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Influence of Aedes aegypti Saliva on the Vertebrate Host Response to Dengue Virus

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INFLUENCE OF *Aedes Aegypti* SALIVA ON THE VERTEBRATE HOST RESPONSE TO
DENGUE VIRUS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Pathobiological Sciences

by
Michael Kevin McCracken
B.S., Louisiana State University, 2010
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ABSTRACT

Dengue virus (DENV) is maintained in a primarily anthroponotic cycle between humans and the mosquito, *Aedes aegypti*. Investigations into DENV infection of the vertebrate host generally do not account for the contribution of vector saliva, an inherent part of the mosquito-borne viral inoculum. Feeding by mosquitoes on vertebrate hosts is initiated by probing, which results in physical damage to the skin and vasculature, and the simultaneous introduction of DENV and saliva into the skin. Saliva contains many individual proteins with the potential to modulate host hemostasis and immune responses, thereby facilitating blood feeding and virus transmission. As exogenous antigens, both DENV and these salivary proteins encounter the vertebrate host immune system and consequently could have an effect on the immunological environment and response of the bite site during viral establishment, as well as the ensuing viremia. My overarching hypothesis is that mosquito saliva aids in the establishment of DENV infections within the vertebrate, and that distinct immunological alterations involved in this enhancement will be attributable to individual salivary proteins. I therefore conducted investigations into the triad of vector-virus-vertebrate interactions aimed at further characterizing 1) the strain-based impact of DENV infection on salivary protein transcript expression in *Ae. aegypti*; 2) the probing-based modulation of vertebrate immune responses during DENV infection in the skin of a murine model of transmission; 3) the effect of individual salivary proteins on DENV production in a human hematopoietic cell line; and 4) the influence of the salivary protein ‘aegyptin’ on DENV infection in the mouse; with emphasis on early, establishment-relevant time points and differences in infection kinetics with the potential to alter transmission success.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General virology

Dengue virus (DENV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. The genome of DENV is a positive sense, single-stranded RNA of approximately 11kb in length. During replication within a host cell, the viral RNA is translated as a single polyprotein that is co- and post-translationally cleaved into ten individual proteins: three structural (capsid, envelope, pre-membrane) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). There are four serotypes of DENV, numbered 1, 2, 3, and 4. DENV serotype 2 (DENV2) is the subject of the novel investigations presented in this dissertation.

Mosquitoes also transmit other viruses. There are three main families to which most of these viruses belong: *Bunyaviridae*, *Togaviridae*, and *Flaviviridae*. Bunyaviruses are enveloped with negative-sense, single-stranded, tripartite RNA genomes. Alphaviruses (*Togaviridae*) are enveloped with positive-sense, single-stranded RNA genomes. Many of these viruses are included below in the sections on modification of viral infection and immune response by saliva.

1.2 Epidemiology

DENV is the etiologic agent of dengue fever. DENV prevalence has increased in recent decades, and estimates place the global incidence at 390 million annual human infections, of which 96 million are clinically or subclinically apparent [1]. Prior to 2009, clinical presentation of DENV disease was classified into one of three categories, progressing from mildest to most severe: undifferentiated fever, dengue fever, and dengue hemorrhagic fever; dengue hemorrhagic fever was further characterized into four grades, with grades III and IV termed dengue shock

syndrome. In 2009, the World Health Organization reorganized these classifications, maintaining three categories: dengue without warning signs, dengue with warning signs, and severe dengue [2]. Signs and symptoms include fever, nausea, vomiting, rash, aches, and leukopenia, with warning signs including persistent vomiting, pleural effusion, ascites, lethargy, and swollen liver. DENV disease is considered severe once one of the following is evident: plasma leakage leading to shock, fluid accumulation with respiratory distress, severe bleeding, elevated liver enzymes (>1000), central nervous system impairment, and organ failure [3]. From 2001-2005, DENV disease resulted in an estimated financial burden of USD \$440 million across eight countries in the Americas and Asia [4]. The health burden of DENV in Asia and the Americas was estimated at approximately 1300 disability-adjusted life years per million in population in 2010 [5].

1.3 Transmission

DENV is an anthroponotic arbovirus, meaning that it is perpetuated in a transmission cycle between humans (the vertebrate) and an arthropod vector (Figure 1.1). The primary vector of DENV is the mosquito *Aedes aegypti*, with additional transmission events attributed to other species in the genus *Aedes* (e.g., *Ae. albopictus*) [2]. Transmission from an infectious female mosquito to a human is achieved during blood meal acquisition [6]. Feeding by the mosquito is initiated by probing, which is the insertion of the stylet fascicle into the skin of the vertebrate in search of a capillary. Probing results in damage to the epithelium and vasculature, and is punctuated by expectoration of saliva containing DENV and numerous individual salivary proteins. Saliva and virus is deposited into the vertebrate skin, primarily within the dermal layer [7]. After the intrinsic incubation period, when virus has replicated and disseminated throughout

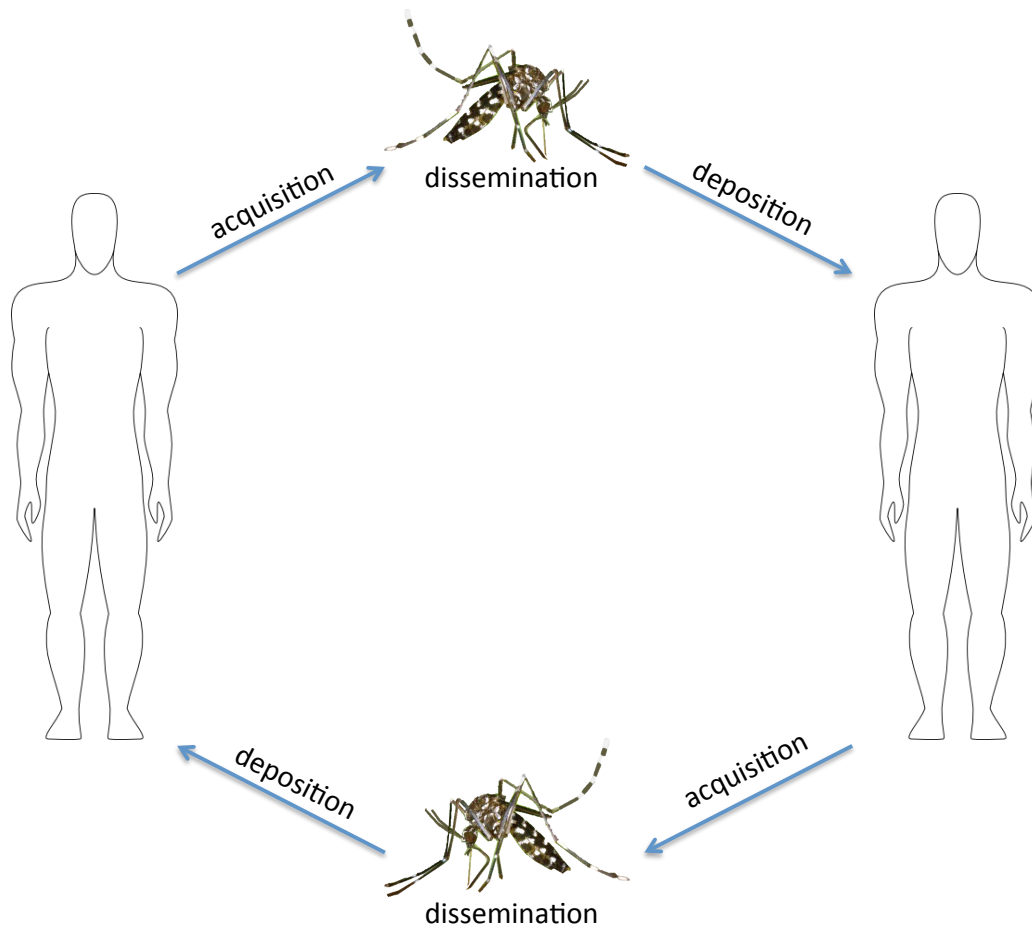


Figure 1.1: Transmission cycle of dengue virus. DENV is perpetuated in a cycle of acquisition, dissemination, and deposition between humans and the mosquito vector, *Aedes aegypti*.

the vertebrate to the point of freely circulating virus particles in the blood (viremia), transmission back to the mosquito is possible. Once again the virus is transmitted during acquisition of a blood meal, this time from vertebrate blood to mosquito [8]. After the extrinsic incubation period, when virus has overcome numerous physical and physiological barriers within the mosquito and disseminated to the salivary gland lumen, the transmission cycle as outlined here can begin again [9].

1.4 Viral deposition

Multiple investigations have shown that infectious mosquitoes inoculate their pathogens substantially, if not primarily, into the extravascular tissue of the vertebrate. Seminal work by Griffiths and Gordon observed and photographed the process of feeding by *Ae. aegypti* on the ear of a mouse, stating that once the fascicle has passed the epidermis, prior to the tapping of a blood vessel, salivation occurred at regular intervals during probing as puffs of clear liquid that would immediately disperse [7]. In another study, four species of West Nile virus (WNV, *Flaviviridae*, *Flavivirus*)-infectious mosquito – *Culex tarsalis*, *Cx. pipiens*, *Ae. japonicus*, and *Ae. triseriatus* – were allowed to probe on a demarcated section of mouse tail. Immediately following probing, the mice were euthanized and the tails were removed. Greater than 99% of the WNV recovered was found within the probed, one centimeter long section of tail, with only small amounts of virus recovered from circulating blood [10]. Similarly, when the tails of mice were fed upon by Rift Valley fever virus (RVFV, *Bunyaviridae*, *Phlebovirus*)-infectious *Cx. pipiens* and then amputated within five minutes post feeding, only 28% of mice died within 72 hours as compared to 92% mortality in the group not receiving amputations [11]. While these investigations do not specifically exclude viral delivery to the epidermis and subcutaneous layer, it seems likely that the salivary and viral inoculum would primarily interact with cells of the dermis.

1.5 Structure of the skin

Mammalian skin is primarily divided into two layers, the epidermis and the dermis, that lie upon a subcutaneous layer primarily composed of adipose tissue. The epidermis is subdivided into four additional layers: stratum corneum, stratum granulosum, stratum spinosum, and stratum basale, which is attached to a basement membrane. The epidermis is avascular, and

is primarily composed of keratinocytes, which contain pattern recognition receptors and are capable of producing cytokines, chemokines, and antimicrobial peptides. The epidermis also contains a few specialized immune cell types, such as Langerhans cells and $\gamma\delta$ T cells. Below the epidermis is the dermis, a highly vascular tissue in which the draining lymphatics also begin. This layer houses numerous resident immune cell types with the potential to interfere with the establishment of a viral infection, including fibroblasts, multiple subtypes of dendritic cells, macrophages, mast cells, $\gamma\delta$ T and $\alpha\beta$ T cells, and NK cells. Upon activation by antigen such as that from salivary proteins or virus, the antigen presenting cells in these layers mature and migrate through the draining lymphatics to the lymph nodes, potentially spreading virus throughout the body [12].

1.6 Vector saliva

Arthropod saliva has been the subject of numerous investigations regarding protein composition, functions in blood meal acquisition, and the impact of saliva on vertebrate immune responses. These investigations encompass the gamut of pathogen-transmitting, ectoparasite arthropod species, including ticks, black flies, sand flies, and mosquitoes [13]. While valuable insights and advances have been achieved through the study of this array of species, the scope of this review shall focus on mosquitoes of the subfamily Culicinae, particularly the genera *Culex* and *Aedes*.

1.6.1 Composition of saliva and blood meal acquisition

The mosquito sialome (salivary transcriptome + proteome) has been well characterized in terms of protein families and broad functionality. Saliva is known to be antihemostatic,

containing proteins that can inhibit clotting, platelet aggregation, and vasoconstriction for the purpose of aiding blood meal acquisition [13]. Among the protein groups of *Ae. aegypti* are D7s, which have been shown to inhibit biogenic amines; protease inhibitors, including serpins; enzymes, including serine proteases and nucleotidases such as apyrase and adenosine deaminase; immunity-related proteins such as C-type lectins, angiopoietins, a defensin, and a putative lysozyme; and a myriad of other proteins described only by their putative size or homology [14, 15]. Additionally, recent studies have described the impact of DENV infection on protein expression in the salivary glands and expectorated saliva of *Ae. aegypti* and postulates how these changes might affect virus transmission and establishment within the vertebrate [16, 17]. Among the proteins found in lower abundance were an adenosine deaminase and an aegyptin (discussed below) [17]. The decreased expression of these proteins during DENV infection could suggest that they impart negative pressure on viral perpetuation, and as such these proteins, along with a putative C-type lectin, were chosen for investigations herein with regard to their impact on DENV infection of the vertebrate. As exogenous antigen, both salivary proteins and virus encounter the vertebrate host immune system and consequently can influence the immunological environment of the bite site during viral establishment and infection.

1.6.2 – Methods and hurdles of investigation

There exists a great deal of variability and in some cases outright contradiction among the data discussed herein regarding salivary modulation of the vertebrate immune response and infection. At present, the confounding factors in existing publications are too numerous to identify the root of this variability, but there are several candidates for consideration. The following tables display many of the possible sources of variability in experimental approach to

examine salivary modulation of the immune response. The enumerations are taken from publications discussed and cited in the sections below.

There are several methods for introducing mosquito saliva and its related mosquito components into a model system, each with its pros and cons (Tables 1.1 and 1.2). In infectious mosquito delivery, mosquitoes (that have either imbibed a blood meal containing virus or have been intra-thoracically inoculated with virus and now have an infection that has disseminated to the salivary glands) are allowed to feed on the vertebrate to initiate infection. This is by far the most natural system, but the quantity of saliva and virus expectorated into each vertebrate is difficult to know and impossible to standardize. In spot-feeding, a naïve (non-infectious, non-exposed) mosquito is allowed to feed on a restricted area of skin and then a known concentration of virus is needle-inoculated into the location of feeding (or in one instance, immediately prior to feeding). The time between feeding and inoculation is usually kept to a minimum, but is occasionally of considerable length. Spot-feeding allows for the delivery of a known, pre-determined quantity and volume of virus, but the quantity of saliva delivered is still not standardized. The uninfected mosquito category is the same as that of the spot-feeding method, with the exception that there is no subsequent viral inoculation. Salivary gland extract or salivary gland homogenate (SGE) is generated by CO₂- or cold-anesthetizing female mosquitoes, dissecting out the salivary glands, and homogenizing the glands in a physiological buffer. The SGE is then needle-inoculated into the vertebrate skin, with or without virus in the same syringe. The SGE method includes additional, intracellular proteins and other components of the salivary glands themselves that are not typically introduced to the vertebrate upon feeding and therefore could alter the vertebrate infection and immune response in unnatural ways. The volume of actual secreted saliva proteins relative to the inoculum is also not known, but with this and all

subsequent needle-inoculation methods, the volume and concentration of virus delivery is easily standardized. Collection of saliva is achieved by prompting female mosquitoes to salivate into a physiological saline buffer or mineral oil held within a capillary tube or artificial feeding apparatus. This saliva is then collected, pooled, sometimes concentrated, and serves as the inoculum in much the same manner as SGE. Collection of saliva is very tedious work and, depending on the application, may require vast numbers of mosquitoes to achieve a usable quantity. The saliva method allows for a more natural protein profile in the inoculum than does SGE, though some of the smaller or less abundant saliva proteins may be lost during concentration. The recombinant protein method utilizes saliva proteins of known sequence expressed *in vitro*. This method allows for precise quantitation of the protein and virus inoculum, but often assumes native/functional conformation state and post-translational modifications, and lacks the influence of other proteins typically found in saliva. However, investigating individual proteins could allow for targeted manipulation of the infection and/or immune response for use in therapeutic development or novel vaccination strategies. The DNA vaccine method, published once in this body of literature, utilizes a vaccine vector containing the sequence for a specific salivary protein. This vector is then injected into the vertebrate for the *in situ* production of the saliva protein under investigation. The pros and cons of this method are similar to those of recombinant proteins, with the exception that quantitation of the saliva protein utilized is difficult at best and a native/functional protein is less tenable. The methods discussed here that involve needle-inoculation also do not adhere to any one inoculation route, or rather depth (Table 1.3), which may have an affect on the subsequent infection and immune response given the differing cell types within each skin layer [12, 18].

