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# Temporal and Biochemical Aspects of Sindbis Virus Dissemination in the Mosquito Host

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# Temporal and biochemical aspects of Sindbis virus dissemination in the mosquito host

By

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Abstract

The prototype *Alphavirus*, Sindbis virus (SIN), relies on cyclic transmission between the mosquito and vertebrate hosts in order to be maintained in nature. This broad host range suggests that alphaviruses use a universally expressed molecule for attachment. Heparan sulfate proteoglycan (HSPG), a ubiquitous SIN receptor present on the cell surface of most eukaryotic cells, has been reported in the salivary glands and midguts of mosquitoes. These organs are essential for virus transmission from this hematophageous invertebrate. Variable host cell response in the mosquito following intrathoracic inoculation with SIN has been documented. In this study, *per os* infection of *Aedine* species with variants of SIN was used to determine organ specific responses to virus as well as the temporal kinetics of SIN dissemination via leg assay. Analysis indicated AR339 virus dissemination in samples at day 14 days post infection (p.i.). TR339 was identified at day 12 in legs of virus fed individuals. AR339, the HSPG adapted variant resulted in SIN-associated pathology in salivary glands of *Aedes albopictus.* This pathology was limited to lateral lobes, while the median lobe remained unaffected. Infection with TR339, a HSPG-independent variant, did not result in virus-associated pathology in the salivary gland to day 28 post infection. Immunohistochemistry determined that HSPG was located in the lateral lobe duct region of the salivary glands. It has been suggested that human lactoferrin (hLF) may interfere with virus receptor attachment and is involved in inhibition of virus infection in vertebrate cells. To that end, the effects of bovine LF inhibition on virus attachment were compared between AR339 and TR339 in the mosquito cell line C7-10. Cytopathic effect was observed earlier and with greater intensity in TR339 infected monolayers when compared to AR339 infected monolayers. This suggests that bovine LF has an inhibitory effect on AR339 infection in invertebrate cells, possibly due to this variants use of HPSG for attachment.

# Chapter 1: Introduction

Arthropod borne viruses (arboviruses) are ubiquitous etiological agents that are significant causes of disease in humans and animals. They are usually maintained by continuously cycling between insect and vertebrate hosts (Fields, 2001). The arbovirus with the widest geographical distribution is Sindbis (SIN) virus (Yuill, 1986). Sindbis, a member of the Family *Togaviridae*, Genus *Alphavirus*, is enveloped with an icosohedral capsid (Fields, 2001). Its genome is a single stranded RNA of approximately 11.7kb, with a 5' cap and 3' polyadenylated tail that serves directly as messenger RNA (mRNA) in a host cell (Strauss and Strauss, 1994). SIN has become the prototype *Alphavirus* used in research applications, partly due to its biological safety level 2 status and lack of pathology in infected humans (Taylor *et al*., 1955)

Sindbis virus was first recognized in *Culex* species of mosquitoes from the town of Sindbis, Egypt in 1952 (Taylor *et al.,* 1955). This arbovirus is ornithophilic in nature, which may account for its widespread geographical distribution. Besides being recovered from seven species of *Culex* (the main vector), SIN has been isolated from two species of *Aedes*, two species of *Mansonia* and one species of *Anopheles* (Yuill, 1986) and is capable of replication in *Ae. aegypti* and *Ae. albopictus* under laboratory conditions (Schiefer and Smith, 1974). *Aedes aegypti* and *Ae. albopictus* are invasive, exotic species that have successfully invaded numerous countries. *Aedes aegypti* is the most important

vector for dengue virus with *Ae. albopictus* being the second most important (Gubler and Kuno, 1997; Lounibous, 2002).

Although *Ae. albopictus*, first detected in the United States in 1985 (Sprenger and Wuithiranyagool, 1986), is not a natural vector of SIN, the recent isolation of the eastern equine encephalitis (EEE) virus from this mosquito in the wild extended the vector range of *Ae. albopictus* to include the *Alphaviruses* (Mitchell *et al.*, 1992). The global distribution, vector status, and previous research conducted to assesses SIN interaction with larval cell clones derived from *Ae. albopictus* and adult *Ae. albopictus* make this an appropriate species for further study (Brown and Condreay, 1985). Additionally, the susceptibility of *Ae. aegypti* and *Ae. albopictus* to SIN, as well as being endemic to Florida, makes them suitable for experimentation.

The hematophageous female mosquito is the biological vector of arboviruses (Fields, 2001). Active replication of arboviruses in the mosquito host is essential for the persistence of virus in a given environment. Horizontal transmission is the primary documented mechanism of *Alphavirus* transmission in the wild and laboratory (Chamberlin, 1980). Lack of evidence for a vertical transmission route for SIN indicates that feeding physiology of female mosquitoes is integral to virus transmission.

For the mosquito to become capable of transmission, virus must first reach and replicate in the salivary glands. Female mosquito salivary glands are paired organs located in the thorax. Each gland consists of three lobes - two identical lateral lobes and one median lobe. The lateral lobes consist of proximal, intermediate and distal regions, while the median lobe consists of a short neck region and a distal region. Each lobe has a

central duct encircled by a layer of epithelial cells that are bound externally by a basal lamina (Clements, 1996). These ducts connect with one another to form a common salivary duct that opens at the base of the hypopharynx (Dhar, 2003). While the male salivary glands are also tri-lobed, they are morphologically and functionally dissimilar due to the lack of distal portions on all three lobes (Clements, 1996). These distal regions produce apyrase involved in blood feeding (Ribeiro *et al.*, 1984; James and Rossignol, 1991) and express a female enriched gene (James *et al*., 1991).

An electron microscopy study of the salivary glands of female *Aedes aegypti* by Janzen and Wright (1971), illustrates that lobes are made up of three glandular and two non-glandular regions. Although the ultra-structural appearance at the distal portions of the lateral and median lobes appears comparable, the increased refractile quality of the median lobe may demonstrate that it is chemically distinct (Janzen and Wright, 1971). Orr and colleagues (1961) suggested that two different components are secreted from the three glandular areas of the salivary gland: a carbohydrate-protein complex from the proximal and distal portions of the lateral lobes and a mucopolysaccharide from the median lobe. These dissimilarities between the lobes suggest that this tissue may provide a means for preventing the spread of arthropod-borne diseases.

Because *Alphaviruses* have a broad host range in nature, replicating in mammalian, avian, arthropod and amphibian species (Fields, 2001), it has been suggested that they use a universally-expressed molecule for attachment. Adaptation of SIN to grow in tissue culture or in animals has generated virus mutants that can be used to evaluate strain-specific differences in receptor usage (Klimstra *et al*., 1998). Following passage in BHK-21 cells, SIN has a positively-charged amino acid substitution in viral

spike protein E2, which allows it to attach to heparan sulfate proteoglycan (HSPG) (Waarts *et al.,* 2005). Heparan sulfate proteoglycans, which are present on the cell surface of most eukaryotic cells, have net negative charges. It has been suggested that a charge interaction might a play significant role in *Alphavirus* attachment (Klimstra *et al.*, 1998).

A number of infectious agents such as Dengue virus, herpes simplex, Hepatitis C, malaria and HIV are known to use the negative charge of cell surface glycosaminoglycans to attach, enter and replicate in animal cells (Sinnis *et al.,* 2007). Dengue virus, which enters mammalian cells using HSPG, is transmitted to humans by mosquitoes and may utilize HSPG in the mosquito to localize to the salivary gland (Chen *et al.,* 1997). A recent study by Sinnis *et al.* (2007) definitively demonstrated that mosquitoes contain HSPGs that are concentrated in the salivary glands as well as the midgut.

The salivary glands and gut-associated visceral muscles are essential to mosquito blood feeding behavior and therefore play a very important role in virus transmission. It has been reported that SIN replicates to a high titer in *Ae. albopictus* organs, including salivary glands, under laboratory conditions (Bowers *et al*., 1995). Structural and immunological evidence of SIN-associated pathology was observed in the salivary glands and insect midgut-associated visceral muscle, tissues known to contain HSPG (Bowers *et al.,* 2003). Proximal lateral salivary gland lobes appeared distended and distal lateral lobes were grossly disrupted in SIN-infected cells. Also, virus antigen and virus-associated pathology were limited to the proximal and distal regions of the lateral lobes, whereas neither morphological damage nor virus was detected in the median lobe.

Since *Alphavius* virions bind to receptors that are highly conserved between species, it was suggested that binding of human lactoferrin (hLF) to cell-surface HSPGs may inhibit virus infection (van der Strate *et al.,* 2001). They hypothesized that lactoferrin can prevent virus cell attachment and entry by binding to the virus particle or by binding to cell-surface molecules that viruses use as receptors or co-receptors. In an effort to prevent the entry of SIN, Waarts *et al*. (2005) successfully demonstrated that hLF prevents infection of a host cell by HSPG-adapted *Alphaviruses* [a tissue culture-adapted strain of SIN (TRSB) or Semliki forest virus (SFV)] via blocking viral attachment receptor HSPG on the surface of baby hamster kidney (BHK) cells. Both SIN and SFV cause gross morphological changes in the salivary glands of *Ae. albopictus* and *Ae. aegypti* respectively (Bowers *et al.,* 2003; Mims *et al.*, 1966).

