm.

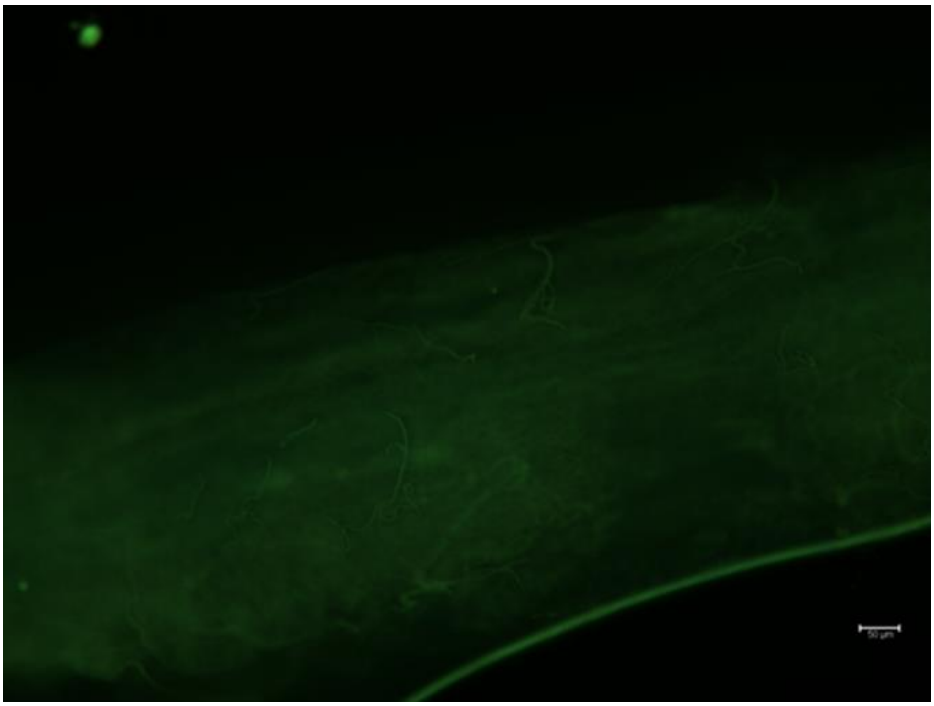

Figure 14. Immune-labeling of *Ae. aegypti* midgut dissected at D30 p.i. with TR339. Note the absence of staining of SINV antigens, indicating a non-disseminated infection. Magnification bar 50µm.

Discussion

Lyski and others (2011) established differences in blood feeding behavior between two similar species of container breeding mosquitoes, *Ae. aegypti* and *Ae. albopictus.* In this investigation, additional differences have been demonstrated that further distinguish these two *Aedine* species. Survivorship differences between species were observed. Greater survivorship was exhibited in *Ae. aegypti* when compared to *Ae. albopictus* at D30 p.i. with SINV, an observation which is consistent with previous studies (Reiskind, et al., 2010). Greater survivorship was observed in *Ae. aegypti* after exposure to both mock and TR339 infected bloodmeals when compared to *Ae. albopictus*. All groups with the exception of TR339 exposed *Ae. aegypti* exhibited an increase in mortality on D30 p.i.. However, if survivorship was assessed at D25, *Ae. albopictus* survival was 75% in both virus-exposed and mock-exposed. While only five days less, twenty five days of possible dissemination culminates in many days of potential transmission of virus in nature.

In the current investigation *Ae. albopictus* exhibited a greater rate of SINV dissemination, indicating that perhaps the higher rate of dissemination comes at a cost to the mosquito host. This is consistent with Reiskind and colleagues (2010), whom observed increased mortality and greater dissemination in *Ae. albopictus* after infection with Chikungunya virus. In the present investigation, after exposure to TR339 both species survivorship remained at 100% until D13 p.i., throughout the extrinsic incubation period, increasing the likelihood of a potential transmission event.

Differences in dissemination rates indicate differences in host response to the same virus variant. Dissemination differences between similar mosquitoes species has

been documented (Reiskind et al., 2010), where *Ae. albopictus* had a higher rate of dissemination after exposure to Chikungunya virus when compared to *Ae. aegypti*. Higher dissemination rates along with greater survivorship can greatly influence the vector capacity of a mosquito species.