Table 1.1: Listing and enumeration of the various methods utilized in the published literature to introduce the mosquito/saliva variable into the experimental system.

Method	# of publications
Infectious mosquito	11
Spot-feeding	6
Uninfected mosquito*	5
Salivary gland extract [†]	14
Saliva	4
Recombinant Protein	3
DNA vaccine	1

* = distinct entries from spot-feeding; utilized in non-infection studies

[†] = includes one entry for “thorax extract”

Table 1.2: Relative value of the five main methods of mosquito/salivary involvement in the modulation of a viral infection *in vivo*. Check marks indicate the magnitude of added value or attribution each option would bring to the understanding in the far left column. Table modified from Mores et al, 2014. J Infect Dis. 2014 Jun 15;209.

	Infectious Mosquito	Co-inoculation with virus			
		Spot-feeding	Saliva	SGE	Proteins
Near natural route of exposure	✓✓✓	✓✓	✓✓	✓	✓
Calculable / known viral titer	✓	✓✓✓	✓✓✓	✓✓✓	✓✓✓
Known concentration of salivary inoculum	✓	✓	✓✓✓	✓✓	✓✓✓
Specificity of immune response modulation	✓✓	✓✓	✓✓	✓	✓✓✓

Table 1.3: Listing and enumeration of the route utilized to deliver needle-inoculated salivary components to the experimental animal model.

Injection Route	# of publications
Intramuscular	1
Subcutaneous	3
Intradermal	6

Another major factor to consider in the investigations of salivary impact on infection and immune response discussed herein is the species and strains of animals used. In terms of the vertebrate animal models, there is considerable diversity among the murine models, with more strains being utilized than there are papers utilizing murine models (Table 1.4). Many of these mice are known to have genetic and immunologic predispositions and, in some cases, resistances that make the comparison of their data more difficult. For example, male and female C57BL/6 mice are known to be predisposed to more prominent innate and adaptive immune responses, respectively [19]. C57BL/6 and BALB/c strains have immune responses that are predisposed toward T_H1 and T_H2 , respectively [20]. C3H/HeJ mice have hyporesponsive TLR4 [21]. C57BL/6, BALB/c, and C3H mice have also demonstrated differential susceptibility and immune response to viral challenge [22]. Among the seven distinct species of mosquito utilized in these investigations, there are at least eleven strains used (and considerably more unspecified; Table 1.5). These mosquitoes have different feeding habits and preferences, and sialome variation has been demonstrated among the different colonies [23, 24].

Table 1.4: Listing and enumeration of the various murine model strains utilized in investigating the effects of mosquito/salivary involvement on the vertebrate immune response. This table does not include the additional model systems utilized: chicken, deer, chipmunk, the non-human primate Vero cell line, and primary and immortalized human cell lines.

Mouse strain	# of publications
129	1
129 IFNAR ^{-/-}	1
BALB/c	2
BALB/c OVA-TCR DO11	1
TCR transgenic BALB/c Thy1.1	1
BALB/c Thy1.2	1
C3H/HeJ	4
C3H/HeN	1
C3H/RV (C3H.PRI-Flv)	1
C57BL/6	3
C56BL/6-NRJ	1
C57BL/6 IFNAR ^{-/-}	1
C57BL/6 IRF3/7 ^{-/-/-}	1
CD-1	1
DBA-1	1
ICR	2
NIH Swiss	2
Swiss Webster	1
Humanized [‡]	1

[‡] = NOD/SCID/IL2R γ null mice transplanted with human cord blood CD34+ cells

Table 1.5: Listing and enumeration of the mosquito species utilized in investigating the effects of mosquito/salivary involvement on the vertebrate immune response. This information includes mosquitoes utilized as sources for primary reagents (e.g., SGE).

Mosquito species	# of publications	# using field caught	# using colony*	# of different colony strains*	Remainder unspecified
<i>Aedes aegypti</i>	16	0	8	≥ 4	8
<i>Aedes albopictus</i>	1	0	1	1	0
<i>Aedes triseriatus</i>	4	1	1	1	2
<i>Aedes vexans</i>	1	0	0	0	1
<i>Culex pipiens</i>	4	0	3	≥ 1	0
<i>Culex quinquefasciatus</i>	1	0	1	1	0
<i>Culex tarsalis</i>	4	0	4	3	1

* = if a strain designation is used, the publication is considered “colony”

The most pertinent potential source of variation to the novel investigations presented in this dissertation is the diversity among DENV strains utilized in saliva-modulated infection models and the very limited number of publications on their investigation (Table 1.6). With regard to DENV2, there are only four publications that experimentally evaluate the impact of mosquito/salivary involvement on the vertebrate infection and immune response (with the possible addition of two more that do not specify a serotype in the published manuscript). Among these four publications, there are seven different strains used: four in one paper, one in each of the other three. These strains vary in their passage history and genetic sequence, which makes comparison all the more difficult.

Table 1.6: Diversity of DENV strains utilized in the limited number of publications investigating the impact of mosquito/salivary involvement on DENV infection and vertebrate immune response.

DENV serotype	# of pubs	# of DENV strains
DENV1	1	1
DENV2	4	7
DENV3	1	1
DENV4	2	2
DENV?*	2	?

* = unspecified serotype and strain

1.6.3 Impact of saliva in the vertebrate – Cellular modulation

Mosquito saliva has been demonstrated to impact vertebrate immune cell functions *in vitro* and *in vivo* outside the context of an infection. *Ex vivo* treatment of murine splenocytes with *Ae. aegypti* SGE reduced antigen-specific (OVA) and non-specific (ConA) splenocyte proliferation overall, as well as decreasing CD4⁺ and CD8⁺ T-cell proliferation and increasing T-cell (both CD4⁺ and CD8⁺) and B-cell (B220⁺) mortality. In contrast, SGE of *Cx. quinquefasciatus* induced no significant changes [25]. In another *ex vivo* experiment utilizing murine splenocytes, pre-incubation with *Ae. aegypti* SGE suppressed proliferation of OVA- or ConA-stimulated T-cells and LPS-stimulated B cells in a dose-dependent manner. Additionally, higher concentrations of SGE (≥ 2.5 SGP-equivalents) again significantly decreased CD4⁺ and CD8⁺ T-cell viability, whereas concentrations tested of up to 25 SGP-equivalents had no effect on dendritic cell viability [26]. In contrast, intradermal injections of *Ae. aegypti* SGE into a mouse one hour prior to ID stimulation with antigen resulted in significantly increased proliferation of CD4⁺ cells [27]. Humanized mice bitten by naïve *Ae. aegypti* exhibited

thrombocytopenia as compared to unexposed, control mice [28], and naïve *Ae. aegypti* feeding on the ears of mice induced recruitment of eosinophils to the bite site [29]. In mice vaccinated with the recombinant salivary protein D7 of *Cx. tarsalis*, the bite of naïve mosquitoes resulted in increased infiltrating polymorphonuclear and mononuclear cells at the bite site as compared to unvaccinated mice [30].

1.6.4 Impact of saliva in the vertebrate – Cytokines, antibodies, & transcripts

Mosquito saliva has been demonstrated to impact vertebrate cytokine production *in vitro* and *in vivo* outside the context of an infection, typically in association with the cellular modulations outlined above. Incubation of monocyte derived dendritic cells *in vitro* with *Ae. aegypti* saliva resulted in increased production of IL12p70 but had no effect on expression of the surface markers CD40, CD83, HLA-ABC, and HLA-DR [31]. *Ex vivo* treatment of murine splenocytes with *Ae. aegypti* SGE resulted in decreased IFN γ and IL10, whereas *Cx. quinquefasciatus* SGE elicited no change [25]. In an *ex vivo* experiment utilizing OVA-stimulated murine splenocytes, pre-incubation with *Ae. aegypti* SGE resulted in decreased production of all cytokines assessed in a dose-dependent manner, with lower concentrations suppressing IL2, IL5, IL12, IFN γ , GMCSF, and TNF α , and higher concentrations additionally suppressing IL4 and IL10 [26]. In contrast, intradermal inoculation of SGE resulted in increased IL4 production at the inoculation site in a mouse [32]. Naïve *Ae. aegypti* feeding on the ears of mice resulted in decreased expression of TLR3 and IFN γ and increased expression of IL4 and IL10 in the skin at the bite site, though these differences were not assessed statistically [29]. *Ae. aegypti* bitten mice exhibited significantly increased IL4 at the location of mosquito bite at three hours post feeding, but demonstrated no significant alterations in the production of IL2, IL10,

IL12p40, IFN γ , and TNF α . Additional investigations in this study revealed that vaccination with a DNA vector containing the sequence for salivary protein SAAG-4 (another designation for the short form of aegyptin) induced elevated levels of IL4 and IL10 and decreased levels of IL12p40, IFN γ , and TNF α . Intradermal injections of SGE or the SAAG-4 DNA vector also yielded a higher percentage of CD4 $^{+}$ T-cells with positive, intracellular staining for IL4 [27]. Splenocytes from mice bitten by *Cx. pipiens* or *Ae. aegypti* collected over several days post feeding and then stimulated with ConA demonstrated a T_H2 cytokine shift, characterized by increased IL4 and IL10 (days 4-7) followed by decreased IFN γ (days 7-10) with similar effects seen for both mosquito species. These splenocyte cytokine expression patterns were mimicked by the injection of mice with the synthesized, salivary peptides sialokinin I and II of *Ae. aegypti* [33]. Splenocytes of mice vaccinated with the recombinant salivary protein D7 of *Cx. tarsalis* produced increased IFN γ and decreased IL10 upon rD7 stimulation two days after the mice received naïve mosquito bites as compared to unvaccinated mice. In contrast, the same study produced elevated levels of serum IFN γ in mice at four days post naïve mosquito bite [30].

1.7 Saliva-mediated modulation of viral infection

Mosquito saliva has been shown to modulate infection of the vertebrate host by a diverse range of viruses. All modulation described below are from the perspective of simultaneous delivery of virus and saliva through the described method versus inoculation of virus alone unless otherwise indicated.

1.7.1 Cellular modulations & distribution

Including mosquito saliva in investigations of viral infection can result in changes to cellular viability, recruitment, and general inflammation. In an *in vitro* experiment using human moDCs derived from primary PBMCs, co-inoculation *Ae. aegypti* saliva and DENV significantly increased cellular survival by decreasing late-stage apoptosis [31]. Co-inoculation of mice with SGE and RVFV significantly decreased total leukocyte and platelet counts, though the leukocyte differential was unaffected. Co-inoculation with SGE also resulted in multifocal hepatitis and increased neutrophil infiltration to these sites [34]. In a murine model of WNV infection, *Ae. aegypti* spot-feeding reduced T (CD3+) cell recruitment to the bite/inoculation site when compared to animals that received only ID injection of WNV, but did not significantly affect the recruitment of neutrophils, Langerhans cells, dendritic cells, or macrophages. There was no change elicited to cell recruitment in the draining lymph node [35]. In mice exposed via WNV-infected mosquito, pre-sensitization to *Ae. aegypti* saliva increased inoculation site tissue size by greater than 200%, exhibiting an increased influx of plasma cells, histiocytic cells, neutrophils, and a greater than two-fold increase in MHCII-positive cells as compared to naïve mice. Draining lymph nodes displayed marked expansion and increased numbers of MHCII+ cells, CD11b+ cells, and decreased numbers of CD3+ cells [36].

1.7.2 Cytokines, antibodies, & transcripts

Much of the work examining the effects of mosquito saliva on viral pathogens suggests that there is a shift away from an antiviral, T_H1 immune response toward a T_H2 response, among other immunological alterations, exemplified best *in vivo*. In an *in vitro* experiment using human moDCs derived from primary PBMCs, co-inoculation of *Ae. aegypti* saliva and DENV

significantly increased production of IL12p70 and TNF α , but did not alter production of IFN α (Ader 2004). In primary human keratinocytes culture infected with DENV, co-inoculation of *Ae. aegypti* SGE significantly reduced production of β -defensin3, IFN α , IFN β , IFN γ , LL-37, Elafin, and S100A7 [37]. In a second *in vitro* study utilizing primary human keratinocytes, co-exposure of cells with DENV and individual, recombinant salivary proteins of *Ae. aegypti*, resulted in decreased expression of the antiviral and antimicrobial peptide transcripts for IFN α , IFN β , IRF3, IRF7, LL-37, S100A7, and RNase7 depending on the protein and concentration utilized. The four proteins utilized were FXa-directed anticlotting serpin-like protein (GenBank Q1HRTV7_AEDAE), an adenosine deaminase (Q179D4_AEDAE), a 34-kDa family secreted salivary protein (Q1HRW0_AEDAE), and a putative secreted protein (Q8T9U5_AEDAE), with the 34-kDa protein eliciting the most marked decreases [38]. *In vitro* assessment of murine peritoneal macrophages revealed that *Ae. aegypti* SGE significantly lowered IFN β , iNOS, and IL12 in response to WNV infection, but did not significantly alter levels of IL10, IFN γ , or IL1 β . As a point of comparison, SGE modulated the response to sindbis virus (SINV, *Togaviridae*, *Alphavirus*) infection in these cells by lowering IFN β and iNOS production, and the initial elevation and subsequent decrease of IL10 production, with no effect on IFN γ , IL1 β , or IL12. Bone marrow derived DCs also produced lower IFN β *in vitro* as a result of *Ae. aegypti* SGE modulation of WNV infection, but had no effect on production of IFN γ , IL2, IL4, IL12, and IL1 β [35]. Co-inoculation of *Ae. triseriatus* SGE and vesicular stomatitis virus (VSV, *Rhabdoviridae*, *Vesiculovirus*) reduced expression of IFN α 2 in the murine fibroblast cell line L929 [39]. Co-exposure of the human keratinocyte cell line HaCaT with *Ae. albopictus* SGE and chikungunya virus (CHIKV, *Togaviridae*, *Alphavirus*) produced significantly decreased levels of IL8 [40].