Human lactoferrin is an 80-kDa cationic glycoprotein produced by epithelial cells. As a result, hLF is present in mucosal secretions such as tears, saliva, gastrointestinal fluids, and in high concentrations in human breast milk (Kanyshkova *et al.*, 2001). Preabsorbtion of cultured vertebrate cells with hLF strongly inhibited infection of cells by HS-adapted alphaviruses (TRSB and SFV), but did not inhibit infection by the non-adapted SIN strain TR339 (Waarts *et al*., 2005). The development of mosquito tissue-culture cell lines that can be serially passaged in the laboratory by Singh (1967) has provided a means for comparative studies of the replication of *Alphaviruses* in vertebrate and invertebrate-derived cells (Brown, 1985). Previous research using the *Ae. albopictus* larval cell line C7-10 (Karpf *et al.,* 1997) demonstrated that the acute phase of SIN infection results in a cytopathic effect (CPE), leading to high death rates of cells that are similar to the SIN pathogenicity observed in BHK-21 cells. The ability of lactoferrin to

decrease infectivity of SIN in this invertebrate cell line was evaluated. Lactoferrin demonstrated the ability to limit infection of C7-10 cells by the SIN tissue culture adapted strain, AR339.

The purpose of this investigation was to correlate the presence of biochemical differences within the salivary gland lobes with the SIN-associated pathology in regions of that organ. This was accomplished by the localization of HSPG to precise regions of the salivary glands. Furthermore, a mode of inhibition of SIN attachment to the cell surface receptor HSPG was studied.

# Chapter 2: Methods and Materials

## Virus Production

AR339 (original isolate) and TR339 (consensus sequence) were used in all virus experimentation and maintained in a biological safety level 2 laboratory. TR339 (kindly provided by Brown/Hernandez Research Group, North Carolina State University) was generated from a cDNA clone. AR339 (ATCC, Manassas, VA) was previously grown and plaque purified, by Dr. Doria Bowers before being aliquoted and frozen at -80°C.

## Growth and Maintenance of Colony Mosquitoes

Colonized *Ae. albopictus* (Lake Charles Strain) and *Ae. aegypti* (Rockefeller Strain) were maintained at 25.5°C $\pm$ 0.5, 70-80% relative humidity and a 16:8 (light/dark) photoperiod in white plastic bucket cages with mesh lids with no more than 350 mosquitoes per cage (Gerberg, 1970). Mosquito eggs were hatched in 1.0% nutrient broth consisting of 1 gram nutrient powder (Becton Dickson Microbiology Systems, Spanks, MD) per 100 ml tap water. First instar larvae were distributed approximately 300/pan of 1.5L tap water and fed a 2% liver powder suspension (ICN Biochemicals, Cleveland, OH). Post emergent adults were supplied with honey-soaked cellucotton as a carbohydrate source and water soaked cotton *ad libitum* (Mustermann, 1985). The carbohydrate meal was placed on top of the cage, allowing mosquitoes to feed through

the mesh while the water source was placed in plastic cups inside of the cage for direct contact.

Artificial Membrane Feeding

Mosquitoes were offered defibrinated bovine blood (Colorado Serum, INC, Denver, CO) at 5 to 7 days post emergence as a protein source. The carbohydrate source was removed from the cages 24 hours prior to a blood feed. A glass, water jacketed artificial membrane feeder was used to maintain the blood at a temperature of 37°C. This glass feeder has a central column and wide base opening through which a blood meal can be accessible to the mosquitoes (Figure 1). Collagen sausage casing (32mm cow hide; www.sausagemaker.com, Buffalo, NY) was hydrated in de-ionized water and used to cover the wide base of the glass feeder. This served as the membrane through which the mosquitoes imbibed their blood meal. This was offered for 1 hour in order to provide a protein source for egg production or as a mode of infection when virus was added. Mated female mosquitoes were used for all experiments.

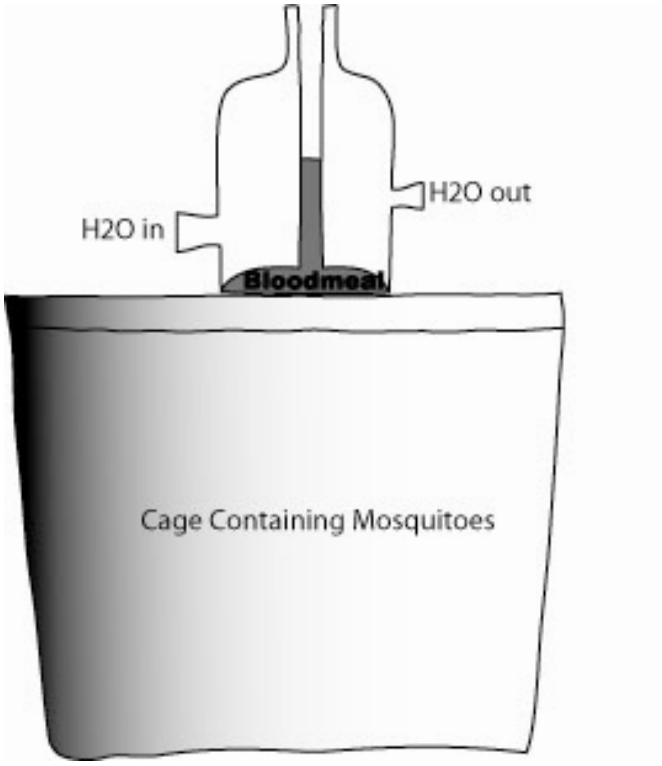


Figure 1. Artificial membrane feeding apparatus. A glass, water jacketed artificial membrane feeder was used to maintain the blood at a temperature of 37°C. This glass feeder has a central column and wide base opening through which a blood meal can be accessible to the mosquitoes.

Virus Infection of Mosquitoes

Adult, female, free-mated mosquitoes were fed a virus/blood suspension 5 to 7 days post emergence via the artificial membrane system described above. Seventy-two hours prior to feeding, females were isolated from the males as follows. Mosquitoes were vacuum aspirated into a collection tube and cold anesthetized by placing the collection tube into a -20° C freezer for 3 minutes. The mosquitoes were subsequently transferred to a glass Petri dish with a watch glass cover and maintained on a chill plate at 4° C. Females were identified and placed into a cage with a final count of 50 female mosquitoes per cage. They were allowed to awaken and were supplied with honey and water. Twenty-four hours prior to feeding, the carbohydrate meal was removed from the cage.

The two variations of SIN, AR339 and TR339, were used at a titer of $7.4 \times 10^7$ plaque forming units (pfu)/ml. Stock virus was diluted in 1X Modified Eagles Medium (MEM; Invitrogen, Carlsbad, CA) as needed. After being allowed to feed on the infected blood for 1 hour, fully engorged mosquitoes were vacuum aspirated, cold anesthetized and carefully isolated. These engorged mosquitoes were then placed in cages labeled "Virus Offered (AR339, TR339 or Control)" and maintained as described.

Cell Culture

BHK-21 cells were cultured at 37º C, 5% $CO_2$ in MEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 10% tryptose phosphate broth, 20ug/ml Gentamycin and 2.5 ug/ml Amphotericin-B to make it complete (MEM-C; Renz and Brown, 1976). Cultured cells were maintained in 25 $cm^2$ flasks until needed.

Cytopathic Effect Assay

A leg from a single virus-offered individual was collected at varying days (0, 1, 5, 7, 9, 12, 14) post feed. An individual was isolated from the cage, cold anesthetized and a single mid leg was removed using alcohol-cleaned forceps. Each isolated leg was placed in an appropriately labeled 1.7 ml pre-lubricated conical microcentrifuge tube along with 10 glass beads. The legs were stored at -80 º C until the assay was performed. At time of assay, the legs were triturated in 500µl 3% Fetal Bovine Serum/Phosphate Buffered Saline (PBS) using a vortex mixer. Preconfluent BHK-21 cells, cultured in 12-well plates, were challenged with 2µl aliquots of virus samples, which served as the positive controls, or 500µl of supernatent from a single triturated leg removed from an individual mosquito. The supernatent was incubated with the cells directly for 1 hour at room temperature on a Vari-Mix (Thermo Scientific, Waltham, MA) platform rocker. After incubation, 1.5 ml of MEM-C was added to the wells and they were incubated at 37º C, 5% $CO_2$. Cytopathic effect (CPE) was monitored at 24 and 48 hours post exposure. Cells were observed through a Leica microscope (Bannockburn, IL) for CPE, characterized by cell rounding and retraction from the substrate, leaving gaps in the monolayer.