The results presented here indicate that replication of SINV in whole mosquitoes for 30 days, can result in the generation of virus variants and indicates that mosquito adapted variants may arise in the field after persistent infection in a mosquito host. Evaluating selective pressures to virus adaptation are important in understanding virus-host interactions and predicting emergence and reemergence of important vector borne viruses. Studies evaluating arbovirus host interactions and virus adaptations *in vivo* systems are largely lacking. These are important to better understand selective forces that drive arbovirus evolution (Ciota et al., 2008). In order to evaluate virus and host specific adaptations phenotypic changes to SINV were evaluated following persistent SINV infection in *Ae. aegypti* and *Ae. albopictus* mosquitoes. Recent endemics of West Nile virus and Chikungunya virus were driven by small genetic changes that greatly increased transmission efficiency (Ciota et al., 2008). This demonstrates the importance of mosquitoes in shaping arbovirus populations.

Little is known about persistent infections within the whole mosquito, but persistent infections in cultured invertebrate cells are commonly associated with temperature sensitive, small plaque variants. (Brown & Condreay, 1986; Davey & Dalgarno, 1974; Igarashi et al., 1977; Renz & Brown, 1976; Shenk et al., 1974). Shenk and colleagues (1974) detected SINV producing small, irregularly shaped plaques measuring 1-2 mm in diameter after several weeks in cultured *Ae. albopictus* cells. This

is noticeably different from the original infecting stock virus which produced large round plaques averaging 8mm in diameter. In addition to small plaque size, the variants were also temperature sensitive, suggesting that the lower temperature at which the mosquito cells were maintained may favor the accumulation of temperature sensitive mutants (Shenk et al., 1974). These same variants failed to replicate efficiently in vertebrate cells at 41^{o}C. It was hypothesized that small plaque, temperature sensitive variants may contain changes to either glycoprotein E2 or its precursor PE2 (Eaton, 1982). In the present investigation, large and medium plaques were isolated from *Ae. aegypti* legs and medium and small plaques were isolated from *Ae. albopictus* legs D30 p.i. with TR339. These TR339-PDIs were grown in vertebrate (BHK) cells at 37^{o}C and in invertebrate (C7-10) cells at 28^{o}C. While these small plaque TR339-PDIs resulted in pinpoint plaques once amplified, a definitive plaque assay was not achieved after titering on BHK cells at 37^{o}C indicating possible temperature sensitivity.

The selection of small plaque SINV variants in our model system was also observed in *Culex* mosquitoes (Theilmann et al., 1984). In addition to persistent studies using cultured cell lines, a study by Theilmann and others (1984) characterized variants that were isolated during persistent SINV infection in *C. tarsalis*. After two consecutive 35 day passages in *C. tarsalis* mosquitoes, 4-7% of isolates were temperature sensitive and failed to replicate in vertebrate cells at 41^{o}C, and 44% of the isolates differed from the wild type virus on the basis of plaque morphology. After three 35 day replication cycles, 9 out of 10 isolates differed from wild type virus in plaque morphology in both vertebrate and invertebrate cells, and 7 out of 10 were temperature sensitive and RNA-. It

appears that variants appear much more slowly *in vivo* than in cultured cells and phenotypic differences take time to establish themselves in the population.

In the present investigation, wild-type (stock) TR339 was proffered to female *Ae. aegypti* and *Ae. albopictus* mosquitoes and allowed to replicate in the mosquito host for 30 days. TR339-PDIs were persistently disseminated isolates harvested from mosquito legs at D30 p.i. From these isolates large, medium, and small plaques were isolated from a plaque assay on vertebrate cells. These plaque size variants represent virus phenotypes different from wild-type TR339 and are an indication of changes to the virus genome brought on by selection in the whole mosquito. According to Pierro and others (2007) as few as 2 single amino acid changes have been shown to greatly affect midgut infection rates in *Ae. aegypti* and a single amino acid change can greatly affect plaque size variation (Pierro, et al., 2007). These mosquito adapted isolates had a titer of ~10^6 PFU, consistent with Bowers and others (1995) who found moderate titers in persistently infected mosquitoes D18 p.i. with SINV via intrathoracic inoculation.

Genetic determinants of midgut infection are often linked to the E2 glycoprotein (Pierro, et al., 2007) which is believed to house a cell receptor binding domain. Borucki and others (2001) determined that 75% of substitutions occurred during infection of the midgut. Once progeny virions escape the midgut these mutants are presumably free floating in the hemolymph and available for infecting secondary tissues such as salivary glands, fat bodies (Bowers, et al., 1995), and haemocytes (Parikh, et al., 2009), where they undergo replication in secondary tissues and potentially encounter additional selective pressures that further alter the virus genome.