Delivery of DENV2 by infected *Ae. aegypti* into humanized mice elicited greater levels of circulating IFN γ , IL8, and MCP1 and increased the number of anti-DENV IgM positive animals. Mosquito delivery also reduced temperature, modulated thrombocyte levels compared to needle or control, and increased the erythema index. The feeding of four or more infected mosquitoes was required for consistent clinical results in this model [28]. Two methods, ID inoculation of DENV2 into humanized mice 30-60 minutes following *Ae. aegypti* feeding at a distal site and ID inoculation of DENV and *Ae. aegypti* saliva simultaneously, lowered erythema index and increased body temperature compared to mosquito-delivered virus infection, but did not significantly affect erythema index or temperature compared to DENV-only treatment. However, simultaneous inoculation of saliva and DENV lowered thrombocyte count relative to normal on the single reported day, whereas the delayed and distal spot-feeding did not elicit any changes to thrombocyte count [28].

Delivery of vesicular stomatitis New Jersey virus (VSNJ, *Rhabdoviridae*, *Vesiculovirus*) to mice by infected *Aedes triseriatus* increased anti-VSNJ antibody production from 13% to 94% in three week old mice and from 11% to 73% in eight month old mice [41]. In a murine model of SINV infection, co-inoculation of *Ae. aegypti* SGE resulted in significantly decreased IFN γ and IFN β with concomitantly increased IL4, IL10, and IL12p40 at the inoculation site [32]. Similarly, infection of mice via CHIKV-infected *Ae. aegypti* resulted in significantly increased IL4 and IL10 production and decreased production of IFN γ , IL2, and TLR3 [29]. In a murine model of WNV infection, *Ae. aegypti* spot-feeding significantly increased IL10 production in the exposed skin and draining lymph node [35]. In mice exposed via WNV-infected mosquito, pre-sensitization to *Ae. aegypti* saliva increased production of IL10 in the inoculation site as compared to naïve mice, as well as increased IL10 and IL4 in the draining lymph node, though

the latter two were not significant [36]. Infected-mosquito delivery of WNV into mice that have been vaccinated against the salivary protein D7 of *Cx. tarsalis* resulted in greater production of IL10 and decreased production of IFN γ , TNF α , IL12p70, and MIP1 from *ex vivo* splenocytes when compared to that of unvaccinated mice [30]. In contrast, infected mosquito delivery of WNV into mice that have been vaccinated against the SGE of *Cx. tarsalis* resulted in increased IFN γ , TNF α , and IL4 in the spleen and increased anti-WNV neutralizing antibody titers when compared to unvaccinated mice [42].

1.7.3 Viral titers in inoculation sites and circulation

With a few exceptions included herein, inoculation via mosquito bite and exposure to saliva have been shown to enhance viral infection or potentiate infection in otherwise non-permissive models. In an *in vitro* experiment using human moDCs derived from primary PBMCs, co-inoculation of *Ae. aegypti* saliva significantly decreased the percentage of DENV2 (40% \rightarrow 18%) and DENV4 (38% \rightarrow 18%) infected cells. Additionally, pre-incubation of moDCs with saliva further inhibited infection with DENV4. Co-inoculation of saliva did not alter yellow fever virus (YFV, *Flaviviridae*, *Flavivirus*) infection in this system [31]. In contrast, *in vitro* infection of mouse embryonic fibroblasts pre-exposed to SGE for ten minutes revealed increased viral RNA in the cells and increased virion production into the supernatant by all four serotypes of DENV [43]. Similarly, in an *in vitro* study utilizing primary human keratinocytes, co-inoculation of *Ae. aegypti* SGE significantly increased DENV titers [37]. In a second study utilizing primary human keratinocytes, co-exposure of cells with DENV and individual, recombinant salivary proteins of *Ae. aegypti*, resulted in greater concentrations of viral RNA. This enhancement occurred at similar levels regardless of the protein tested (listed above in

subsection 1.7.2), and may occur in a dose-dependent manner, though this was not assessed statistically [38]. Co-exposure of the human keratinocyte cell line HaCaT with *Ae. albopictus* SGE and CHIKV produced significantly increased levels of CHIKV RNA [40]. In an *in vitro* study of VSV infection, co-inoculation of *Ae. triseriatus* SGE significantly increased production of VSV in the murine fibroblast cell line L929, but not in the type I IFN-deficient Vero cell line [39]. Interestingly, *in vitro* assessment revealed that *Ae. aegypti* SGE had no effect on WNV or SINV titers in murine peritoneal macrophages, nor on WNV titers in bone marrow derived DCs [35].

Delivery of DENV2 by infected *Ae. aegypti* into humanized mice resulted in a longer duration of viremia, reaching up to 56 days and thereby far exceeding that of a natural human infection. The feeding of four or more infected mosquitoes was required to extend detection of circulating viral RNA beyond that of needle inoculation in this model. Additionally, ID inoculation of DENV2 into humanized mice 30-60 minutes following mosquito feeding at a distal site resulted in a viremia comparable to that of infected mosquito delivery. ID inoculation of DENV2 and *Ae. aegypti* saliva simultaneously into humanized mice also resulted in a viremia statistically comparable to that of infected mosquito delivery, though it trended lower [28]. In contrast, in mice co-inoculated with DENV2 and *Ae. aegypti* SGE, no difference was found between groups regarding the inoculation site, spleen, or viremia DENV titers at the single time point assessed, 24 hours post exposure. However, DENV titers were found to be significantly elevated in the draining lymph node, and this enhancement was found to be significantly inhibited by the addition of a serine protease inhibitor [43].

Delivery of La Crosse virus (*Bunyaviridae*, *Orthobunyavirus*) by infected *Ae. triseriatus* into deer and chipmunks significantly increased the magnitude and duration of viremia when

compared to IM needle-inoculation of virus alone [44]. Spot-feeding by naïve *Ae. aegypti*, *Ae. triseriatus*, or *Cx. pipiens* on mice potentiated infection of Cache Valley virus (*Bunyaviridae*, *Orthobunyavirus*) and led to neutralizing antibody production in these otherwise non-permissive vertebrates after subcutaneous injection of virus. This potentiation of infection occurred with up to a four hour delay between mosquito feeding and injection, but did not occur when injection of virus was administered in a site distal (greater than four centimeters) to the feeding site. In contrast, injection of virus plus “thorax extract” (an SGE approximation) did not lead to infection or production of neutralizing antibodies [45]. In mice infected with RVFV, spot-feeding and intradermal co-inoculation with SGE led to an earlier median day of death. Co-inoculation with SGE also significantly increased RVFV viremia titers, and titers in the brain, liver, inguinal lymph node, spleen, thymus, lungs, kidneys, bladder, and heart. In contrast, RVFV titers were decreased in the cerebellum and pancreas, and unaffected in the mesenteric, aortic, and popliteal lymph nodes and salivary glands [34]. Infection of mice via CHIKV-infected *Ae. aegypti* resulted in infiltration of neutrophils and abundant eosinophils to the inoculation site, as compared to undetectable recruitment in mice bitten by naïve mosquitoes or those needle-inoculated with CHIKV alone [29].

The bite of WNV-infected *Culex spp.* significantly increased WNV viremia in chickens and increased viral shedding to the oral mucosa and cloaca. Additionally, chickens bitten by multiple infected mosquitoes (n= 3-11) exhibited early viremias with WNV titers far exceeding that of a strictly additive or viral dose-dependent effect (25-50 times greater than that resulting from a single infected mosquito). This enhancement is likely attributable to other factors involved in viral inoculation, such as the damage caused by probing or the proteins in the salivary inoculum [46]. Spot-feeding of mice by *Ae. aegypti* that are then inoculated

intradermally with WNV exhibited viremias with greater magnitude and duration, as well as significantly increased WNV titers in the brain. Spot-feeding decreased survival rates from 24.9% to 0% in mice inoculated with 10^2 PFU of WNV, and mice co-inoculated with SGE exhibited a survival rate between that of spot-feeding and needle-inoculation alone mice. Spot-fed mice also displayed a consistent trend toward elevated WNV titers in the exposed skin and draining lymph node, though not significant [47]. In a later study by this group, *Ae. aegypti* spot-feeding significantly increased viral titers in the exposed skin and draining lymph node in a murine model of WNV infection [35]. Infection of mice by a single WNV-infected *Cx. tarsalis* resulted in decreased WNV titers at the inoculation site at a single time point (24 hours) post exposure, whereas spot-fed mice inoculated subcutaneously initially presented decreased WNV titers (below limit of detection) at twelve hours post exposure that then surpassed needle-inoculation alone titers at 24 hours post exposure. No differences were observed in the draining lymph node in these mice. Infected-mosquito delivery, spot-feeding, and co-inoculation with SGE all resulted in significantly increased WNV viremia titers, up to ten times greater on days one and two post exposure, with spot-feeding and SGE extending this elevation to day three post exposure. Spot-feeding and infected-mosquito delivery led to faster dissemination of virus to other tissues, a greater proportion of mice experiencing neuroinvasion, and increased WNV titers in the spleen and spinal cord. Additional IV inoculation of WNV did not affect viremia or tissue titers, nor was there a notable difference between spot-fed viremia titers and infected mosquito viremia titers, suggesting that variations in the location of viral deposition itself did not cause the enhancement of WNV in these mice. SGE inoculation in a site distal to WNV inoculation did not lead to enhancement, indicating once again a local effect by SGE on the pathogenesis of infection enhancement. Importantly, spot-fed enhancement still occurred in mice with antibodies

against saliva (due to pre-exposure to *Cx. tarsalis* feeding), with these mice exhibiting two-times the WNV viremia of mice without antibodies against saliva at 24 hours post exposure [48]. Pre-sensitization of mice to *Ae. aegypti* saliva enhanced WNV titers in the draining lymph node and WNV-induced mortality in mice inoculated via infected mosquito when compared to naïve mice. Additionally, a greater number of pre-sensitization treatments resulted in greater mortality rates [36]. Infected mosquito delivery of WNV into mice that have been vaccinated against the salivary protein D7 of *Cx. tarsalis* resulted in significantly increased mortality when compared to unvaccinated mice [30]. In contrast, infected mosquito delivery of WNV into mice that have been vaccinated against the SGE of *Cx. tarsalis* resulted in delayed neuroinvasion, decreased WNV titers in the brain, and decreased (zero) mortality when compared to unvaccinated mice [42].

1.8 Aegyptins

Aegyptins, the subject of the fourth chapter of experimental investigations, are a family of GE-rich 30-kDa proteins that possess multiple direct and indirect physiological effects. There are two subclades of these proteins within *Aedes*, designated subclade I and subclade II. Prior analysis of expectorated saliva from DENV2 infected *Ae. aegypti* using 2D gel electrophoresis and LC-MS/MS identified a subclade I aegyptin (gi|18568322) that was reduced 14.1-fold when compared to saliva from uninfected mosquitoes [16]. This aegyptin has also been termed “SAAG-4,” and has been shown to suppress IFN- γ expression and increase IL-4 expression by CD4⁺T cells outside the context of a viral infection [27]. The archetypal protein of the aegyptin family in *Ae. aegypti* is a member of subclade II, and has been shown to perform two distinct roles within the vertebrate. First, as an allergen, this protein (termed “Aed a 3,” gi|205525920)

has been shown to induce positive skin-test reactions, and IgG and IgE antibody responses in sensitized mice and humans [49, 50]. Researchers have also found an association between serum reactivity to this protein and the mild DENV disease state in clinical patients in Thailand [51]. Second, aegyptin (gi|94468546, identical in sequence to Aed a 3) has demonstrated the capacity to bind to collagen, inhibiting its interaction with glycoprotein IV, integrin $\alpha 2\beta 1$, and von Willebrand factor, which could facilitate blood feeding by reducing the formation of blood clots [52, 53]. Indeed, transgenic suppression of aegyptin expression in *Ae. aegypti* significantly reduced blood feeding success. Probing time increased from a range of 15-21 seconds to a range of 78-300 seconds before initiation of successful blood acquisition, and those mosquitoes that did feed acquired smaller blood meals [54].

While the two aegyptin groups represent distinct subclades, the acidic (glycine-, aspartic acid-, and glutamic acid-rich) aminoterminal domain and the more complex carboxyterminal domain characteristic of this protein family remain conserved [13]. Due to the involvement of aegyptins with both the vertebrate immune response and hemostasis, we explored the impact of a recombinant, subclade II aegyptin on the vertebrate immune response within the context of a DENV infection.

1.9 Hypothesis & research overview

The overarching hypothesis governing the novel investigations presented in this dissertation is that mosquito saliva will aid in the establishment of DENV infections within the vertebrate, and that distinct immunological alterations involved in this enhancement will be attributable to individual salivary proteins. I therefore conducted investigations into the triad of vector-virus-vertebrate interactions aimed at further characterizing the following:

- 1) the strain-based impact of DENV2 infection on salivary gland protein transcript expression in *Ae. aegypti*. This is the subject of Chapter II, where high-throughput RNA-sequencing technology was implemented to quantify transcriptional abundance in Rockefeller colony mosquitoes with disseminated infections. Potential differences in protein production may provide selective advantage to the perpetuation of specific DENV strains through enhancements in establishment of infection and in viremia within the vertebrate.
- 2) the probing-based modulation of vertebrate immune responses in the skin and the subsequent viremia during DENV2 infection of a murine model of transmission. In Chapter III, modulations of the immune response in *Ae. aegypti* spot-fed inoculation sites of C57BL/6 IRF3/7^{-/-} mice were assessed via transcript arrays and targeted qRT-PCR analysis. These modulations were then associated with a greatly enhanced viremia that could potentially support increased acquisition rates among naïve mosquitoes.
- 3) the effect of individual salivary proteins from *Ae. aegypti* on DENV2 replication in a human hematopoietic cell line. In Chapter IV, three recombinant salivary proteins with known and putative functions (an aegyptin, a C-type lectin, and an adenosine deaminase) were generated and co-inoculated alongside DENV2 into K562 cell culture for assessment of DENV2 replication kinetics.
- 4) the influence of the salivary protein aegyptin on DENV2 infection in the mouse. In Chapter V, modulations in the C57BL/6 IRF3/7^{-/-} murine immune response, inoculation site viral titers, and viremia due to co-inoculation of DENV2 and recombinant aegyptin were assessed. The results of this investigation provide a rationale for the decreased abundance of aegyptin in the expectorated saliva of DENV2-infected *Ae. aegypti*.