Salivary Gland Isolation

Non-blood-fed female mosquitoes were cold anesthetized as described. Mosquitoes were placed on a glass microscope slide with a few drops of cold PBS while viewed through a Leica dissection stereomicroscope (Bannockburn, IL). Thumb forceps and insect pins were used to remove the salivary glands. Following removal of salivary glands, the PBS was wicked away from the salivary gland with filter paper (Whatman,

London, England). Tissues were then incubated in 4% paraformaldehyde/0.1 M Na Cacodylate buffer for 1 hour at room temperature followed by 3 rinses with PBS. The microscope slides containing the fixed tissue were stored in a slide box at -20 °C and sealed with parafilm for up to a month prior to immunohistochemistry.

Localization of HSPG on Salivary Glands

All immunohistochemistry assays were performed in a humidity chamber to prevent desiccation of tissue. Salivary glands, on glass slides, were incubated in blocking solution of PBS containing 10% Normal Goat Serum (NGS) for 30 minutes at room temperature. The blocking solution was wicked away with filter paper and the tissue was incubated in 250 µl of the primary antibody, HSPG C17 (Hybridoma Bank, Iowa City, IA), at a 1:100 dilution in PBS containing 2% NGS, for 2 hours at room temperature. The tissue was washed with PBS and incubated with 250 µl of the secondary antibody, FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), in a 1:60 dilution of PBS containing 2%NGS, for 30 minutes at room temperature. Again, the tissue was washed and slides were mounted with 0.25 ml Vecta Shield (Sigma-Aldrich, St. Louis, MO), covered with 1.5mm thick coverslips and observed using an Olympus BX60 epifluorescence microscope (Tokyo, Japan) equipped with a U-MWU dual filter. A KE/SE digital camera captured the images, which were processed using a SPOT/RT program (Diagnostic Instruments Inc., Flint, MI).

Label of SIN in Midgut of *Ae. aegypti*

Midguts were dissected out of cold-anesthetized TR339 virus-offered individuals at consecutive days (0, 1, 5, 7, 10, 12, 14) post feed and placed into ice-cold acetone for 5 minutes. Organs were placed on microscope slides and frozen as described. At time of labeling, midguts were incubated in a blocking solution consisting of PBS containing 10% NGS for 30 minutes at room temperature. The blocking solution was wicked away with filter paper and the tissue was incubated with 250 µl of the primary antibody rabbit anti-SINV, diluted 1:100 in PBS containing 2% NGS, for 2 hours at room temperature. The tissue was washed as described and incubated with 250 µl of the secondary antibody, FITC-conjugated goat anti-rabbit IgG (Millipore, Billerica, MA), at a 1:40 dilution in PBS containing 2% NGS, for 30 minutes at room temperature. The tissue was washed as described and slides were mounted with Vecta Shield and observed using an Olympus BX60 epifluorescence microscope.

Lactoferrin Inhibition in C7-10 Cells

*Ae. albopictus* larval cells (C7-10; the C7-10 clone is from the laboratory of Victor Stollar [1977]) were grown in MEM-C at 28°C in 5% $CO_2$. Cells were plated in 12-well plates and grown to preconfluency. Bovine lactoferrin (LF; Sigma-Aldrich) was diluted in MEM supplemented with 10% fetal bovine serum to 100 *u*g/ml, 200 *u*g/ml and 300 *u*g/ml. The solutions were incubated on the cells for 1 hour at RT on a platform rocker. The LF was removed and viral variants AR339 or TR339 were diluted to 2.7 x $10^7$ pfu/ml in the LF/MEM suspension. This was done for each concentration of LF. The diluted virus/LF/MEM suspension was then added to corresponding wells and allowed to

adsorb to cells at RT for 1 hour on a platform rocker. After incubation, the cells were washed five times with PBS and 1.5 ml of MEM-C was added. Cells were incubated overnight and CPE was monitored at 24 and 48 hours post infection through a Leica microscope (Bannockburn, IL). CPE is characterized by cell rounding and retraction from the substrate, which leaves gaps in the monolayer.

# Chapter 3: Results and Discussion

## Dissemination of virus variants

Temporal analysis of virus dissemination following engorgement on an infectious blood meal was conducted. Female *Ae. aegypti* mosquitoes were allowed to ingest artificial blood meals containing either AR339 or TR339. A control group was offered a mock blood meal containing MEM at an equal volume as the virus inoculum. Imbibed virus particles must somehow escape the midgut of the mosquito and travel to the salivary gland through the hemolymph. Virus escape was measured through leg assays at days 0, 1, 5, 7, 9, 12 and 14 post feed (Table 1). Analysis indicated AR339 virus dissemination in samples at day 14 days post infection (p.i.). TR339 was identified at day 12 in legs of virus fed individuals. Analysis of legs from control groups never exhibited CPE. These results were reproduced in three separate trials.

Table 1. Dissemination of virus variants. Temporal analysis of virus dissemination from the midgut of the mosquito *Ae. aegypti*. A single leg from a virus fed mosquito was removed at varying time-points p.i. and triturated in media. This suspension was then adsorbed on BHK-21 cells and CPE was monitored. Dissemination of AR339 was first detected at day 14 p.i. and TR339 at day 12 p.i. +, CPE; -, No CPE

| | Days Post Infection | | |
|---|---|---|---|
| | 0-9 | 12 | 14 |
| AR339 | --- | --- | + |
| TR339 | --- | + | + |
| Control | --- | --- | --- |

TR339 infection of *Ae. aegypti* midgut

Within the mosquito, SIN encounters barriers to infection and dissemination that are critical determinants of vector competence. The first barrier that the virus must overcome is the midgut. Posterior midguts are constructed of a monolayer of simple cuboidal/columnar epithelium and a basal lamina all surrounded by visceral musculature (Clements, 1996). Tracheal branches and nerve branches surround this peristaltic organ (Clements, 1996). Following dissection of midguts from virus-fed individuals, essentially no contaminating tissues such as fat bodies were observed in any of the dissected tissues. Temporal and spatial analysis of TR339 infection was detected by immunofluorescence labeling of SIN antigen on whole-mount tissue preparations of the *Ae. aegypti* midgut at days 2, 5 and 10 p.i. Virus antigen was not present at day 2 p.i. (results not shown). Virus antigen was also not detected in tissues from the control mock-infected group (Figure 2). TR339 was detected in the midgut of *per os* infected *Ae. aegypti* at day 5 p.i. (Figure 3B). A focus of infected tissue was restricted to a small patch at the posterior midgut. By day 10 p.i. the area of tissue containing virus was extended greatly to include a large portion of the posterior midgut (Figure 4B).

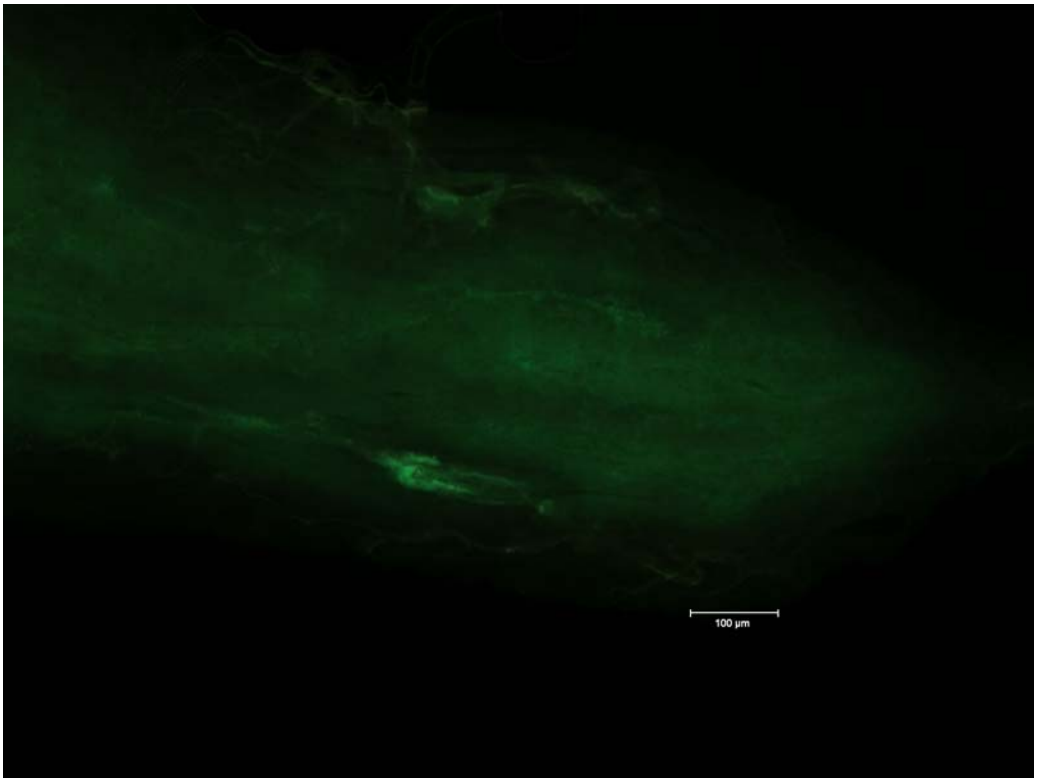


Figure 2. Midgut immunohistochemistry following artificial blood meal. Fluorescence image of *Ae. aegypti* midgut at 5 days post feed *per os* to which only MEM without virus was offered. Immunolabeled for SIN. Magnification Bar – 100 µm