Cell type used in virus amplification has also been shown to have a great effect on midgut infection rates (MIR). Pierro and others (2007) fed C6/36 (*Ae. albopictus* cell line) propagated virus to mosquitoes and noticed a decrease in MIR. This was conducted to mimic the spread of infection between midgut cells. The effect of cell type amplification on MIR indicates the importance of viral protein processing. In this investigation, TR339-PDIs were isolated from CPE leg assays on BHK-21 cells, and then amplified in both C7-10 (invertebrate) and BHK-21 (vertebrate) cells.

In addition to persistent infection in the mosquito host for 30 days, a mechanical mosquito was devised to mimic a persistent infection without cellular amplification. Can disseminated virions survive for 30 days in the mosquito microenvironment without cellular amplification? This experiment provided the investigator with a plaque size variance control as well as offered insight into virus viability during a chronic infection. Large, medium, and small plaque size variants were isolated from mosquitoes at D30 p.i. indicating multiple virus phenotypes presumably due to cellular amplification and selection. After 30 days in the mechanical mosquito no variance in plaque morphology was observed, D30 virus exhibited plaque size morphology like that of the stock virus. The virus persisted in the mechanical mosquito under insectary conditions; indicating that virus can exist freely in the haemolymph, without cellular amplification. Presumably the same virus that crosses the midgut escape barrier can float along in the hemolymph maintaining titer until it encounters secondary targets such as haemocytes, fat bodies, salivary glands and/or midgut peristaltic muscles as indicated by this study. This observation supports the concept that while persistence is not necessarily dependent upon

infection of secondary target tissues, transmission is dependent upon infection of salivary glands.

Once an infectious bloodmeal enters the lumen of the midgut, virus encounters the midgut, a simple monolayer of epithelial cells along with the peritropic membrane. The exact mechanism for SINV entry into cells is unclear, and a definitive receptor has not been located within the mosquito midgut. Peirro and others (2007), speculated that viruses are not only restricted by potential receptors in the midgut at the initial site of infection, but also by biochemical processing within the midgut cells that have the potential to affect the spread of infection from the initial site to other midgut epithelial cells. This initial site of infection is evident by SINV antigens localized in a patch of epithelial cells (Ciano, 2010; Pierro et al., 2007). Ciano (2010), observed a single foci of SINV antigens in *Ae. aegypti* midguts D5 p.i. with TR339. Pierro and colleagues (2007) observed 1-4 foci of SINV antigens in *Ae. aegypti* midguts, D9 p.i with TR339 along with minimal muscle banding. In this investigation SINV labeling was extensive in muscle bundles at D30 p.i. in *Ae. aegypti*. Bowers and others (2003) identified SINV associated pathology in midgut associated visceral muscles of *Ae. albopictus* at D18 p.i. via intrathoracic inoculation. In the current investigation, SINV antigens were observed in patches throughout the midgut epithelium, following 30 days p.i. with TR339. SINV antigens were observed in peristaltic muscles, but not as prominent as labeling seen in *Ae. aegypti*. The difference of SINV localization between species could be due to a greater distribution of receptors or biochemical differences in the midgut environment that effect initial site of infection. Biochemical differences between the two *Aedine* species could account for the differences in SINV antigen distribution at D30 p.i. Additionally, while

virus antigen was still present at D30 p.i., it is possible that antigens are cleared more rapidly in *Ae. albopictus* when compared with *Ae. aegypti.*