As detailed above, these investigations focus on early, establishment-relevant time points regarding DENV2 infection in the vertebrate, and differences in infection kinetics with the potential to alter transmission success.

1.10 References

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CHAPTER 2

A VIRAL STRAIN-BASED HIGH-THROUGHPUT RNA-SEQUENCING ANALYSIS OF *Aedes aegypti* SALIVARY GLAND TRANSCRIPTOME UPON INFECTION WITH DENGUE VIRUS

2.1 Introduction

Dengue virus (DENV), the etiologic agent of dengue fever, is an enveloped, positive-sense, single-stranded RNA virus of the genus *Flavivirus*. DENV is maintained in a primarily anthroponotic cycle between humans and the *Aedes aegypti* mosquito [1]. Feeding by *Ae. aegypti* on vertebrate hosts involves the deposition of saliva, and thereby virus, into the skin [2-5]. Mosquito saliva contains many proteins that modulate host hemostasis and immune responses, facilitating blood feeding and virus transmission [6, 7]. *Ae. aegypti* saliva also has been shown to contain allergenic proteins [8]. The vertebrate immune response to DENV infection is altered as a result of mosquito probing and the introduction of these immunogenic salivary proteins, thereby altering DENV infection kinetics and the likelihood of viral perpetuation [9].

As part of the investigation into the dynamics of the interface between mosquito, virus, and vertebrate, researchers have analyzed the composition of *Ae. aegypti* salivary glands at the transcriptional and protein expression levels [7, 10-16]. Work has been done to ascribe function to some of these salivary components and investigate their individual effects on vertebrate hemostasis and immune response, with many disrupting specific events in hemostasis (e.g., collagen-binding to platelets or vasoconstriction) or modulating the production of T_H1 and T_H2 associated cytokines [10, 17-25]. Additional work into individual mosquito salivary proteins has shown that some serve as a boon to arboviral infection of the vertebrate, while other(s) may prove to be only a detriment [26-28]. *Ae. aegypti* saliva has been shown to also elicit protein-

specific IgG and IgE responses in humans [16, 29]. The functionality of these individual salivary proteins in the context of establishment of a DENV infection in the vertebrate is an area that still requires thorough investigation.

Of particular importance are recent studies describing the effect that DENV serotype 2 (DENV2) infection has on transcript and protein expression in the salivary glands or expectorated saliva of *Ae. aegypti*, each conducted with a different strain of virus and varying strains of mosquito. One study using microarrays at the transcript level primarily demonstrated an increase in salivary gland transcript quantity upon DENV2, strain New Guinea C infection of Rockefeller strain mosquitoes [30]. In contrast, a high-throughput RNA-sequencing (RNA-seq) investigation primarily demonstrated a decrease in salivary gland transcript quantity upon DENV2, strain Jam1409 infection of Chetumal strain mosquitoes [31]. As a point of comparison, two studies utilizing two-dimensional gel electrophoresis at the protein level measured protein levels that are predominantly decreased or unaltered upon infection of Rockefeller strain mosquitoes with DENV2, strain 1232 [32, 33]. The disparity between these results indicates the need for an investigation into the strain-based influence of DENV infection on salivary gland gene expression in a unified setting.

As such, we examined the salivary gland transcriptome of female *Ae. aegypti* during infection with each of two strains of DENV2 in comparison to a sham-exposed control. This investigation utilizes high-throughput RNA-seq analysis and is the first study to conduct a side-by-side analysis of transcriptome modulation by multiple strains within one serotype. The elucidation of strain-based differences, particularly in the abundance of expectorated salivary proteins, will provide a foundation from which to more accurately examine the interplay of

DENV and saliva during establishment of viral infection within the vertebrate host and how these interactions impact DENV perpetuation.

2.2 Materials and Methods

2.2.1 Viruses

DENV serotype 2 (DENV2) strains 1232 and 16803 were propagated as described previously [9]. Briefly, we inoculated T-75 flasks of confluent Vero cells with 100 μ l of viral stock and incubated them for 30 min. Eight milliliters of M199E medium with 10% fetal bovine serum and 2% penicillin-streptomycin-amphotericin B (Fungizone) (P/S/F) was added. The flask was incubated at 37°C with 5% CO₂ for 5 days, and subsequently the supernatant was collected for virus. The supernatants were found to contain 1.36×10^7 PFU/ml for 1232 and 2.61×10^7 for 16803 by plaque assay as described previously in the supplemental material of reference [34], with modification. M199E medium containing P/S/F was used, and incubations occurred at 37°C.

2.2.2 Mosquito rearing and inoculation

Laboratory strain *Ae. aegypti* (Rockefeller) were maintained in an environmental chamber at 28°C and 75 to 80% humidity and subjected to a 16:8 light-dark photoperiod regimen until the time of use. Mosquitoes were provided with a water and 10% sucrose solution ad libitum, which was removed 24 h prior to blood feeding and replaced thereafter. Approximately one week post emergence, female mosquitoes were allowed to feed on bovine blood in Alsever's anticoagulant via Hemotek feeding device (Discovery Workshops, Lancashire, England), after which the blood-fed females were sorted. After 48 hours, these were then inoculated

intrathoracically with either DENV2 strain 1232 or strain 16803 as previously described [33].

The control group mosquitoes, also previously blood fed, received an inoculation of BA1 media (M199E, 10% bovine serum albumin, 0.1g/L L-glutamine, 2.2g/L sodium bicarbonate, 25mM HEPES, 2% P/S/F, titrated to 7.4 pH with Tris and HCl) without virus. All mosquitoes were collected from the same rearing cohort to eliminate the influence of the potential variables of larval density, resource competition, and temperature or humidity fluctuations on salivary gene expression.

2.2.3 Salivary gland collection and dissemination testing

After an 11-day extrinsic incubation period, mosquitoes were dissected, and their salivary glands were removed and stored individually at -80°C in 70 μl RLT buffer. Legs were removed and placed in 400 μl BA1 media for assessment of DENV positivity as a proxy for dissemination, as performed previously [32]. Legs were disrupted and homogenized using the TissueLyser (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. DENV RNA was extracted using the MagMax-96 Viral RNA Isolation Kit (Ambion/Life Technologies, Carlsbad, CA) and detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay with the Superscripts III® Platinum® One-Step qRT-PCR system (Life Technologies, Carlsbad, CA) on the LightCycler 480 (Roche Diagnostics Corp., Indianapolis, IN) and DENV concentration standards derived from plaque assays as previously described [26, 35]. Only the salivary gland pairs from the mosquitoes with disseminated infections confirmed by qRT-PCR were combined to create a pool of infected salivary gland extract: 10 pairs pooled for each BA1 and 16803, 9 pairs pooled for 1232.

2.2.4 RNA processing and quality assessment for sequencing.

Upon confirmation of dissemination as above, the RLT solutions containing salivary gland pairs were pooled and then RNA was extracted using the RNeasy Tissue Mini kit (Qiagen, Valencia, CA). A portion of this elution was then DNase I treated with the TURBO DNA-free™ Kit (Ambion/Life Technologies, Carlsbad, CA) and then processed with the NucleoTrap® mRNA Mini kit (Clontech Laboratories Inc., Mountain View, CA) to select for mRNA.

cDNA libraries were then constructed using the Ion Total RNA-Seq Kit v2 (Life Technologies, Carlsbad, CA) following the low input protocol to be analyzed in the Ion Proton System (Life Technologies, Carlsbad, CA). Library quality was analyzed using the Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer Instrument (Agilent Technologies, Inc., Santa Clara, CA). Average library size for the 1232 sample was found to be approximately 156 bp; for BA1 approximately 122 bp, and for 16803 approximately 133 bp. Libraries were diluted to the 11 pM concentration each (1232 was diluted 1: 570, BA1 was diluted 1:319, and 16803 was diluted 1:1500) and used for sequencing template preparation. Template-positive Ion Sphere Particles (ISPs) containing clonally amplified DNA was produced using the Ion PI Template OT2 200 Kit v2 (for 200 base-read libraries) with the Ion OneTouch 2 instrument. Ion OneTouch ES was used to enrich ISPs intended for Ion Proton System using the Ion PI Sequencing 200 Kit v2. Individual Ion PI sequencing chips (no barcoding) were used for each of the sequencing samples.

2.2.5 Data analysis

The *Ae. aegypti* reference genome assembly utilized for Ion Proton output alignment and further analysis is AaegL3, specifically the VectorBase scaffold file *Aedes-aegypti*-

Liverpool_SCAFFOLDS_AaegL3.fa. BAM files resulting from a standard Ion Proton processing and alignment of reads were uploaded to Galaxy (<https://usegalaxy.org>) [36-38]. A gene transfer format (GTF) file was generated for each BAM file using Cufflinks (Galaxy Tool Version 0.0.7) with a maximum intron length of 300000, minimum isoform fraction of 0.1, pre-mRNA fraction of 0.15, effective length correction set to “yes,” and using the VectorBase reference annotation *Aedes-aegypti*-Liverpool_BASEFEATURES_AaegL3.1.gtf as guide [39]. As the GTF file generated for strain 1232 repeatedly encounters an unknown error and returns as “empty,” only the GTF files for BA1 and strain 16803 were combined using Cuffmerge (Galaxy Tool Version 0.0.6) and the reference annotation utilized above [39]. Differential expression analysis was performed using Cuffdiff (Galaxy Tool Version 0.0.7) on these three BAM files with the merged GTF file, a geometric library normalization method, blind dispersion estimation method, a false discovery rate of 0.05, and a minimum alignment count of 10 [39]. Because the above samples are pooled, each pool only represents a statistical sample size of one and therefore the p value will be utilized as a guide rather than to strictly assess significance.

2.2.6 RT-qPCR confirmation of transcript levels

The transcript levels of clade I and clade II aegyptins in the salivary gland pools above were analyzed using two-stage reverse transcription quantitative polymerase chain reaction on the LightCycler 480 (Roche Diagnostics Corp., Indianapolis, IN). cDNA creation was performed using the SuperScript® III First-Strand Synthesis System (Life Technologies, Carlsbad, CA) following the random hexamer protocol. qPCR was performed using the GoTaq® qPCR Master Mix (Promega, Madison, WI). Cycling conditions were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s. Resultant

crossing point (Cp) values were compared using the $\Delta\Delta C_p$ method [40, 41]. The normalization gene used for this method was 40S ribosomal protein S5 (RPS5). Primer sequences are listed in Table 2.1.

Table 2.1: Primer sequences for RT-qPCR confirmation of high-throughout sequencing results.

Gene ID	Forward (5'-3')	Reverse (5'-3')
AAEL010228 (clade I aegyptin)	CTT AGG TGT TCG CTA CAT TAT GAA AT	CGT GAG CAT TAA GTT CAC TAG GC
AAEL010235 (clade II aegyptin)	GAA GGT GAA GAA CAT GCT GGA	CAG CAT CGT CAT GTC CTG TAT T
AAEL013625 (RPS5)	CGT CGT GTC AAC CAG GCT A	GTT ACG GAA TGC AGC TTC G

2.3 Results

2.3.1 Ion Proton General Output

Using the Ion Proton high-throughput sequencer for RNA-seq analysis, we analyzed the transcript levels of pooled salivary glands of female *Ae. aegypti* mosquitoes. These salivary glands were infected with DENV2 strain 1232 or strain 16803, or were uninfected (BA1 media-inoculated) controls. A total of 61,831,241 reads were generated for the 1232 sample with total output of 7.4 G. A total sequence output of Q20 quality that is derived from the predicted per-base quality scores and corresponds to an error rate of 1% generated approximately 4.1x coverage of the reference genome. A total of 41,395,724 reads were generated for the 16803 sample with total output of 4.7 G. A total sequence output of Q20 quality generated approximately 2.3x coverage of the reference genome. A total of 54,753,501 reads were

generated for the BA1 sample with total output of 6.1 G. A total sequence output of Q20 quality generated approximately 2.5x coverage of the reference genome.

2.3.2 Differential transcript levels

There were a total of 236,181 comparisons generated, with 78,727 unique test IDs, and 74,736 unique gene loci (including four mitochondrial loci). Of these, 49,974 were differential comparisons ($p \leq 0.05$). There were 22,362 loci with differential transcript levels between strain 16803 and BA1, of which 288 had fragments per kilobase of exon per million fragments mapped (FPKM) values ≥ 1 in both data sets. There were 17,865 loci with differential transcript levels between strain 1232 and BA1, of which 232 had FPKM values ≥ 1 in both data sets. There were 9,747 loci with differential transcript levels between strains 16803 and 1232, of which 166 had FPKM values ≥ 1 in both sample sets.

Of particular interest were the proteins previously identified with differential abundance in the expectorated saliva of *Ae. aegypti* with disseminated DENV2 strain 1232 infections [33]. The amino acid sequences of these proteins were compared to the VectorBase database using BLAST to obtain the VectorBase designations that likely correspond. The remainder of this investigation will focus on said proteins, as well as other expectorated salivary proteins of putative importance in the literature. The aforementioned expectorated proteins, their FPKM values, and any differential transcript levels are displayed in Table 2.2. Other expectorated proteins, as well as putatively secreted proteins and related proteins, their FPKM values, and any differential transcript levels are displayed in Table 2.3.

2.3.3 Confirmation of high-throughput sequencing results

We set out to confirm the differential transcript levels for clade I and clade II aegyptins observed in the high-throughput sequencing output by utilizing a more traditional RT-qPCR assay. In DENV2 strain 1232 infected salivary glands relative to BA1 control, clade I aegyptin transcript was detected in 19.97-fold greater quantities and clade II aegyptin transcript was detected in 6.28-fold greater quantities. These transcript levels reflect the directionality and relative magnitude of change observed in the sequencing FPKM data, but to a greater degree (Figure 2.1).

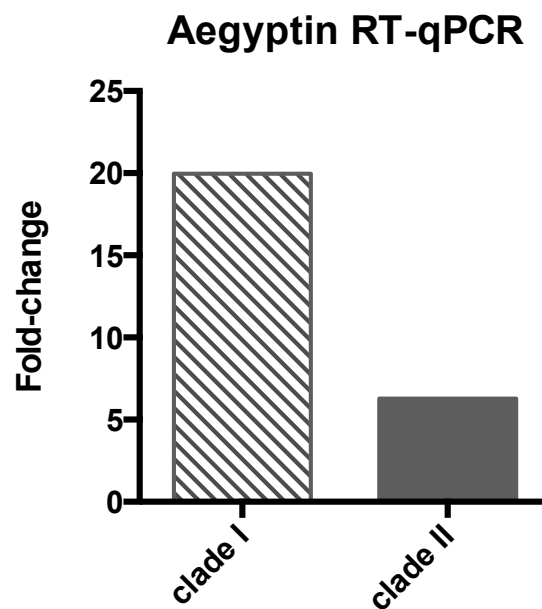


Figure 2.1: Confirmation of differential transcript levels of clade I and clade II aegyptins between DENV2 strain 1232 infected salivary glands and BA1 control salivary glands by qRT-PCR.