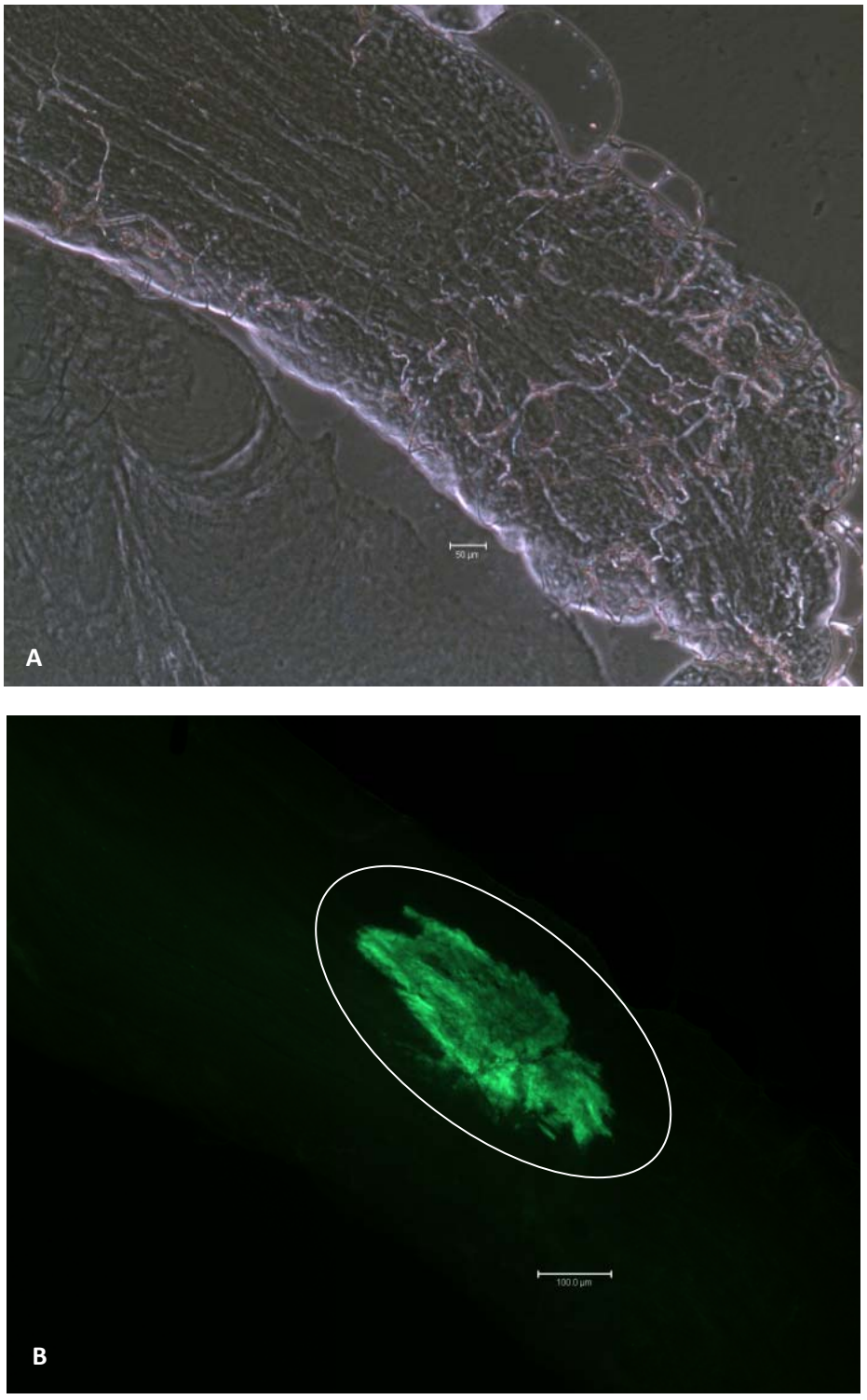


Figure 3. SIN localization in midgut following *per os* infection. A. Phase contrast image of *Ae. aegypti* at 5 days p.i. *per os* with TR339. B. Fluorescence image of *Ae. aegypti* midgut at 5 days p.i. *per os* with TR339. Immunolabeled for SIN. A Focal patch SIN infected tissue is indicated by the oval. Magnification Bar for A – 50 µm. Magnification Bar for B – 100 µm

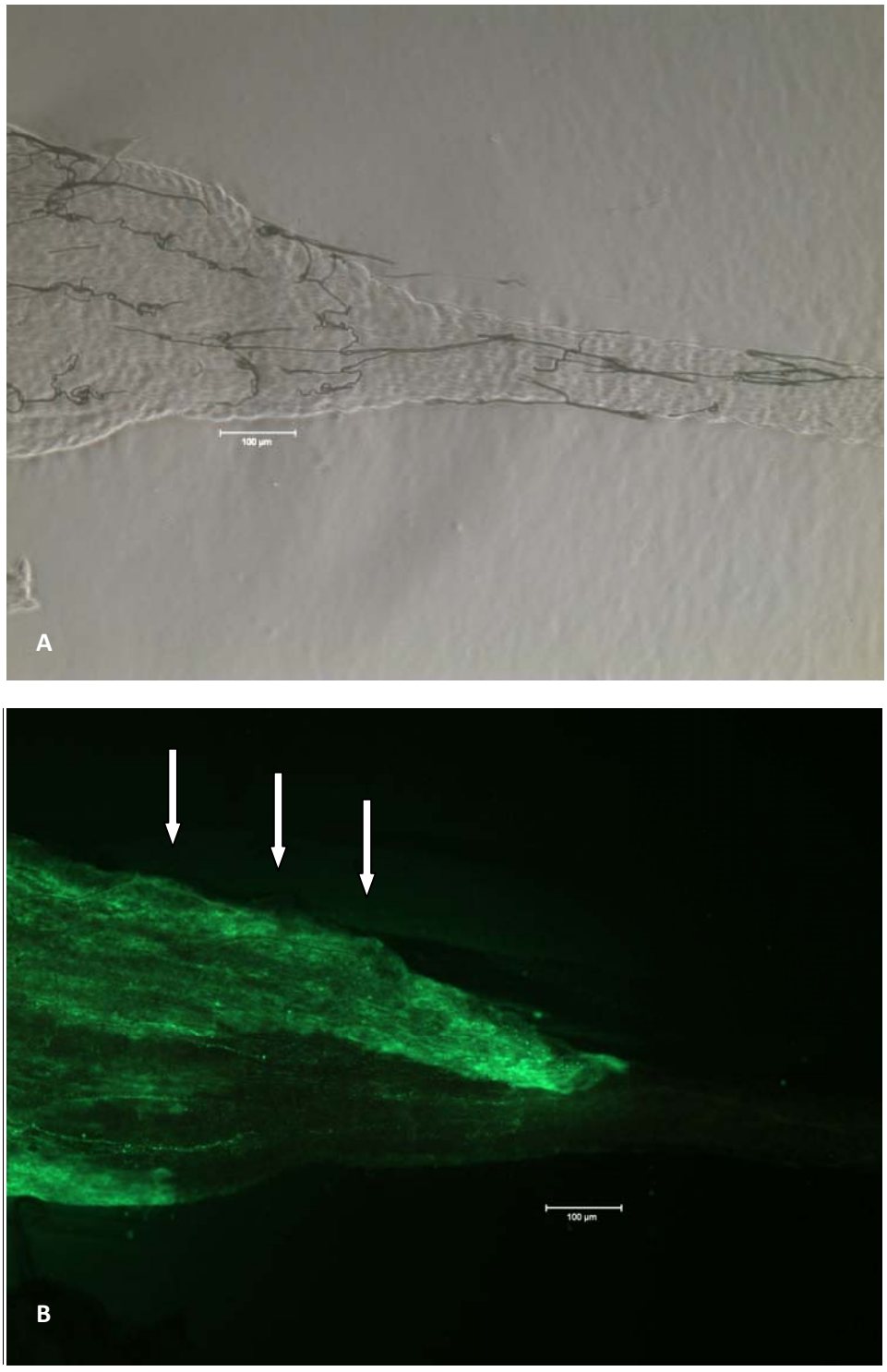


Figure 4. SIN infection of *Ae. aegypti* midgut. A. Phase contrast image of *Ae. aegypti* midgut/hindgut at 10 days p.i. *per os* with TR339. B. Fluorescence image of *Ae. aegypti* midgut/hindgut at 10 days p.i. *per os* with TR339. Immunolabeled for SIN as indicated by the arrows. Magnification Bar – 100 µm