Spatial patterns of SINV localization appear different in disseminated verses non-disseminated mosquitoes; this is different from that observed in (Peirro et al., 2007) whom were looking at *Ae. aegypti* mosquitoes 9 days p.i. with TR339. In the current investigation different patterns of SINV antigen localization were observed between disseminated and non-disseminated mosquitoes at D30 p.i. with TR339. Most often, non-disseminated mosquito guts labeled for SINV looked like mock infected mosquitoes, however in some cases unusual discrete patches and stippling of SINV distribution was observed. In *Ae. aegypti* non-disseminated mosquito, SINV antigens were located inside cells, potentially within the rough endoplasmic reticulum or Golgi. Within several *Ae. albopictus* non-disseminated mosquitoes, SINV antigens were localized within unidentifiable structures. Within several *Ae. albopictus* non-disseminated mosquitoes, SINV antigens were localized within unidentifiable globular structures. Fat body is essential for storage and metabolism and is responsible for synthesizing properties of the hemolymph and vitellogenin for egg maturation (Martins, et al., 2011). Fat body cells line the body cavities, surrounding digestive and reproductive organs. The globular nature of fat bodies increases surface area and aides in the exchange of substances between digestive organs and the haemolymph. The fat body (Bowers, et al., 1995) and haemocytes (Parikh, et al., 2009) were both were found to be susceptible to SINV infection after intrathoracic inoculation. It is suspected that the unidentified globular structures staining for the presence of SINV antigens are fat body, however these warrant further investigation.

In the present investigation a structure that appears to be globular in nature was positive for SINV antigens. These non-disseminated virus phenotypes could not be isolated and investigated any further, this investigation focused on persistent disseminated virus, however persistent non-disseminated virus can be of great importance to study as well. Non-disseminated virus is trapped in the mosquito gut, and therefore cannot escape to infect the salivary gland. Whatever changes were made to the genome that made the virus unable to disseminate, could be very beneficial to researchers and potentially helpful in vaccine development. The TR339-PDIs isolated from this investigation can provide further insight into the establishment of chronic infections in the whole mosquito.

References

Alto, B.W., Lounibos, L.P., & Juliano, S.A. (2003). Age-dependent blood feeding of *Aedes aegypti* and *Aedes albopictus* on artificial and living hosts. *Journal American Mosquito Control Association, 19*, 347-352.

Andreadis, T.G. **(**2009). Failure of *Aedes albopictus* to overwinter following introduction and seasonal establishment at a tire recycling plant in the northeastern USA. *Journal American Mosquito Control Association, 25,* 25-31.

Borucki, M. K., Kempf, B.J., Blair, C.D., & Beaty, B.J. (2001). The effect of mosquito passage on the La Crosse virus genotype. *Journal General Virology, 82,* 2919-2926.

Bowers, D.F., Abell, B., & Brown, D.T. (1995). Replication and tissue tropism of the alphavirus Sindbis in the mosquito *Aedes albopictus. Virolology, 212*, 1-12.

Bowers, D. F., Coleman, C.G., & Brown, D.T. (2003). Sindbis virus-associated pathology in A*edes albopictus* (diptera: Culicidae). *Journal of Medical Entomology, 40,*698-705.

Brown, D. T. & Condreay. L.D. (1986). Replication of alphaviruses in mosquito cells. S.Schlesinger and M. J. Schlesinger (eds.), *Togaviridae and Flaviviridae*. Plenum Press, NewYork, NY.

Ciano, K.A. (2010). Temporal and Biochemical Aspects of Sindbis Virus Dissemination in the Mosquito Host *UNF Theses and Dissertations,* 67, http://digitalcommons.unf.edu/etd/67

Ciota, A.T., Lovelace, A.O., Jia, Y., Davis, L.J., Young, D.S., & Kramer, L.D. (2008). Characterization of mosquito-adapted West Nile virus. *Journal General Virology, 89,* 1633-1642.

Clements, A.N. (1992). *The Biology of Mosquitoes: development, nutrition, and reproduction.* Vol. 1. CABI Publishing, New York, New York.

Clements, A. N. (1996). *The Biology of Mosquitoes; Adult food and feeding mechanisms; Structure of the adult alimentary canal*. Champman and Hall London.

Davey M.W., & Dalgarno L. (1974). Semliki Forest virus replication in cultured *Aedes albopictus* cells: studies on the establishment of persistence. *Journal General Virololology, 24,* 453–463

Delatte, H., Desvars, A., Bouetard, A., Bord, S., Gimonneau, G., Vourc'h, G., & Fontenille, D. (2010). Blood feeding behaviour of *Aedes albopictus*, a vector of Chikungunya on LaReuion. *Vector Borne Zoonotic Disease, 10,* 249–258.

Eaton, B.T. (1982). Sindbis virus variants from a persistently infected mosquito cell culture contain an altered E2 glycoprotein. *Virology,* 122, 486-49l.

Fields, B.N., Howley, P.K., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Strausm, B., & Snipe D.M. (2001). *FieldsVirology*. Lippincott, Williams & Wilkins, NewYork.