Table 2.2: Expecterated salivary proteins of *Ae. aegypti*. Gene IDs listed here were obtained by BLAST into VectorBase using peptide sequences from a previous publication [33]. † = significantly different protein abundance between strain 1232 and media control in previous publication. * = $p \leq 0.05$ in current investigation.

VectorBase Gene ID	Description	strain 1232 FPKM	strain 16803 FPKM	control BA1 FPKM	1232 vs. BA1	16803 vs. BA1	1232 vs. 16803
AAEL000533	C-Type Lectin	503	1218	323	---	*	---
AAEL000732 [†]	conserved hypothetical protein	208	252	55	*	*	---
AAEL000793	Venom allergen	1694	2884	576	---	*	---
AAEL002704 [†]	Serine Protease Inhibitor (serpin) homologue	9321	920	181	*	*	---
AAEL003600	conserved hypothetical protein (putative 34kD family secreted salivary protein)	965	904	449	---	---	---
AAEL005672 [†]	adenosine deaminase	390	1103	76	*	*	---
AAEL006347	Apyrase precursor (Allergen Aed a 1)	1166	2280	272	*	*	---
AAEL006417	D7 protein, putative	3380	6033	571	*	*	---
AAEL006424	37 kDa salivary gland allergen Aed a 2 Precursor (Protein D7)(Allergen Aed a 2)	1904	1904	344	*	*	---
AAEL007041 [†]	Low-density lipoprotein receptor (ldl)	0.58	1.14	3.14	---	---	---
AAEL008463	DEAD box ATP-dependent RNA helicase	2	0.47	0.09	---	---	---
AAEL010228 [†]	conserved hypothetical protein (short-form aegyptin; SAAG-4; clade I aegyptin)	2150	530	229	*	---	*

Table 2.3: Expecterated salivary proteins and related or putatively secreted proteins of *Ae. aegypti*. * = $p \leq 0.05$.

VectorBase Gene ID	Description	strain 1232 FPKM	strain 16803 FPKM	control BA1 FPKM	1232 vs. BA1	16803 vs. BA1	1232 vs. 16803
AAEL000028	Clip-Domain Serine Protease family B	17	88	10	---	*	---
AAEL000229	Prosialokinin Precursor {Contains Sialokinin(Sialokinin-1)(Sialokinin I)(Sia I)(Sialokinin-2)(Sialokinin II)(Sia II)}	1815	810	323	*	---	---
AAEL000556	C-Type Lectin	390	977	26	*	*	---
AAEL001098	clip-domain serine protease, putative	99	40	187	---	*	---
AAEL002610	serine protease	96	20	18	*	---	---
AAEL003057	allergen, putative	474	1011	34	*	*	---
AAEL003182	Serine Protease Inhibitor (serpin) homologue – unlikely to be inhibitory	330	370	55	*	*	---
AAEL005641	C-Type Lectin (CTL) - galactose binding.	78	16	49	---	---	*
AAEL005997	allergen, putative	123	20	58	---	---	*
AAEL006333	salivary apyrase, putative	100	275	32	---	*	---
AAEL007420	Serine Protease Inhibitor (serpin) homologue – unlikely to be inhibitory	71	191	13	---	*	---
AAEL008620	D7 protein, putative	27	43	6	---	*	---
AAEL010235	30 kDa salivary gland allergen Aed a 3 Precursor (Allergen Aed a 3; clade II aegyptin)	7233	1530	1573	*	---	*

2.4 Discussion

The differential changes in transcript levels among salivary glands infected with DENV2 strain 1232 or strain 16803 as compared to those of control, BA1 media-inoculated female *Ae. aegypti* suggest the potential for saliva to play a role in strain-based differences in viral perpetuation success. For example, co-exposure of strain 1232 and the salivary proteins aegyptin (clade II; AAEL010235) or C-type lectin (AAEL000533) are shown in chapter 4 of this dissertation to have a detrimental effect on DENV production in a human hematopoietic cell line when compared to DENV-only exposure. Additionally, co-exposure of strain 1232 and aegyptin (clade II) led to alterations in cytokine production, inoculation site titers, and viremia titers in mice when compared to DENV-only exposure, as outlined in chapter 5 [26]. As displayed in Tables 2.2 and 2.3 above, infection with strain 16803 led to much greater changes in C-type lectin transcript level (3.77x) and essentially no change in the level of aegyptin (clade II; 0.97x) transcript relative to BA1 media control, when compared to the modulation elicited by infection with strain 1232 (1.56x and 4.60x, respectively). Additionally, a previous study found that infection with DENV2 strain Jam1409 resulted in no change to the level of clade II aegyptin, whereas the level of clade I aegyptin (AAEL010228) was more than 2x different from control levels (in comparison to the 9.39x and 2.31x differences seen here for strains 1232 and 16803, respectively) [31]. The salivary protein profiles of *Ae. aegypti* mosquitoes may be differentially pressured by the various strains of DENV2 as a compensation mechanism for differences in viral fitness.

Accordingly, it has been shown previously that these two strains of DENV2 exhibit differences in infection kinetics in the vertebrate and efficiency in the vector [35, 42]. With regard to the vertebrate infection kinetics, infection with DENV2 strain 1232 elicited a longer

viremia (four days versus two) with significantly greater magnitude than did strain 16803 (Figure 2.2). Additionally, strain 16803 elicited significantly greater concentration of IFN γ when compared to strain 1232 (Figure 2.3), among other cytokine alterations [42]. With regard to efficiency in the vector, strain 1232 had a higher dissemination rate (0.35) compared to strain 16803 (0.06) on day seven post exposure in female *Ae. aegypti*, and strain 16803 then surpassed the dissemination rate of strain 1232 on day 9 post exposure (0.58 and 0.44, respectively) [35]. The existence of these strain-based phenotypic differences and the relatively poor success of strain 16803 in the vertebrate (without the influence of saliva) lend credence to a differential reliance on salivary proteins for viral perpetuation.

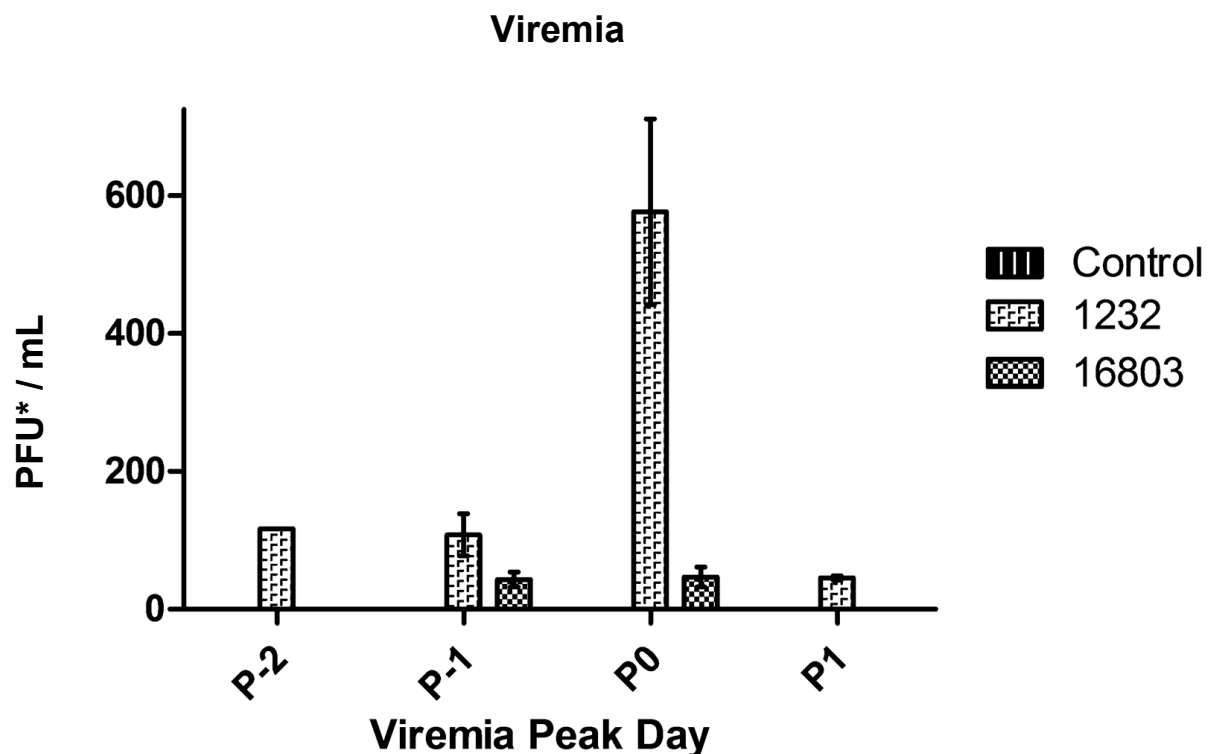


Figure 2.2: Mean viremia levels of DENV infections by different strains from interferon regulatory factor 3 & 7 double knock-out mice. Mean viremia levels are aligned by day of peak viremia (P0). Figure reproduced with permission from the authors.

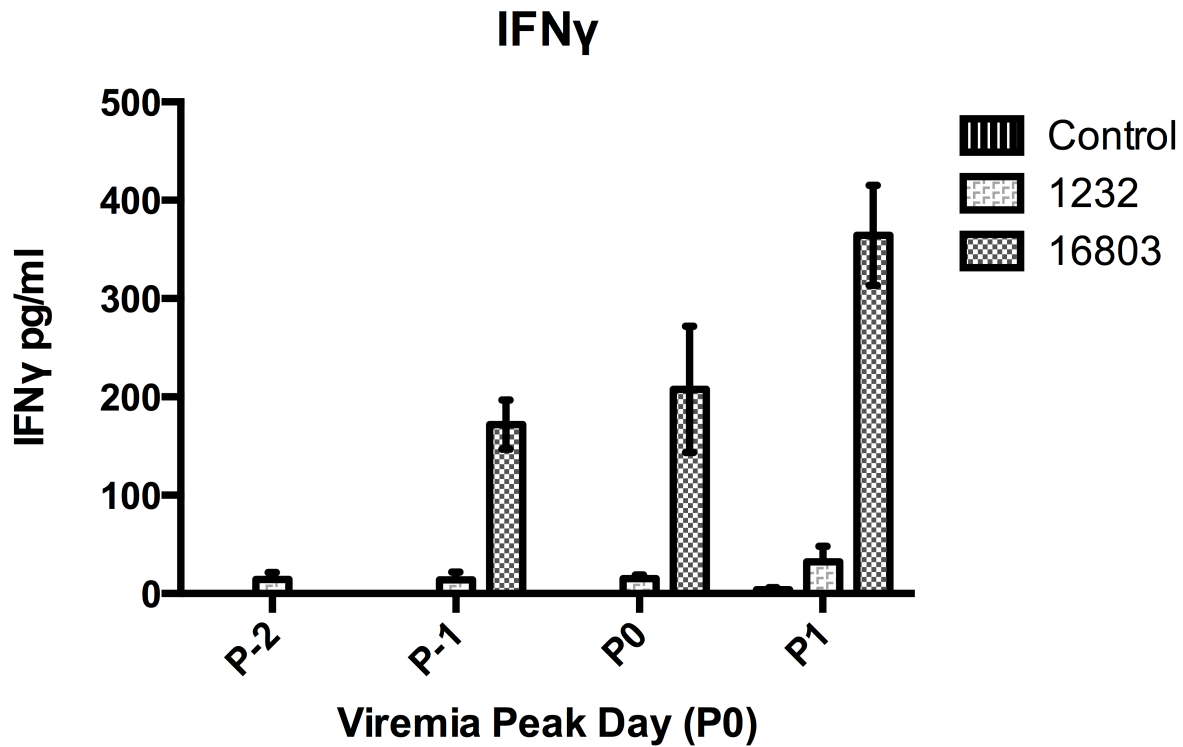


Figure 2.3: IFN γ response of DENV infections by different strains from interferon regulatory factor 3 & 7 double knock-out mice. Mean concentrations are aligned by day of peak viremia (P0). Figure reproduced with permission from the authors.

Importantly, the transcript levels of some salivary proteins were altered to similar degrees by both strains of DENV2, and some were not substantially altered from control values. An example of a protein whose transcript level was altered similarly by both strains is the D7 protein allergen Aed a 2 (AAEL006424). The two strains elicited identical FPKM values herein for the transcript of this protein, and were 5.53x greater than BA1 media control. Further, a previous investigation demonstrated that immunization of mice with a D7 protein from *Culex tarsalis* significantly increased neurovirulence of West Nile virus when exposed by infectious mosquito. The potential necessity to maintain at least a certain level of some proteins is exemplified by the lesser degree of difference between infected pools, and the relative similarity of their FPKM

values to those of control samples, in the transcript levels of the putative 34kDa family member protein (AAEL003600) measured herein and its effect on DENV infection observed previously. This protein has been shown to significantly decrease the production of IFN α and IFN β transcript levels, as well as that of the transcription factors IRF-3 and -7, and the antimicrobial peptides LL-37, S100A7, and RNase7 in DENV-infected human keratinocytes. These modulations were concurrent with a significant increase in the concentration of DENV genomic RNA [27]. The nature of these transcript levels and others speaks to the potential commonality of importance (or a lack thereof) of certain salivary proteins in the perpetuation of DENV.

The variety of differential, salivary protein transcript levels observed in this study among the two DENV2 strains and the control, as well as the potential impact of these differences on DENV infection of the vertebrate, speak to a dynamic nature of the interaction between DENV strains, the mosquito vector, and the vertebrate in DENV perpetuation. However, it is important to note here that transcript levels are not always an accurate depiction of protein levels. This is evident in Table 2.2, where clade I aegyptin (AAEL010228) was observed with dramatically increased transcript levels upon infection with strain 1232, whereas the aforementioned previous publication found the expectorated protein levels to be significantly reduced upon infection with this DENV strain at a similar time point post inoculation [33]. Therefore, further work into the strain-based protein profiles of expectorated saliva, the mechanism underlying the disparity between transcript accumulation and protein production, and the impact of differing salivary protein profiles on DENV transmission and pathogenesis are warranted.