Virus-associated pathology in the salivary glands

Previous research by Bowers *et al.* (2003) showed that gross pathology is co-localized with virus antigen in the proximal and distal lateral lobes of the salivary glands following intrathoracic inoculation with SIN. These results were reproduced in *per os* AR339 infected *Ae. aegypti* in the present study. Salivary glands were dissected out of virus offered individuals and observed at the light microscopic level for signs of virus-associated pathology. At days 14 (Figure 5A) and 28 (Figure 5B) p.i. pathology was sequestered to the proximal and distal regions on the lateral lobes. Tissues of AR339 offered individuals showed distention and disruption of the lateral lobes, while the median lobe remained intact. Attempts to label morphologically disrupted tissue were unsuccessful due to the fragility of the specimens. There was a correlation between a positive leg assay for the dissected individuals and the noted pathology. Lobe specific pathology was not observed in TR339 *per os* infected mosquitoes (Figure 6) 28 days p.i. and the control group (Figure 7).

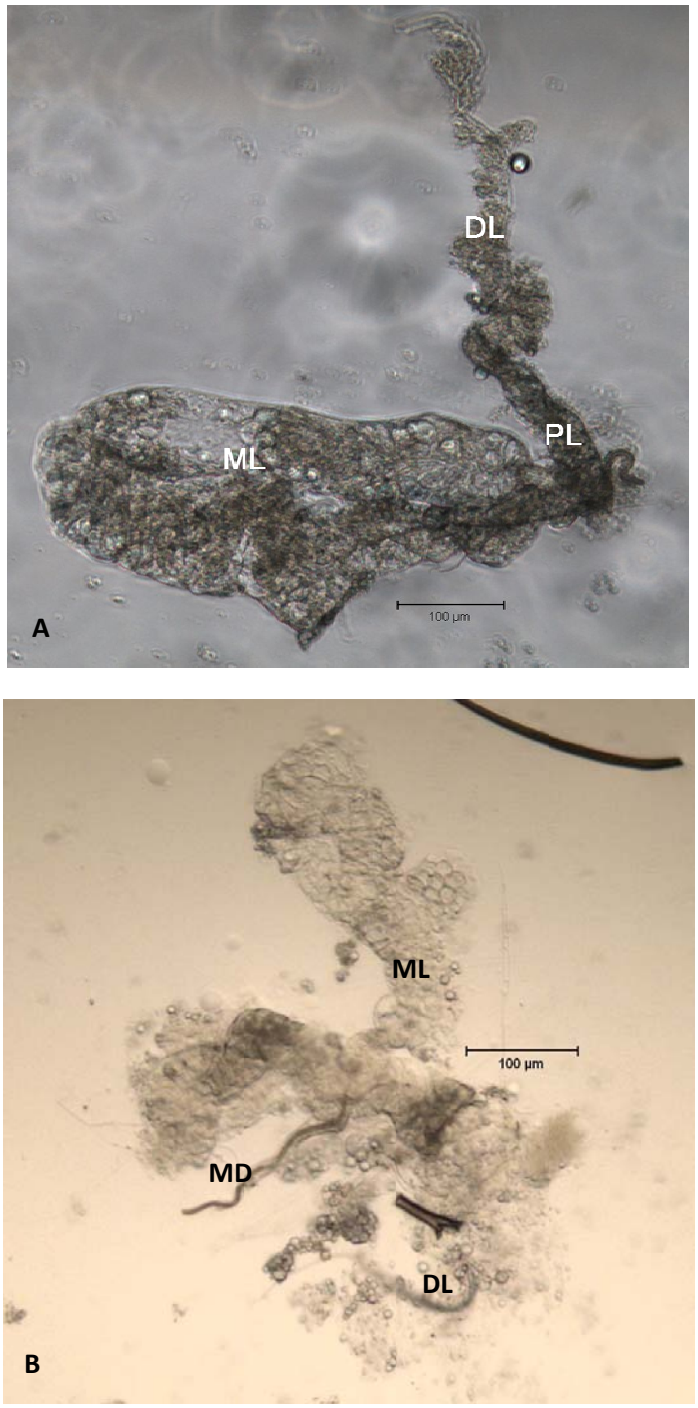


Figure 5. AR339 associated pathology in salivary glands. A. Phase contrast image of the salivary gland of *Ae. aegypti* at 14 days p.i. with AR339 *per os*. The distal lateral lobe (DL) appears grossly disrupted, while the median lobe (ML) remains intact. The proximal lateral lobe (PL) also shows evidence of virus-associated pathology. B. Light microscopy image of the salivary gland of *Ae. aegypti* at 28 days p.i. with AR339 *per os*. The distal lateral lobes (DL) are not apparent, while the median lobe (ML) remains intact. The main salivary duct (MD) is also visible. Magnification Bars – 100 µm

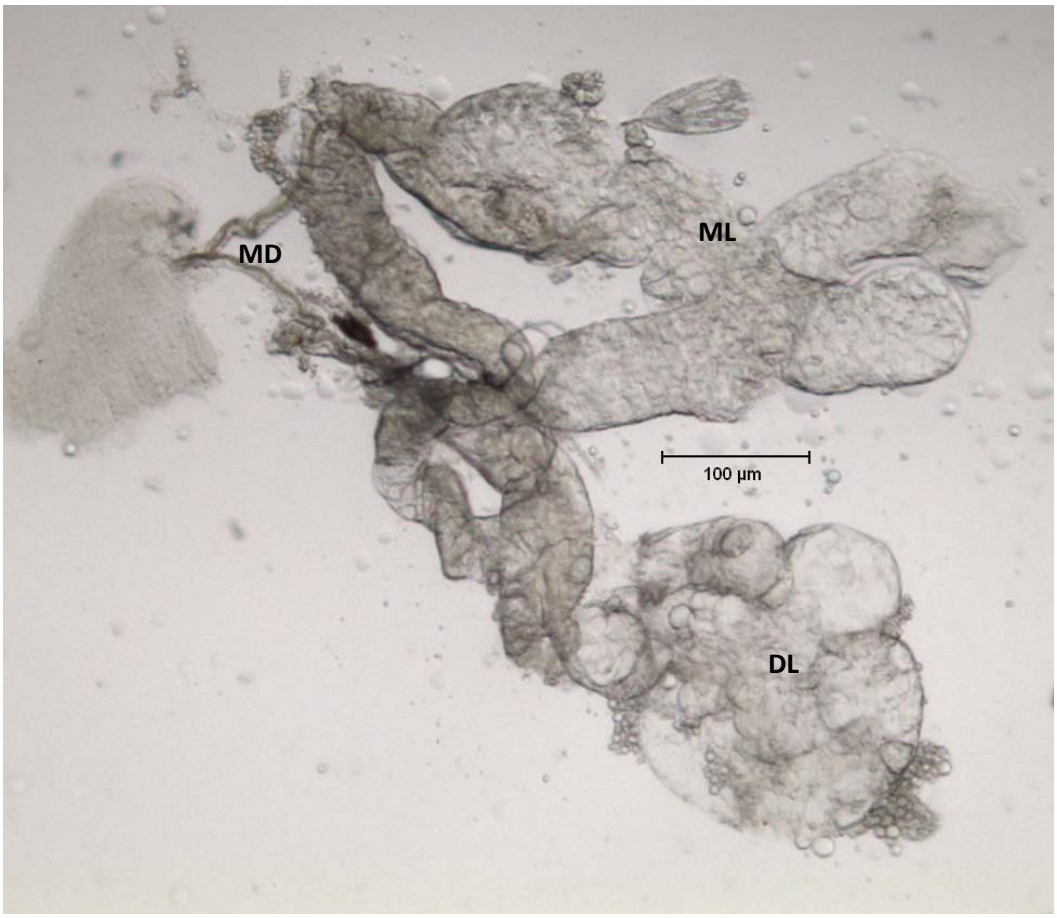


Figure 6. Effects of HSPG independent virus on salivary glands. Light microscopy image of the salivary gland of *Ae. aegypti* at 28 days p.i. with TR339 *per os*. Two sets of salivary glands are displayed, connected to the main duct (MD). The distal lateral lobes (DL) and the median lobe (ML) remain intact. Magnification Bar – 100 µm