Gerberg, E.J., Barnard, D.R. & Ward, R.A. (1994). Manual for Mosquito Rearing and Experimental Techniques. Lake Charles, LA: *American Mosquito Control Association*.

Gratz, N.G. (2004). Critical review of the vector status of *Aedes albopictus*. *Medical Veterinary Entomol*ogy, *18,* 215-227

Harrington, L.C., Edman, J.D., & Scott T.W. (2001).Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? *Journal Medical Entomol*ogy, 38, 411-422.

Hernandez, R., Sinodis, C., Horton, M., Ferreira, D., Yang, C., & Brown, D.T. (2003). Deletions in the Transmembrane Domain of a Sindbis Virus Glycoprotein Alter Virus Infectivity, Stability, and Host Range. *Journal of Virology*, 77, 12710-12719.

Hernandez, R., Sinodis, C., & Brown, D.T. (2005). Sindbis virus: propagation, quantification, and storage. *Current protocols in microbiology*; Chapter 15:Unit 15B.1.

Higgs, S., & Beaty, B.J. (1996). Rearing and containment of mosquito vectors. In: The Biology of Disease Vectors (ed. B.J. Beaty and W.C. Marquardt). 595-605. Niwot, CO: University Press of Colorado.

Holland, J.J., Grabau, E.A., Jones, C.L., & Semler, B.L. (1979). Evolution of multiple genome mutations during long-term persistent infection by vesicular stomatitis virus. *Cell, 16,*495-504

Houk, E.J., & Hardy, J.L. (1979). In vivo negative staining of the midgut continuous junction in the mosquito *Culex tarsalis* (Diptera: Culicidae). *Acta Trop*, *36*, 267-276.

Igarashi, A., Koo, R., & Stollar, V. (1977). Evolution and properties of Aedes albopictus cell cultures persistently infected with Sindbis virus. *Virology,* 82, 69-83.

Johnson, D.A., Gautsch, J.W., Sportsman,J.R., & Elder, J.H. (1984). Improved Technique Utilizing Nonfat Dry Milk for Analysis of Proteins and Nucleic Acids Transferred to Nitrocellulose. *Gene Analysis Techniques, 1,* 3-8.

Jones, H.J., & Potter, H.W., Jr. (1972). A six-position artificial feeding apparatus for *Culicoides variipennis*. *Mosquito News, 32*, 520-528.

Khoo, C.C.H., Piper, J., Sanchez-Vargas, I., Olson, K.E., & Franz, A.W.E. (2010). The RNAi pathway affects midgut infection and escape barriers for Sindbis virus in *Aedes aegypti*. *BMC-Microbiology*, 10, 130-141.

Klimstra, W. B., Ryman, K.D., & Johnston, R.E. (1998). Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *Journal Virology,* 72, 7357-7366.

Kramer, L.D., Hardy, J.L., Presser, S.B., & Houk, E.J. (1981). Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low virus dose. *American Journal Tropical Medicine and Hygiene, 30,* 190-197.

Lyski, Z.L., Saredy, J.J., Ciano,K.A., Stem,J., & Bowers, D.F. (2011). Blood Feeding Position Increases Success of Recalcitrant Mosquitoes. *Vector Borne Zoonotic Disease, 11,* 1165–1171.

Martins G.F., Serrão J.E., Ramalho-Ortigão J.M., & Pimenta P.F.P. (2011). A comparative study of fat body morphology in five mosquito species. *Memórias do Instituto Oswaldo Cruz, 106,* 742-747.

Moore, C.G., & Mitchell, C.J. (1997). *Aedes albopictus* in the United States: ten-year presence and public health implications. *Emerging Infectious Dis*ease, *3,* 329-334.

Mourya, D.T., Gokhale, M.D., Barde, P.V., & Padbidri, V.S. **(**2000). A simple artificial membrane-feeding method for mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 94, 460-460.

Mudiganti, U., Hernandez, R., Ferreira, D., & Brown, D.T. (2006). Sindbis virus infection of two model insect cell systems—A comparative study. *Virus Research, 122,* 28-34.

O'Meara, G.F., Evans, L.F. Jr.,Gettman, A.D., & Cuda, J.P. (1995). Spread of *Aedes albopictus* and decline of *Ae. aegypti* (Diptera:Culicidae) in Florida. *Journal of Medical Entomol*ogy, *32,* 554-562.