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CHAPTER 3

ANALYSIS OF EARLY DENGUE VIRAL INFECTION IN MICE AS MODULATED BY *Aedes Aegypti* PROBING¹

3.1 Introduction

Dengue virus (DENV), the etiologic agent of dengue fever, is an enveloped, positive-sense, single-stranded RNA virus of the genus *Flavivirus*. DENV prevalence has increased in recent decades and now infects an estimated 50-100 million humans world wide annually of approximately 2.5 billion at risk for infection [1]. The increased risk for infection can be attributed, in part, to the continued growth and urbanization of human populations and an expanded global distribution of the primary vectors of DENV: the mosquitoes *Aedes aegypti* and *Ae. albopictus* [2]. Feeding by these vectors on vertebrate hosts is initiated by probing that results in physical damage to the epithelium and vasculature, as well as the simultaneous introduction of virus and saliva into host tissues [3-6]. This saliva contains many pharmacologically important proteins that modulate host haemostasis and innate immune responses, which in turn facilitate blood feeding and virus transmission [7-9]. As exogenous antigen, both salivary proteins as well as virus encounter the vertebrate host immune system and consequently could have an effect on the environment of the bite site during viral establishment.

To investigate the impact of the vector on mosquito-borne viral infection, researchers have analyzed viral titers and/or immunological response markers utilizing various methods of mosquito involvement, including salivary gland extract (SGE) [10-16], infected mosquitoes [14, 17-23], and spot-feeding [11, 12, 14, 24]. The immunological effects of mosquito saliva and/or

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probing in these arboviral infection experiments were examined at time points of 24 hours post exposure or later, with three notable exceptions. Limesand et al. assayed IFN α/β expression four to 48 hours post exposure in response to vesicular stomatitis virus infection *in vitro*, following treatment with SGE [10]; Thangamani et al. assessed the response to chikungunya-infected *Ae. aegypti* mosquito bites in mice at three and six hours post exposure [22]; and Surasombatpattana et al. examined the effect of *Ae. aegypti* SGE on DENV infection in human keratinocytes *in vitro* at six and 24 hours post exposure [15]. Three other studies have investigated the effects of *Aedes* saliva on DENV infection of the vertebrate host [17, 25, 26], with observations again gathered 24 hours post exposure or later.

The immunological modulations observed in these DENV studies would likely affect the progression of DENV infection and disease. However, the time points utilized are distinctly separate from the act of transmission or time of inoculation and therefore may not describe the events that occur *in vivo* during the early hours of DENV infection. The investigation of potential differences at earlier time points will clarify the acute dynamics of DENV transmission at the bite site in the context of mosquito probing and expectorated saliva. As such, we examined the early *in vivo* transcriptional changes of murine innate immune recognition and response pathways at sites of DENV inoculation due to contemporaneous *Ae. aegypti* probing.

Specifically, we assessed potential differences in the murine Toll-like receptor (TLR), nod-like receptor (NLR), and RIG-I-like receptor (RLR) signaling pathways between skin samples that had or had not been probed by mosquitoes immediately prior to DENV inoculation. TLRs, NLRs, and RLRs are pattern recognition receptors critical to the detection of viral pathogens by the innate immune system. The subsequent signaling pathways initiate a cascade of events that include activation and nuclear translocation of transcription factors, increased

transcription of antiviral genes, and the production of effector proteins with the ultimate goal of creating an antiviral immune environment. We utilized commercial qPCR-based transcript arrays of 84 genes each as broad screens for differential gene expression in these pathways at 3 hours post inoculation. Following these arrays, we assayed transcript of four differentially expressed genes and two housekeeping genes using gene-specific primer sets at 10 minutes, 3 hours, and 6 hours post inoculation. The four differentially expressed genes assayed were Toll-like receptor 7 (TLR7), v-rel reticuloendotheliosis viral oncogene homolog A (avian) / transcription factor p65 (RelA), interferon (IFN)- γ , and interferon- γ -inducible protein 10 / CXCL10 (IP-10). The two housekeeping genes assayed were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ribosomal protein L-32. Taken together, the differential regulation of these transcripts, as modulated by mosquito probing, informs on a vertebrate host environment that may benefit the establishment of DENV immediately following transmission.

3.2 Materials and Methods

All experiments met the approval and conditions of the LSU Institutional Animal Care and Use Committee (protocol # 12-079). LSU IACUC procedures and policies adhere to and comply with the guidelines stated in the NIH Guide for the Care and Use of Laboratory Animals.

3.2.1 Mosquitoes

Mosquitoes utilized in this study are field-caught *Aedes aegypti* from New Orleans, LA generously provided by City of New Orleans Mosquito, Termite, & Rodent Control Board and by Dawn Wesson (Tulane University School of Public Health and Tropical Medicine, New Orleans, LA). Mosquitoes were maintained in an environmental chamber at 28°C, 75–80%

humidity, and subjected to a 16:8 light:dark photoperiod regime until time of use. Mosquitoes were provided with water and 10% sucrose solution *ad libitum*, which was removed 24 hours prior to experimentation.

3.2.2 Mice

Mice were the generous gift of Dr. M. Diamond (Washington University, St. Louis, MO) with permission from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). These IRF-3/7 ^{-/-} ^{-/-} mice are on a C57Bl/6 background and lack functional IRF 3 and 7, and as such have a deficient, but not abrogated, type I IFN response. [27]. These mice have been demonstrated previously to be a suitable model for DENV transmission by both mosquito and needle inoculation, with cytokine responses, DENV replication, and resulting viremia evaluated [28].

3.2.3 Virus

Dengue serotype 2, strain 1232 was propagated as described previously [29], with modification. Briefly, we inoculated a T-75 flask of confluent Vero cells with 100 µl of viral stock and incubated for 30 minutes. Eight mL of M199E medium with 10% fetal bovine serum and 2% penicillin / streptomycin / fungizone (P/S/F) was added. The flask was incubated at 37°C with 5% CO₂ for 5 days and subsequently the supernatant was collected for virus at peak titer. The supernatant was titered to contain 6.7x10⁶ PFU/mL using a plaque assay as described previously in [30] supplemental material, with modification. M199E medium containing P/S/F was used and incubations occurred at 37°C. This strain was originally isolated from a patient in Indonesia in 1978 (personal communication, R. Tesh), and examined previously in mice [28].

3.2.4 Exposure & sample collection

Three *Aedes aegypti* females were allowed to feed on the pinna of one restricted ear of each mouse for 10 minutes from initiation of probing. Mice then immediately received intradermal needle-inoculation (10uL) of 6.7×10^4 PFU of DENV in the location of mosquito probing and in a similar location in the opposite ear. Thus, both the treatment group – the one ear where mosquitoes had probed – and the control group – the ear where mosquitoes had not probed – were located on the same mouse in order to control for mouse-to-mouse variation. At 10 minutes (n=10), 3 hours (n=8), and 6 hours (n=10) post inoculation, mice were CO₂ euthanized, then the pinnae of their ears were removed and homogenized. Subsequently, RNA was purified and cDNA then created for each sample. A separate group of mouse ears exposed only to mosquito probing were collected at these same time points (7, 6, and 7 ears respectively). Ten additional, unexposed mouse ears were collected for comparison.

Two groups of four additional mice were exposed as above to DENV with and without mosquito probing. These mice were bled via submandibular vein puncture daily for the next six days. This blood was collected in microcentrifuge tubes, allowed to clot for thirty minutes at room temperature, and then centrifuged at 8000 rcf for four minutes at 4°C. Clarified serum was placed into clean microcentrifuge tubes and stored at -80°C until nucleic acid extraction and subsequent measurement of viremia.

3.2.5 RNA purification and cDNA creation

Individual mouse ears were disrupted and homogenized in 600ul RLT + β -ME buffer using the TissueLyser (QIAGEN, Valencia, CA) as per manufacturer's instructions. RNA was then purified using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA) with on

column DNase I treatment (QIAGEN, Valencia, CA). Total nucleic acid was extracted from clarified mouse serum using the MagMax-96 Total Nucleic Acid isolation kit (Ambion/Life Technologies, Carlsbad, CA).

A uniform quantity of total RNA per mouse ear, as determined by NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE) A260 reading, was used for each pair of ears to create cDNA. cDNA creation was performed using the RT² First Strand Kit (SABiosciences, QIAGEN, Valencia, CA) for the RT² Profiler PCR Arrays or the SuperScript® First-Strand Synthesis System for RT-PCR (Life Technologies, Carlsbad, CA) with oligo-dT primer for follow-up testing.

3.2.6 Viral titer assessment

RNA was extracted from all ear tissue and serum samples as described above. Titers of DENV were measured by qRT-PCR assay using the One-Step TaqMan qRT-PCR system (Life Technologies, Carlsbad, CA) and DENV concentration standards derived from plaque assays, as previously described [29]. As such, viral titers are expressed as PFU-equivalents/mL, symbolized as PFU*/mL.

3.2.7 Transcript assays

All samples were tested using real-time quantitative PCR (qPCR) on the Roche LightCycler480 in 384-well format.

The first assays performed used the TLR (PAMM-018A) and Antiviral (PAMM-122Z) RT² Profiler PCR Arrays (SABiosciences, QIAGEN, Valencia, CA) as low-replicate screens of some 3 hours post inoculation samples for TLR and antiviral responses in the mouse. These

commercial plate arrays assay the expression of 84 different genes along various innate immune pathways as well as housekeeping genes and quality controls. Samples were prepared according to manufacturer's instructions.

Follow-up testing of all samples was conducted with individual primer sets (Integrated DNA Technologies, Coralville, IA) for TLR7, IFN- γ , IP-10, RelA, L-32, and GAPDH using EXPRESS SYBR GreenER qPCR SuperMix Universal (Life Technologies, Carlsbad, CA). Primer sequences are shown in table 3.1. Samples were assayed in triplicate using 10ul reaction volumes and a cDNA concentration per reaction equivalent to 3.34ng starting total RNA (to equilibrate cDNA loads to the RT² Profiler PCR Arrays). The run parameters were as follows: 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, 58°C for 20 seconds, 72°C for 20 seconds.

Table 3.1: Primer sequences listed 5'-3', source publications, and NCBI reference genes.
*Modified from original sequence publication

Gene	NCBI Gene: #	Forward Primer	Reverse Primer	Source
TLR7	Tlr7: 170743	GGC ATT CCC ACT AAC ACC AC	TTG GAC CCC AGT AGA ACA GG	[31]
GAPDH	Gapdh: 14433	TGC ACC ACC AAC TGC TTA GC	TGG ATG CAG GGA TGA TGT TC	[31]
IP-10	Cxcl10: 15945	CAG TGA GAA TGA GGG CCA TAG G	CTC AAC ACG TGG GCA GGA T	[31]
IFN- γ	Ifng: 15978	CCA TCG GCT GAC CTA GAG AA	ATG AGG AAG AGC TGC AAA GC	[32]
RelA	Rela: 19697	CTT CTG GGC CTT ATG TGG AGA T	TCG CAT TTA TAG CGG AAT CG	[33]*
L-32	Rpl32: 19951	TAA GCG AAA CTG GCG GAA ACC	AGG ATC TGG CCC TTG AAC CT	[33]*

3.2.8 Analysis

The $\Delta\Delta\text{Cp}$ method [34, 35] was used in order to calculate fold-change and fold-regulation differences between the treatment and control groups. The $\Delta\Delta\text{Cp}$ method is discussed here briefly. The average Cp for all qPCR technical replicates is calculated first. For our study, this was done on a per ear basis (Equation 1). Next, the ΔCp is calculated, which is the Cp value for the gene of interest minus the Cp value for the designated housekeeping gene, again on a per ear basis (Equation 2). To get the $\Delta\Delta\text{Cp}$, the control ΔCp is subtracted from the treatment ΔCp . For our study, this was done by subtracting the control ear ΔCp from the treatment ear ΔCp , on a per mouse basis (Equation 3). Fold-change values are then equal to 2 raised to the negative $\Delta\Delta\text{Cp}$ (Equation 4).

$$\text{Equation 1: } \text{Cp}^{\text{Avg}} = (\text{Cp}_1 + \text{Cp}_2 + \dots + \text{Cp}_n)/n, \text{ per ear}$$

$$\text{Equation 2: } \Delta\text{Cp}^{\text{Avg}} = \text{Cp}^{\text{Avg}}_{\text{GOI}} - \text{Cp}^{\text{Avg}}_{\text{HKG}}, \text{ per ear}$$

$$\text{Equation 3: } \Delta\Delta\text{Cp} = \Delta\text{Cp}^{\text{Avg}}_{\text{TRT}} - \Delta\text{Cp}^{\text{Avg}}_{\text{CTRL}}, \text{ per mouse}$$

$$\text{Equation 4: } \text{Fold-change} = 2^{(-\Delta\Delta\text{Cp})}, \text{ per mouse}$$

Since a fold-change value of 1 indicates that the values between the treatment and control groups did not change, any fold-change value greater than one is an increase in the amount of transcript produced. Similarly, a fold-change value less than one indicates a decrease in the amount of transcript produced. Fold-regulation is a manipulation that allows for better conceptualization of the data. This adjusts the relative scale of the data such that a 2-fold increase and a 2-fold decrease would have the same absolute value, making zero on the y-axis the turning point between the directionality of change, rather than one (Equation 5 & 6).

Equation 5: Fold-regulation = fold-change, for values ≥ 1

Equation 6: Fold-regulation = $-(1/\text{fold-change})$, for values < 1

Statistical analysis was then performed on fold-change values using SAS 9.2 (Cary, NC). Student's t-test was utilized with a null hypothesis of the mean of the differences between control and treatment ears equal to 1. Significance was assessed at, and confidence intervals of the differences were calculated based on, an α value of 0.05. Differences in viral titers between treatments at each time point were compared using a one-way ANOVA with repeated measures to control for intra-mouse variation on the two treatment categories. For the serially bled mice, a one-way ANOVA was performed per day post exposure, but without repeated measures. Again, significance was assessed at an α value of 0.05.

3.3 Results

3.3.1 DENV titers

Based on the qRT-PCR assay, the titer of DENV in ears of mice that received mosquito probing were not found to differ significantly ($p > 0.05$) from DENV titers in ears that received DENV injection alone at 10 minutes, 3 hours, and 6 hours post inoculation (Figure 3.1). However, mice that received mosquito probing demonstrated a significantly greater viremia titer on days 3 and 4 post exposure. In addition, DENV RNA was detectable through day 6 post exposure in the mosquito probing group, whereas DENV RNA was evident in only one mouse on day 5 and undetectable on day 6 post exposure in the virus only group (Figure 3.2).