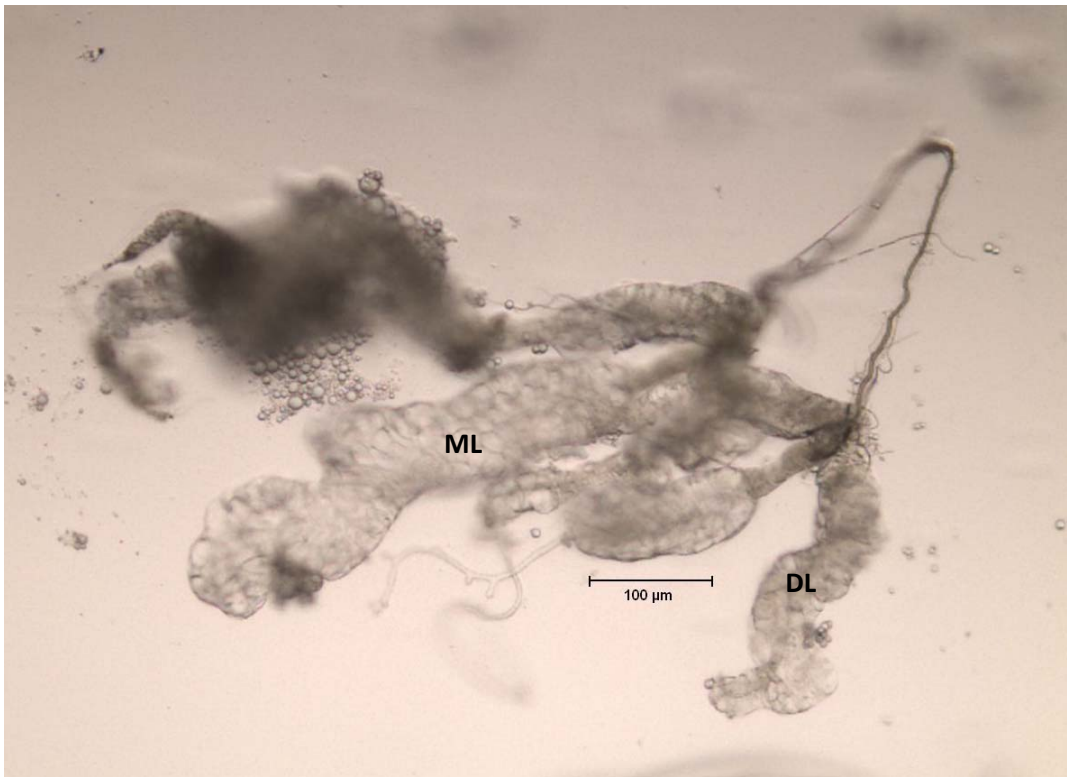


Figure 7. Uninfected *Ae. aegypti* salivary gland. Light microscopy image of a control salivary gland of *Ae. aegypti* at 28 days post mock infection *per os*. The distal lateral lobes (DL) and the median lobe (ML) remain intact. Magnification Bar - 100 µm

Localization of HSPG on salivary glands

Previous findings of proteomic differences between the lobes of the salivary glands (Ribeiro *et al.*, 2007), as well as the significant finding of HSPG in the salivary glands of mosquitoes (Sinnis *et al.*, 2007) offer plausible explanations to the lobe specific pathology shown after virus infection. In an attempt to further pin point structural differences between salivary gland lobes, an immunohistochemistry label of HSPG was performed on whole mount *Ae. albopictus* salivary gland dissections. HSPG was localized to the lateral ducts of the salivary glands in *Ae. albopictus* mosquitoes (Figure 8). The HSPG antibody appears to have attached to the filamentous extensions encircling the duct lumen in the lateral lobes (Figure 9A), while the median duct appears HSPG deficient.

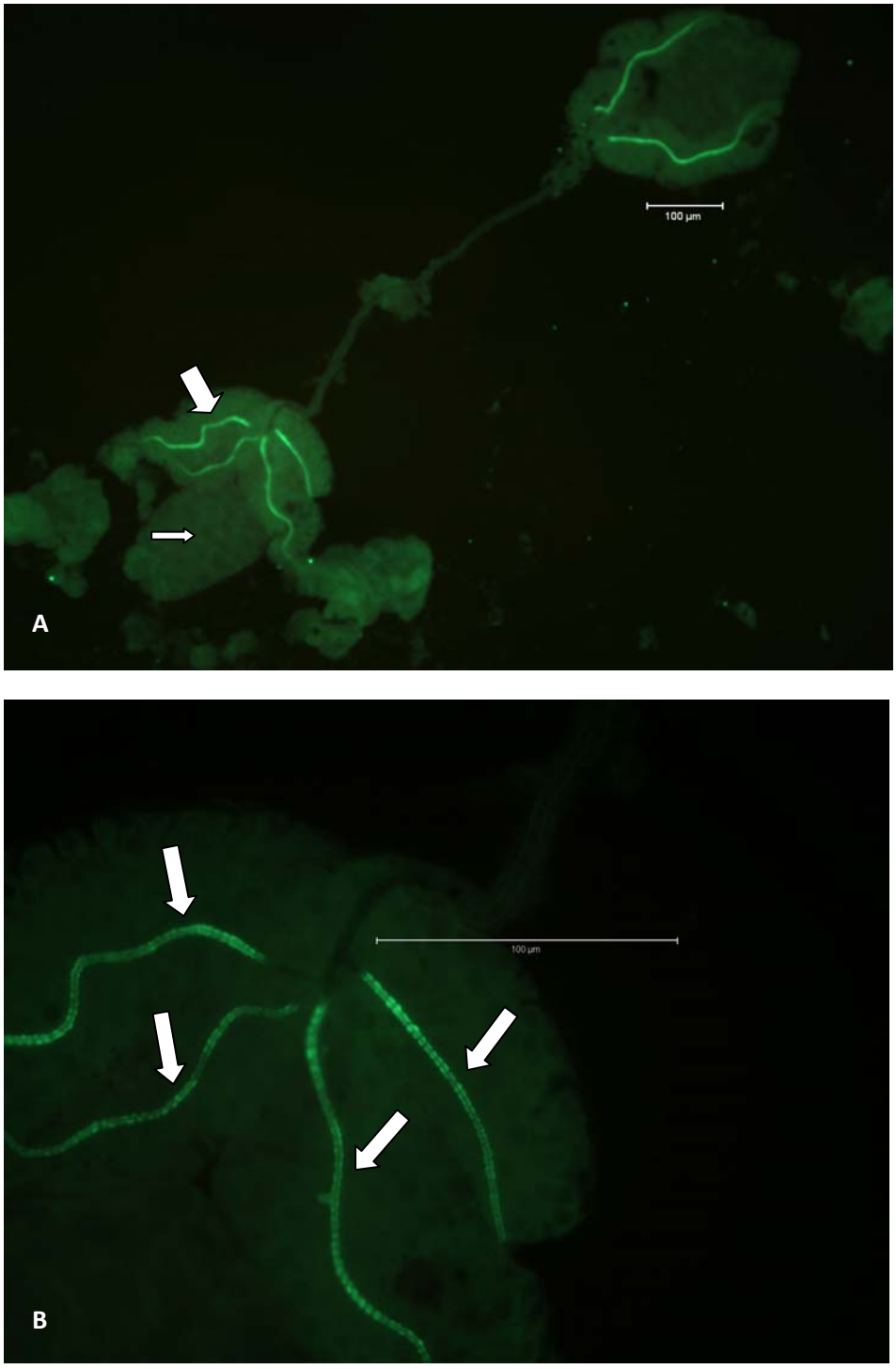


Figure 8. HSPG localization on salivary glands. A. Fluorescence image of two sets of *Ae. albopictus* salivary glands immunolabeled with HSPG antibody C17. The lateral lobe ducts show positive labeling of HSPG (thick arrow), while median lobes appear to be deficient in HSPG (thin arrow). B. Higher magnification view of a salivary gland labeled with HSPG. Filamentous extensions of duct cuticle in lateral lobes show positive immunofluorescence for HSPG (arrows). Magnification Bars – 100 µm