Parikh, G.R., Oliver, J.D., & Bartholomay, L.C. (2009). A haemocyte tropism for an arbovirus. *Journal General Virology, 90,* 292–296.

Peleg, J. (1969). Inapparent persistent virus infection in continuously grown *Aedes aegypti* mosquito cells. *Journal General Virology, 5,* 463-571.

Pierro, D.J., Powers, E.L., & Olson, K.E. (2007). Genetic determinants of Sindbis virus strain TR339 affecting midgut infection in the mosquito *Aedes aegypti*. *Journal General Virol*ogy, *88,* 1545–1554.

Reiskind, M.H., Westbrook, C.J., & Lounibos, L.P. (2010). Exposure to chikungunya virus and adult longevity in *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse). *Journal Vector Ecology, 35,* 61-69.

Renz, D., & Brown, D.T. (1976). Characteristics of Sindbis Virus Temperature-Sensitive Mutants in Cultured BHK-21 and *Aedes albopictus* (Mosquito) Cells. *Journal Virology,* 19, 775-781.

Rosen, L., & Gubler, D. (1974). The use of mosquitoes to detect and propagate Dengue viruses. *American Journal Tropical Medical Hygiene, 23,* 1153-1160.

Shenk, T.E., Koshelnyk, K.A., & Stollar, V. (I974). Temperature-sensitive virus from *Aedes albopictus* cells chronically infected with Sindbis virus. *Journal of Virology, 13*, 439-447.

Sprenger, D, & Wuithiranyagool, T. (1986). The discovery and distribution of *Aedes albopictus* in Harris County, Texas. *Journal American Mosquito Control Association, 2,* 217-219.

Strauss, J.H, & Strauss, E.G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiology Reviews, 58,* 491-562.

Tseng, M. (2003). A simple parafilm M-based method for blood-feeding *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). *Journal Medical Entomo*logy, *40,* 588-589.

Turell, M.J., Gargan, T.P., & Bailey, C.L. (1984). Replication and dissemination of Rift Valley Fever virus in *Culex pipiens. American Journal Tropical Medical Hygiene, 33,* 176-181.

Vo, M., & Bowers, D.F. (2006). Arbovirus lifecycle: links in a chain. *Technical Bulletin Florida Mosquito Control Association,* 7, 31- 34.

Vita

Zoë L. Lyski

2013—Completed degree requirements for Masters of Science in Biology from the University of North Florida.

2009—Graduated from University of North Florida with a Bachelors of Science in Biology.

Employment History

2010-Present—Graduate Research Assistant, Doria F. Bowers P.I. at the University of North Florida

2009-2010—Research Assistant, Doria F. Bowers P.I. at the University of North Florida

Publications

Zoe L. Lyski, Saredy, J.J., Ciano, K.A., Stem, J., Bowers, D.F. 2011. Blood Feeding Position Increases Success of Recalcitrant Mosquitoes. *Vector Borne Zoonotic Disease*.11(8): 1165–1171.

Conference Presentations

Zoe L. Lyski and D.F. Bowers. "Arbovirus persistence and selection of persistent variants following chronic infection in *Aedine* mosquitoes: A comparative study between *Ae. aegypti* and *Ae. albopictus* 30 days post infection with Sindbis." Poster presentation. American Society for Virology 32nd annual conference. State College, Pennsylvania 2013 (accepted).

Zoe L. Lyski and D.F. Bowers. "Vertical blood feeding and arbovirus persistence in mosquitoes." Poster presentation. Society for Vector Ecology annual conference. Saint Augustine, Florida 2012.

Zoe L. Lyski, J.J. Saredy, and D.F. Bowers. "Feeding Position Increases Blood Feeding Success of Recalcitrant Mosquito." Oral presentation. Florida Mosquito Control Association annual fall meeting. Jacksonville, Florida 2011

Zoe L. Lyski and D.F. Bowers. "Blood feeding position increases success of recalcitrant mosquitoes." Oral presentation. STARS Research Symposium. Jacksonville, Florida 2011.

Zoe L. Lyski, K.A. Ciano, J. Stem, and D.F. Bowers. "Novel Modifications to Membrane Blood Feeding Increases Compliance of Recalcitrant Mosquitoes." Poster presentation. American Society for Virology 29th Annual conference. Bozeman, Montana 2010.