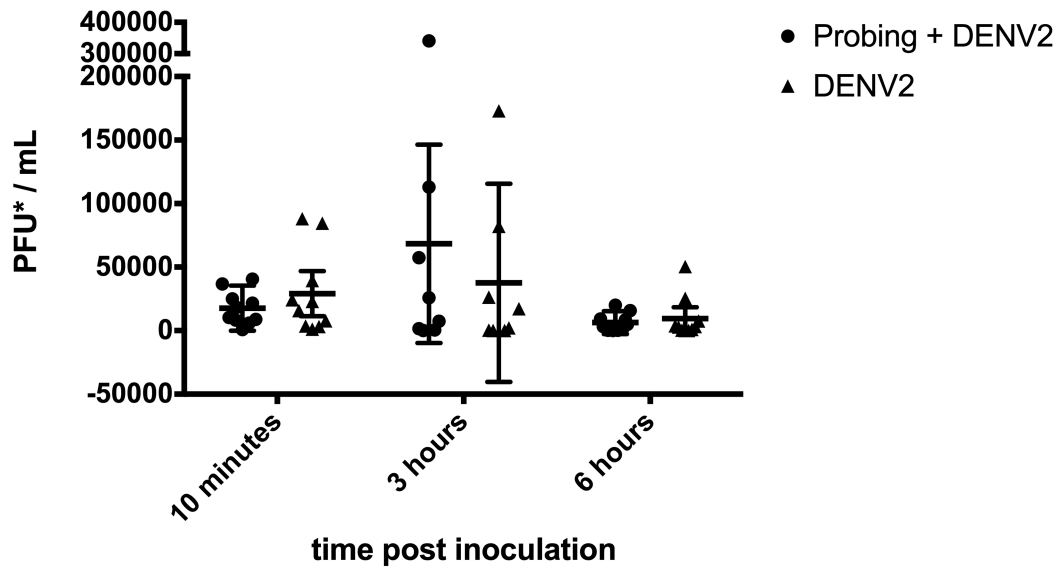


Figure 3.1: DENV titers in ear samples at 10 minutes, 3 hours, and 6 hours post inoculation. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL), did not differ significantly between samples exposed to mosquito probing and those injected with DENV alone ($p>0.05$).

3.3.2 Preliminary transcript arrays

Based on the SABiosciences RT² Profiler PCR Arrays, there was differential regulation of many genes for innate immune receptors including TLR 7, Ifih1/MDA5, and Nod2; the MAP kinases; activators, inhibitors, and adaptors of signaling such as Ticam1 and Nfkbia; transcription factors such as the subunits of NF- κ B, Rel and RelA; enzymes such as caspase 1; and cytokines including IFN- γ and IP-10 (data shown in supplement). In the *Ae. aegypti* spot-feeding treatment group relative to the virus only control group, the differential expression observed for the transcripts of TLR7 (3.88x down-regulated), RelA (3.4x up-regulated), IFN- γ (6.13x down-regulated), and IP-10 (2.86x down-regulated) was chosen for further study. In total, 41 transcripts were at least 2-fold down-regulated and 12 transcripts were at least 2-fold up-regulated out of a total 130 targets (Figure 3.3).

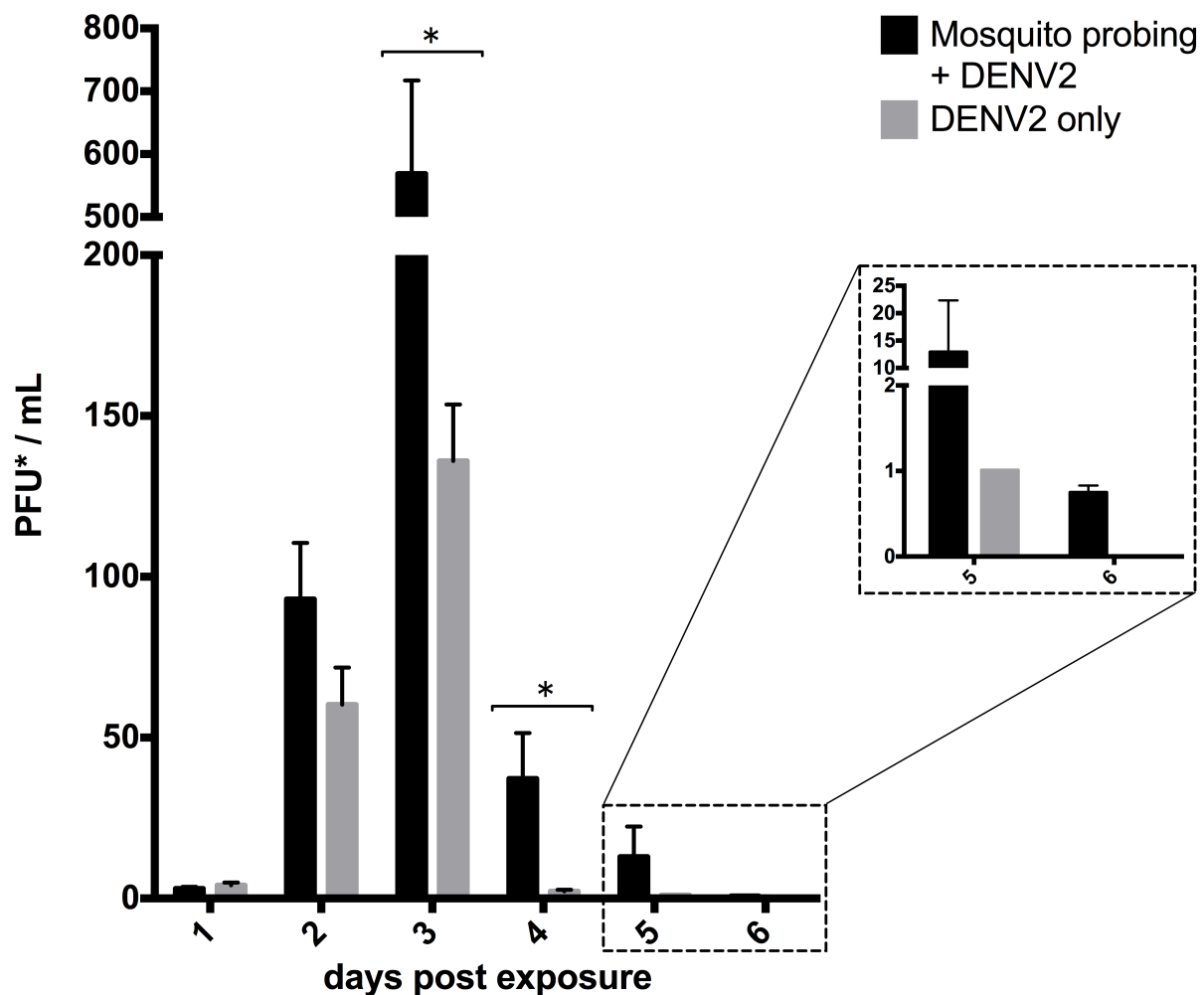


Figure 3.2: DENV viremia titers in serum samples on the first six days post exposure. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL), were significantly higher in mice exposed to mosquito probing + virus compared to those that received only DENV inoculation on days 3 and 4 post exposure, as indicated by asterisks ($p < 0.05$). Associated bars represent standard error of the means.

During this initial investigation, we observed that the housekeeping genes normally used in these transcript arrays (e.g. GAPDH) had a more variable response between the two treatments than we thought appropriate for a standardizing method. In some cases, subsequent assays revealed this Cp difference to be indicative of over a log-fold difference in GAPDH expression.

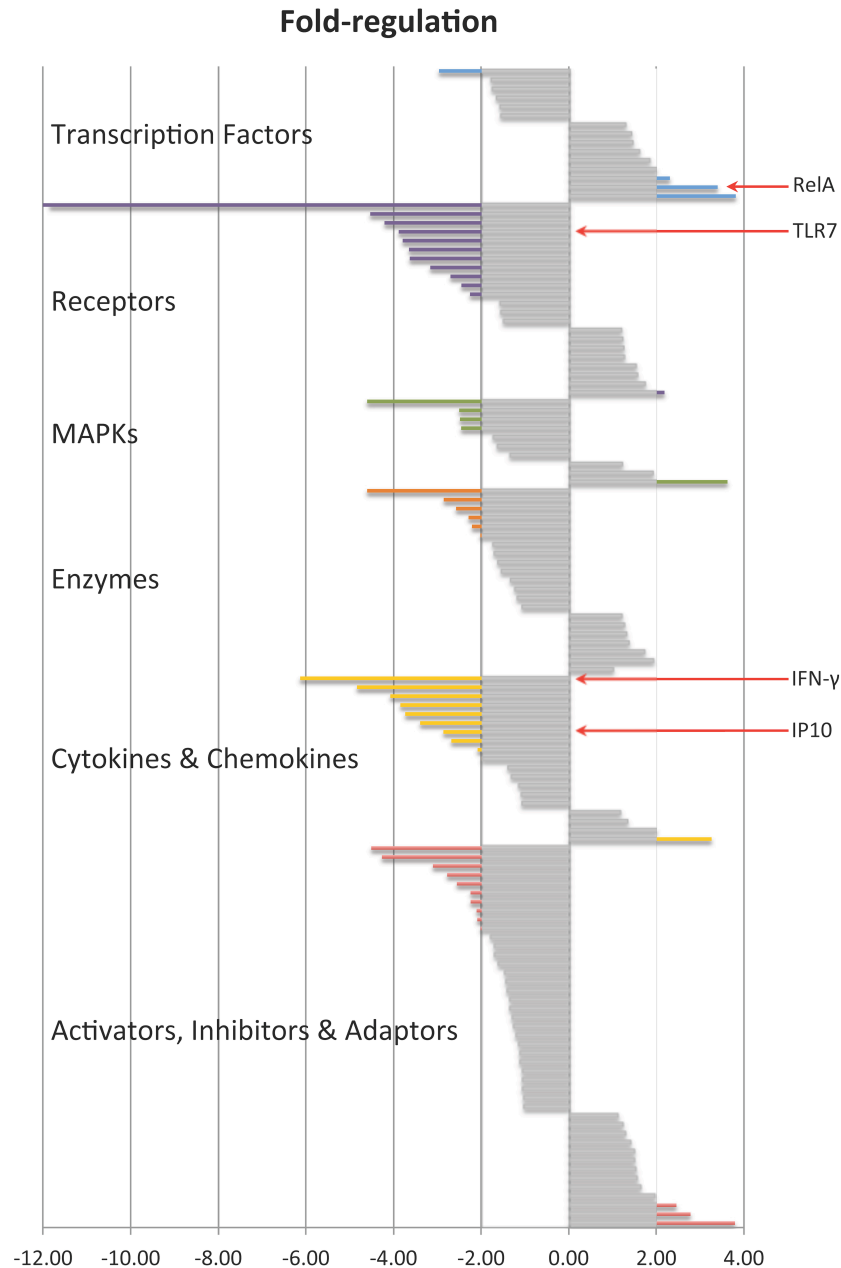


Figure 3.3: Preliminary transcript arrays. Differential regulation of genes for transcription factors, innate immune receptors, the MAP kinases, enzymes, cytokines, chemokines, and activators, inhibitors, and adaptors of signaling was detected in mouse ear samples that received mosquito probing + DENV2 inoculation relative to DENV2 inoculation alone. A cutoff of 2-fold up- or down-regulated was used to assess which genes were differentially expressed (colored bars surpass the grey 2-fold cutoff). The transcripts of RelA (3.4x up-regulated), TLR7 (3.88x down-regulated), IFN- γ (6.13x down-regulated), and IP-10 (2.86x down-regulated) are marked in their respective categories. Forty-one transcripts (31.5%) were at least 2-fold down-regulated and 12 transcripts (9.2%) were at least 2-fold up-regulated out of a total 130 targets. The colors of each category correspond to the similarly colored sections in the data supplement.

Thus, we assessed other housekeeping genes and determined L-32 was the most stable, and as such was used as the housekeeping gene for the remainder of the study (Figure 3.4).

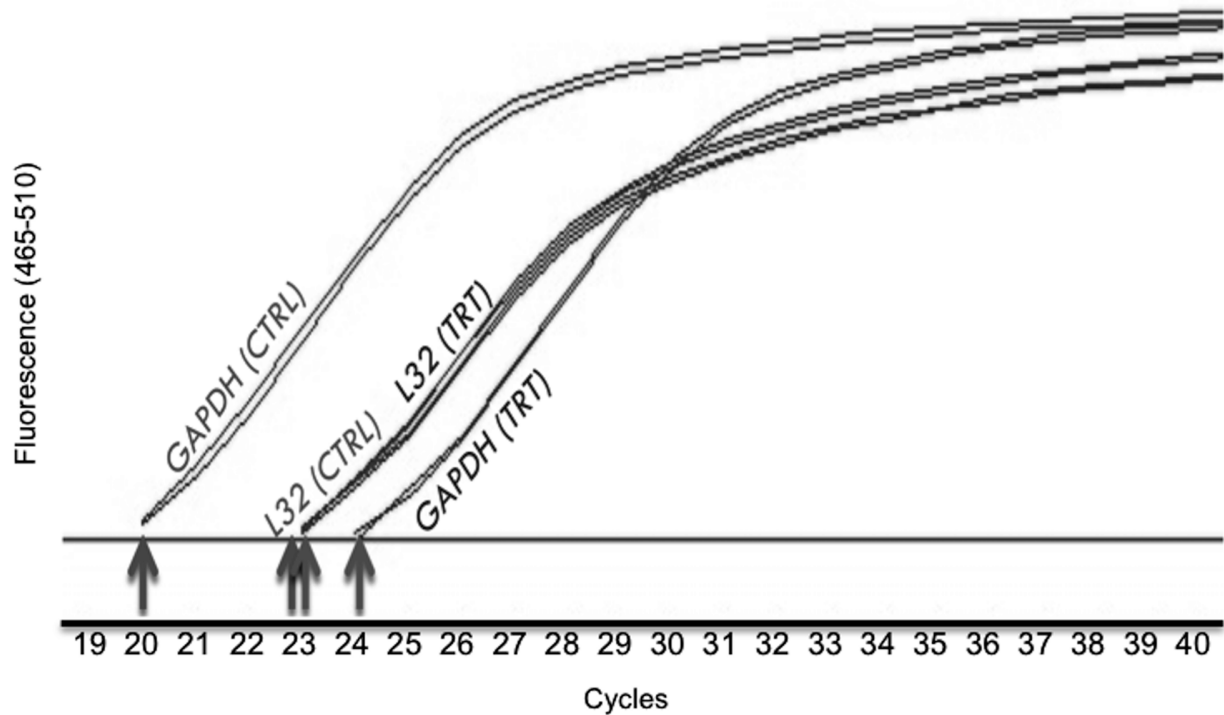


Figure 3.4: qPCR amplification curves representing GAPDH and L-32 fluorescence and C_p values for a set of treatment and control samples. Arrows represent the approximate cycle where amplification curves cross the threshold.