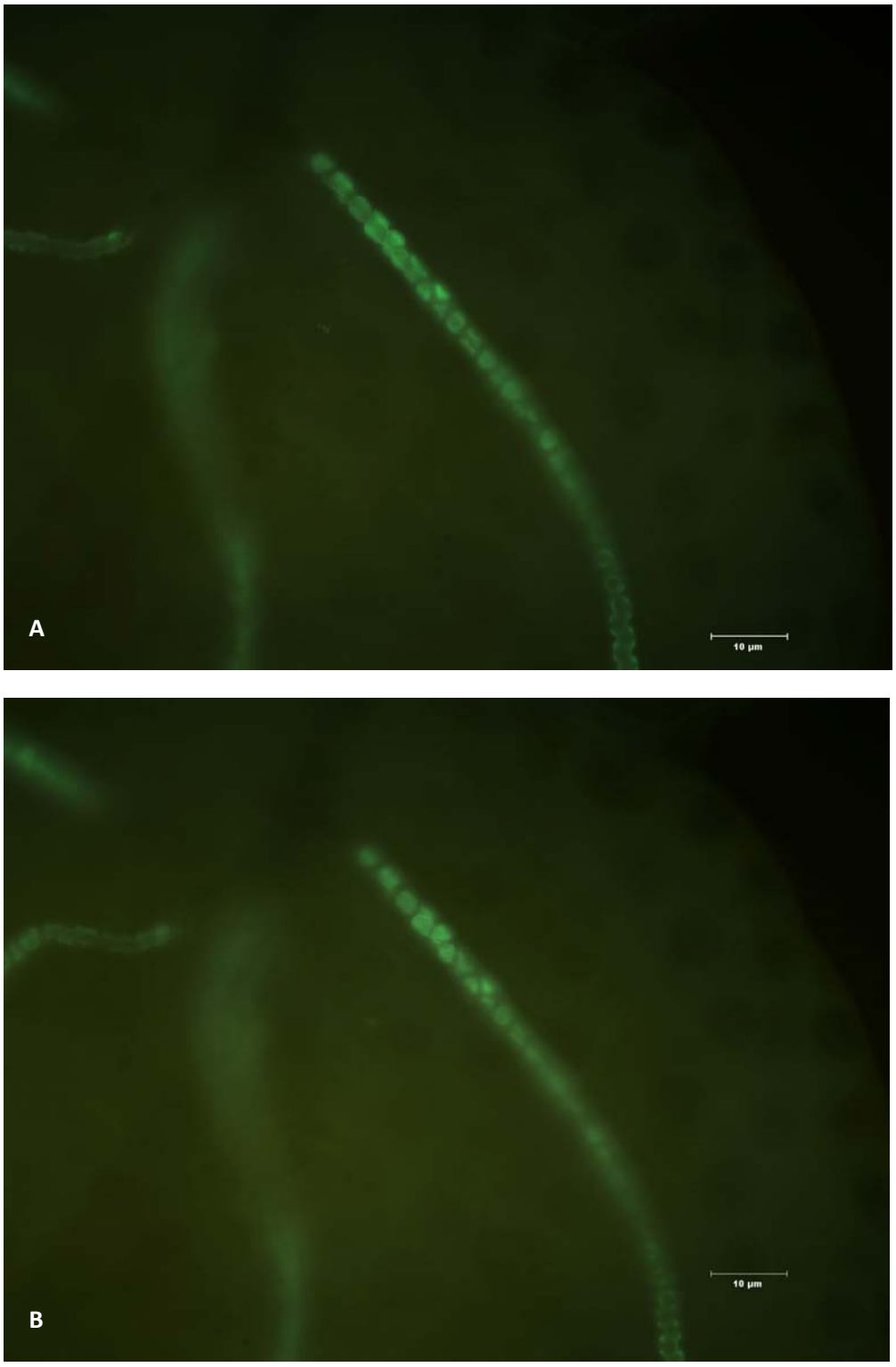


Figure 9. Filamentous extensions of duct cuticle. Higher magnification views of salivary gland lateral lobes of *Ae. albopictus* immunolabeled with the HSPG antibody C17 localizes to the duct. The lumen is shown in A. whereas the ventral aspect of the duct is shown in B. Magnification Bars – 10 µm

Lactoferrin inhibition of SIN infection in C7-10 cells

It is presumed that lactoferrin can prevent virus cell entry by binding to the virus particle or by binding to cell-surface molecules that viruses use either as receptors or co-receptors. In either case, it has been suggested that binding of hLF to cell-surface HSPGs is involved in inhibition of viral infection (van der Strate *et al*., 2001). Preadsorption of vertebrate cells with hLF inhibited infection of cells by HSPG-adapted *Alphaviruses* (AR339), but did not inhibit infection by the non-adapted SIN strain TR339. Here, the effects of bovine LF inhibition on virus attachment are being compared between mosquito cultured cells. C7-10 cells were preincubated with bovine LF in three concentrations (100, 200 and 300 μg/ml) and virus ($10^7$ pfu/ml) was adsorbed onto cell monolayers. Initial results suggested a working concentration of 200 μg/ml to be most effective at virus inhibition. This concentration resulted in optimal inhibition of virus with no deleterious effects on the cell culture (Figure 10A). Subsequent studies utilized this concentration. Cytopathic effect was observed earlier and with greater intensity in TR339 infected monolayers (Figure 10C) when compared to AR339 infected monolayers (Figure 10B). This suggests that bovine LF has an inhibitory effect on AR339 infection in invertebrate cells, possibly due to this variants use of HPSG for attachment. These results were reproducible in two trials.

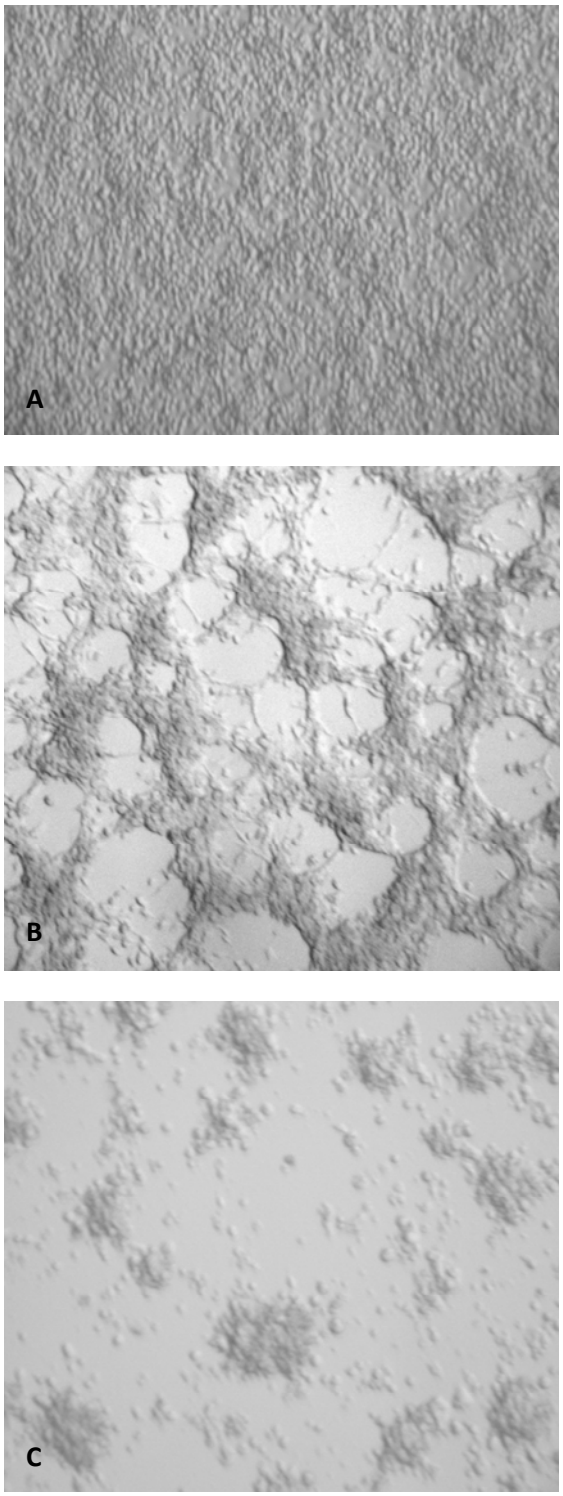


Figure 10: Inhibition of virus-associated CPE in C7-10 cells at 48 hours p.i. A. Control, no virus added. Cells remain healthy after addition of 200 µg/ml of LF. B. AR339 infected cells preincubated with 200 µg/ml of LF. CPE is present, but not as extensive as C. TR339 infected cells preincubated with 200 µg/ml of LF.

Previous studies on SIN-associated pathology in the mosquito did not represent an "in the wild" acquisition of virus. Here, *per os* infection of *Aedine* mosquitoes with SIN variants more accurately portrays virus-host interactions. While the method of infection was altered to include a more natural entrance into the host, the effects of the virus on the organs of the mosquito were comparable to previous research by Bowers *et al.* (2003). The initial hypothesis was that attempts to label HSPG on the salivary gland would result in localization at the basal membrane of the lateral lobes. Instead data show HSPG studded filamentous extensions of the duct cuticle (Figures 8 & 9). While this does not offer an initial route of SIN entry into the salivary glands, these experiments provide a clearer picture of biochemical differences between the lateral and median lobes of the salivary glands in female *Aedine* mosquitoes.

Millions of people die of arthropod-borne diseases including malaria, yellow fever and dengue fever each year (Hill *et al.,* 2005). *Alphaviruses* cycle alternately between vertebrates and hematophagous insects suggesting that virions bind to receptors that are highly conserved between species. The normal function of the cell surface molecule HSPG is to bind a diverse group of growth factors, chemokines, enzymes, and matrix components. In addition, it is important in the cell surface binding of a number of bacteria, parasites, and viruses (Rostand and Esko, 1997). HSPG can serve as an attachment receptor for SIN, but contributes significantly to binding and infection only when using cell culture-adapted strains (Klimstra, Ryman, and Johnston, 1998). This adaptability suggests that SIN rapidly mutates to utilize HSPG when grown in cell culture. While binding to cell surface, HSPG is not equivalent in interactions between natural SIN isolates and cells in vivo. It does offer a means to study the mechanisms of

HSPG adapted viruses such as HIV, Dengue Virus and eastern equine encephalitis. This study provides evidence of HSPG localization in the salivary glands of *Aedine* mosquitoes. More exacting, HSPG is located in the duct cells of the lateral lobes and was absent from the median lobe duct, triad and common duct cells (Figure 8B).

The proximal and distal ducts of the lateral lobes were analyzed through electron microscopy by Janzen and Wright, 1971. They describe their morphology as containing a duct whose lumen is about 1.2 pi in diameter, while the duct wall is about 0.5 pi thick. There were duct thickenings seen by light microscopy which are regions of fine filamentous extensions of the duct cuticle. Maze-like canals (0.1 pi in diameter) penetrate the duct in the regions of the filaments. It is these filamentous extensions that label positive for HSPG (Figure 9). Prior unpublished research by Dr. Doria Bowers showed labeling of the same filamentous extensions by phalloidin, an actin binding toxin. This co-localization can be expected because of the documented relationship between actin and HSPG (Carey, 1997). The median lobe lacks the filamentous projections of the duct cuticle and therefore did not result in a positive antibody label against HSPG.

Previous studies (Bowers *et al*., 2003) showing regional discrepancy in virus-associated pathology within the salivary gland of the mosquito may provide clues to the transmission of these diseases between species. The post infection obliteration of the lateral lobes of the salivary gland while the median lobe stays intact suggests an important biochemical and structural difference. The current study resulted in gross morphologic changes of the proximal and distal lateral lobes in AR339 infected *Ae. aegypti* following *per os* infection at a titer of $7.4 \times 10^7$ pfu/ml. However, this virus-

associated pathology was not observed in the median lobe (Figure 5). Another *Alphavirus*, SFV, also causes gross morphologic changes in the salivary glands of *Ae. aegypti* (Mims *et al.,* 1966).

This prominent lateral lobe-specific pathology would appear to be a disadvantage to the female mosquito. Her ability to blood feed, an important physiological requirement for egg development, (Clements, 1996) is potentially compromised. Previous studies (James and Rossignol, 1991; Marinottinotti and James, 1990; Riberiro, 1992) suggest, however, that the median lobe may be essential for insect survival and reproduction. Therefore, significant virus-associated pathology of the lateral lobes might not be as lethal to insect survival and fitness. Additionally, acinar cells destroyed by the virus may not constitute the sole source of saliva in the mosquito as suggested by Rossignol and Spielman (1981). They established that the salivary ducts of mosquitoes contribute volumetrically to the saliva. Transection of the lateral ducts from the salivary gland demonstrated the ability of the wall of the duct to produce a significant amount of fluid. Muangman (1968) successfully transmitted SIN from virus-infected mosquitoes having transected salivary ducts to suckling mice, suggesting the passage of secretions across the duct wall. Findings that duct-transected mosquitoes imbibe blood conclude that saliva is not a prerequisite for blood feeding and virus transmission. Rossignol and Spielman (1981) even went so far as to hypothesize that the transmitted virus may have not been aspirated from the hemolymph near the open ends of the transected ducts, but instead originated in the duct wall. This would coincide with the results presented in the current study, illustrating obliteration of the gland acinar cells, while the duct remains intact (Figure 5A).

Transmission of SIN from a vertebrate host to a mosquito is initiated by the mosquito probing the skin of an infected host. Their proboscis penetrates the vasculature and the blood pools are siphoned, allowing SIN to enter the lumen of the mosquito midgut. The virus initially infects the midgut epithelial cells, replicates, and then exits the midgut to infect secondary tissues such as fat body and salivary glands (Woodring *et al.,* 1996). Productive infection of the midgut is critical for a mosquito species or population to be competent to transmit an arbovirus (Black *et al.,* 2002). The original isolate strain of SIN, AR339, has limited dissemination and transmission potential in *Ae. aegypti* after oral infection (Jackson *et al.,* 1993; Seabaugh, 1998). Seabough reported an average virus dissemination rate of 39.5% in *Ae. aegypti* 14 days p.i. This corresponds to the late stage dissemination (day 14 p.i.) and lack of virus foci on the midgut p.i. reported in this study. Myles *et al.* (2004) characterized the infection rate and distribution of TR339 virus in the midgut of *Ae. aegypti* mosquitoes and found a greater then 90% midgut infection rate 7 days p.i. These results correspond to SIN antigen detected in the midgut of TR339 virus infected individuals at day 5 p.i. noted in this study (Figure 3A). The virus initiated infection of the midgut with a discrete patch of infection (Figure 3A) that spread, resulting in an expanding focus (Figure 4B). Myles *et al*. (2004) also reported that the spatial and temporal midgut infection pattern for mosquitoes having a disseminated infection appeared similar to those with only a midgut infection, but no dissemination. This offers a possible explanation to the late dissemination (day 12 p.i. in TR339 infected individuals) shown in the current study even though infected midgut tissue is observed at day 5 p.i. (Table 1; Figures 3 and 4).

The consensus SIN strain, TR339, derived from an infectious clone of the original natural AR339 isolate, attaches initially to cells through a low affinity, primarily HS-independent mechanism (Klimstra *et al.,* 1998). This is in contrast to AR339, which utilize cell-surface HSPG molecules as receptors to varying degrees (Byrnes and Griffin, 1998; Klimstra *et al.,* 1998). The HSPG-dependent cell-surface attachment phenotype occurs because of the positive charge amino acid mutations in the E2 attachment protein. This mutation is rapidly selected for during passage in cultured cells and is believed to either form part of a conformation-dependent, nonlinear, HSPG-binding sequence or to promote the exposure of another such site (Klimstra *et al.,* 1998). Interaction of viruses with HSPG as a result of tissue-culture adaptation has been reported for several viruses (Bernard *et al*., 2000; Heil et al., 2001; Jackson *et al*., 1996). Clinical isolates of other viruses showed the ability to bind HSPG as well (Goodfellow *et al.*, 2001; Trybala *et al.,* 2002).

It has been suggested that binding of LF to cell-surface HSPGs is involved in inhibition of viral infection (van der Strate *et al.,* 2001). In order to determine whether LF results in antiviral activity through interference with virus binding to HSPG receptors in the invertebrate host, the effects of LF on SIN-associated pathology of C7-10 cells was studied. Lactoferrin treatment of *Ae. albopictus* cells, C7-10, did result in a reduced virus-associated pathology in AR339 exposed cells (Figure 10B). This result was restricted to the HS-dependent variant, however, as TR339 exposed cells presented advanced characteristics of CPE 48 hours p.i. (Figure 10C). This is similar to the results of Waarts *et al*. (2004) in the vertebrate cell line, BHK-21. They showed that hLF strongly inhibited infection of cells by HS-adapted *Alphaviruses,* but did not inhibit

infection by the non-adapted SIN strain TR339. At a concentration of 200 μg/ml, hLF caused a 70% reduction in viral plaques for TRSB, 90% reduction for SFV, and no reduction for SIN TR339.

Supplementary supportive evidence of LF anti-microbial activities includes the findings by Di Biase *et al.* (2003). They showed that mixtures of bovine lactoferrin and heparin abolished the anti-adenovirus effect of each compound alone and concluded that the anti-adenovirus activity of bovine lactoferrin involves blocking cell-surface HSPGs. Additionally, Marchetti *et al.* (1996) studied the antiviral activity of lactoferrins on HSV-1 and proposed that lactoferrins block cell receptors for HSV-1, including HSPGs.

Sindbis virus is an important tool to study the interaction between viruses and mosquitoes. This is because of the availability of the full-length infectious cDNA clones and because SIN can infect *Aedes aegypti*, a mosquito vector, which is important in the transmission of dengue and yellow fever viruses. The results of this study offer insight into important interactions between the virus and its vector.

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## Published Abstracts

Zoe L. Lyski, Kristen A. Ciano and Doria F. Bowers. Novel modifications to membrane blood feeding increases compliance of recalcitrant mosquitoes. Abstract submitted. 29th Annual Meeting of The American Society for Virology, Poster Session, Bozeman, MT; July 2010.

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