3.3.3 Subsequent targeting of transcripts

We then used individual primer sets to specifically target the four genes outlined above, as well as our chosen housekeeping gene, L-32. Once the $\Delta\Delta C_p$ were calculated, we observed significant transcript down-regulation of all four of our genes of interest for the spot-feeding treatment group relative to the virus only control group at 3 hours post inoculation. The fold-

regulation values for these transcripts were calculated to be as follows: TLR7 = -2.33x (p=0.008), RelA = -2.59x (p=0.001), IP-10= -2.75x (p=0.004), IFN- γ = -2.17x (p=0.036). These values and the associated 95% confidence intervals are shown in figure 3.5.

When the study window was expanded out to 6 hours post inoculation, we observed no difference in fold-change values relative to 1, indicating a convergence in expression of the two groups by this time point. We then looked at expression 10 minutes post completion of viral inoculation. Shown in figure 3.5, this cohort exhibits a great deal of variation in the expression levels of the two groups. While there may be a trend toward up-regulation at this time point, only IFN- γ shows significant up-regulation (3.04x, p=0.034).

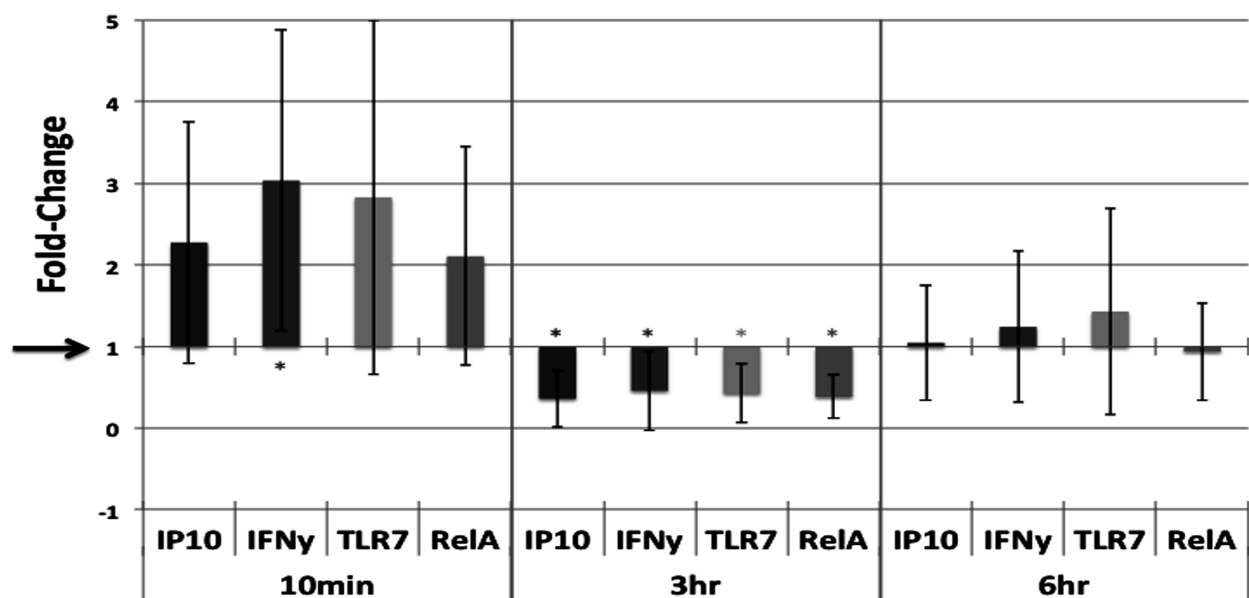


Figure 3.5: Fold-change values for each of the four genes of interest at 10 minutes, 3 hours, and 6 hours post inoculation in mouse ear samples that received mosquito probing + DENV2 inoculation relative to DENV2 inoculation alone. Asterisks represent significance of fold-change values as different from 1 (p<0.05). Associated bars represent 95% confidence intervals for each gene per time point, thus bars that do not overlap 1 indicate significance.

Additionally, the relative abundance of expression in all samples (mosquito + virus, virus only, mosquito only, and unexposed ears) was calculated for each gene of interest relative to the normalization gene L-32, shown in figure 3.6. The expression level of each gene in unexposed ears tended to be lower than the other three groups, while the expression level of the mosquito only group in relation to the virus only and mosquito + virus groups varies depending on the gene.

3.4 Discussion

The preliminary immune pathway transcript arrays revealed 31.5% of target transcripts were at least 2-fold down-regulated whereas only 9.2% of transcripts were at least 2-fold up-regulated. Importantly, 11 of the down-regulated transcripts were receptors and 9 were proinflammatory cytokines and chemokines. The down-regulation of these transcripts suggests a propensity for mosquito-induced, generalized suppression of the inflammatory response and a reduction in infiltrating leukocytes.

The transcripts for TLR7, RelA/p65, IFN- γ , and IP-10/CXCL10 were examined individually due to the changes in expression observed in our preliminary transcript arrays and the potential for these genes to affect dengue viral establishment. Plasmacytoid dendritic cells expressed increased levels of the ssRNA receptor TLR7 in response to DENV exposure and recognize DENV genomic RNA in a replication-independent manner, resulting in production of type I interferon that was inversely proportional to DENV titer [36]. Additionally, PBMCs from clinical patients in Thailand exhibited down-regulation of TLR7 in secondary DHF cases [37]. RelA is part of the NF- κ B complex and is responsible for the induction of many inflammatory cytokines, as well as DENV-induced expression of MHC class I [38] and early production of

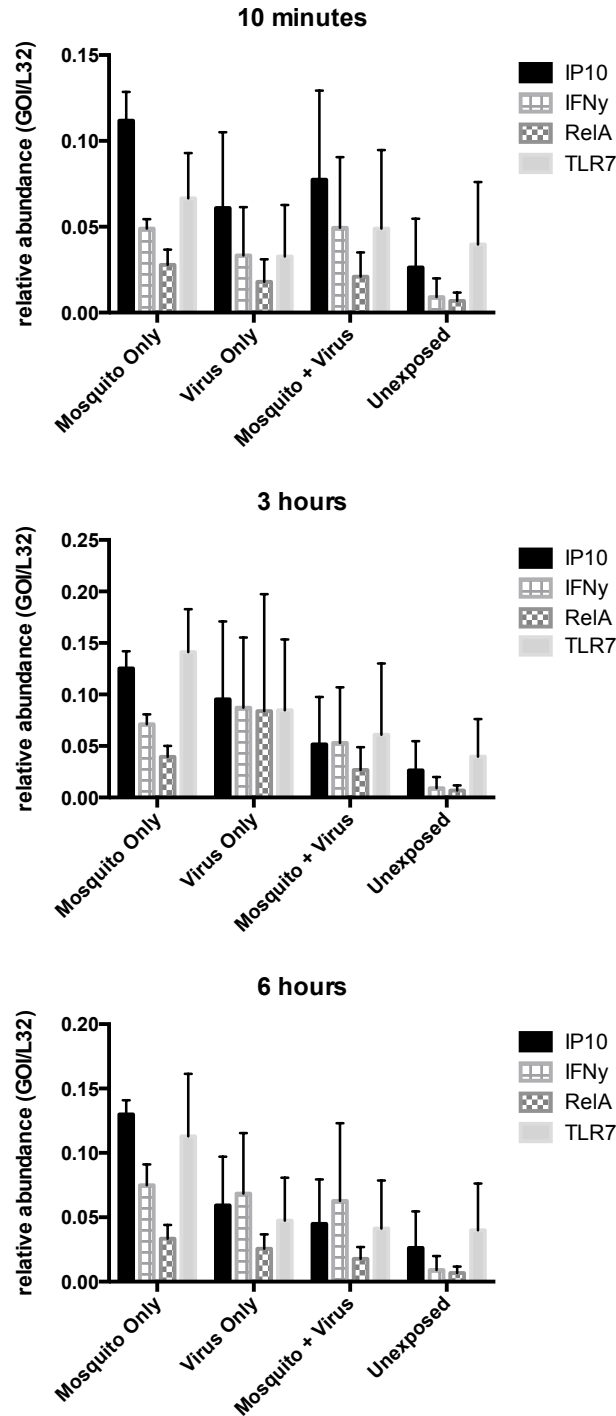


Figure 3.6: Relative abundance of expression for each gene of interest normalized to L32 expression in mouse ears. The groups depicted are unexposed, mosquito probing only, virus inoculation only, and virus inoculation + mosquito probing at 10 minutes, 3 hours, and 6 hours post inoculation. Values depicted represent mean relative abundance and associated bars represent the standard deviation.

IFN- β , leading to resistance to RNA viral replication [39]. IFN- γ has manifold effects, including enhanced activation of DENV-infected dendritic cells [40] and, in combination with IFN- β , inhibition of DENV RNA translation[41]. The vital importance of IFN- γ is further exemplified by one of the primary mouse models for DENV infection, the AG129 mouse, which lacks a functional IFN- γ response and thereby suffers clinical illness and increased DENV titers [42]. IP-10 is an antigen-presenting cell chemokine induced by IFN- γ that was also shown to competitively inhibit DENV attachment to new cells, resulting in decreased DENV titer [43]. Similarly, an approximately two-fold decrease in average IP-10 concentration was demonstrated to increase the DENV titer in monocyte-derived dendritic cells [44]. As potential internal controls for normalization we also measured GAPDH and L-32 transcript levels. GAPDH is a traditional housekeeping gene involved in the glycolysis cycle. L-32 is a component of the 60S ribosomal RNA subunit and a less traditionally used transcription control.

In reference to the choice of L-32 as our housekeeping gene for the individual transcript assays (as opposed to those as used in the preliminary transcript arrays), it is important to note that in addition to the variation in GAPDH expression we observed between the treatment and control groups, the literature indicates that increased GAPDH expression is often observed in studies of cellular activation and stress-induced apoptosis [45, 46]. There is also literature stating that certain viral infections, such as Newcastle disease, influenza, and vaccinia viruses, can modulate GAPDH expression [47-49]. As such, we opted to use the more stably expressed L-32 as the gene of reference for data normalization, and suggest that this gene is a better choice for future transcript studies involving DENV and mosquito feeding.

This study provides the first transcriptional analysis of the effects of mosquito probing on the *in vivo* immune response to DENV infection at time points prior to 24 hours post exposure.

In 2010, Thangamani et al. worked to assess the immune response to early chikungunya virus infection by measuring transcript levels at 3 and 6 hours post inoculation. Notably, they found that expression of IFN- γ and the double-stranded RNA receptor TLR3 was lowered in the infected mosquito inoculation group compared to the needle inoculation group [22], which parallels our IFN- γ and TLR7 results from 3 hours post inoculation. Although we initially anticipated that RelA would be up-regulated based on our preliminary transcript arrays, ultimately specific investigation of this gene revealed a significant down-regulation due to probing – perhaps due to the change in housekeeping gene utilized to normalize the data.

Interestingly, we found a general down regulation observed at 3 hours post inoculation, while there was no significant difference between the treatment and control groups at 6 hours post inoculation. Similarly, in an *in vitro* experiment by Surasombatpattana et al., expression of IFN types I and II was not found to differ during DENV infection between SGE exposed and unexposed keratinocytes at 6 hours post exposure. However, differential expression of other immune effectors was observed [15]. It must be considered, then, that the convergence of expression we observed at 6 hours post inoculation could be due to two things.

First, the convergence could be due to the waning effects on our targets of salivary components temporally removed from the initial expectoration event. That is, probing and salivary proteins may have a more immediate effect on viral recognition and response components that diminishes with time. Accordingly, Edwards et al. observed that when mice were inoculated with Cache Valley virus into areas of *Ae. triseriatus* spot-feeding with a delay between one and four hours post feeding, there was an enhancement of infection and serum antibody production. When inoculation was delayed greater than four hours, or when inoculation was performed without delay but at a site distal to the location of spot-feeding, no

enhancement was observed [24]. Similarly, Styer et al. demonstrated that *Culex tarsalis* SGE-induced enhancement of West Nile virus infection in mice was absent when SGE was injected into a distal location [14]. These experiments would suggest that the influence of salivary components on downstream immunological and infection characteristics is clustered both spatially and temporally at the bite site. In contrast, DENV-2 inoculation delayed 30-60 minutes after *Ae. aegypti* spot-feeding on a distal location resulted in viremia levels similar to those elicited by infected-mosquito delivery in hu-NSG mice [17], warranting further study into the temporospatial nature of salivary influence.

Second, our observed convergence could be due simply to the interference of systemic responses reaching the opposing inoculation site, stemming from both the treatment and control groups existing on the same mouse. Importantly, significant differences in expression were observed between the mosquito spot-feeding treatment group and the virus only control group at 10 minutes and 3 hours post inoculation, indicating that the difference in observed expression, while likely conservative, is due to treatment differences and not the result of a systemic response at these time points.

We did not detect a significant difference in DENV titers in the inoculated ears between treatments, as shown in figure 3.1. This lack of difference may be due to residual DENV RNA from the initial viral inoculum clouding the detection of meaningful changes in virion production. Alternatively, DENV replication may not be occurring at detectable levels this early in the course of infection [50, 51]. Importantly, we did detect significantly greater levels of virus in the serum of mice that received mosquito probing concurrent with DENV inoculations in the days following infection, as shown in figure 3.2. This finding is consistent with a previous

DENV infection in this IRF3/7 double knockout mouse model utilizing this same strain of DENV introduced via infected mosquito [28].

Much of the work examining the effects of mosquito saliva on viral pathogens suggests that there is a shift away from an antiviral, T_H1 immune response toward a T_H2 response characterized by increased production of IL-4 and IL-10, among other immunological alterations [11, 13, 20, 22]. Additionally, inoculation via mosquito bite and exposure to saliva have been shown to enhance viral infection or potentiate infection in otherwise non-permissive models [10-12, 14, 15, 17-21, 24]. The observed down-regulation of TLR7, RelA, IFN- γ , and IP-10 transcripts at 3 hours post inoculation appears to reflect a shift away from an antiviral immune response, potentially generating an environment with reduced DENV recognition and antigen presentation, and diminished inhibition of viral replication and spread (depicted in figure 3.7). Upon superficial examination, the up-regulation of IFN- γ at 10 minutes post inoculation might seem to contradict this argument. However, mosquito probing causes tissue injury during the search for capillaries, and IFN- γ is known to be up-regulated during skin injury repair [52]. Additionally, IFN- γ is known to cause increased expression of and antigen presentation by MHC classes I and II [53]. Therefore, it is plausible that the tissue damage caused by mosquito probing is the initial stimulus for production of IFN- γ . This IFN- γ may in turn cause an increased presentation of mosquito salivary proteins, some of which are known to have potent allergen potential, thereby instigating a shift away from an antiviral immune response toward a mosquito-allergen-based, T_H2 immune response. An obvious next step would be to assess these MHC complexes and immune effectors such as IL-4 and IL-10 that could indicate a T_H2 shift, as well as other markers of tissue injury repair and allergen-based immune responses. Based on the implications of our findings, down-regulation of our target transcripts early in the infection

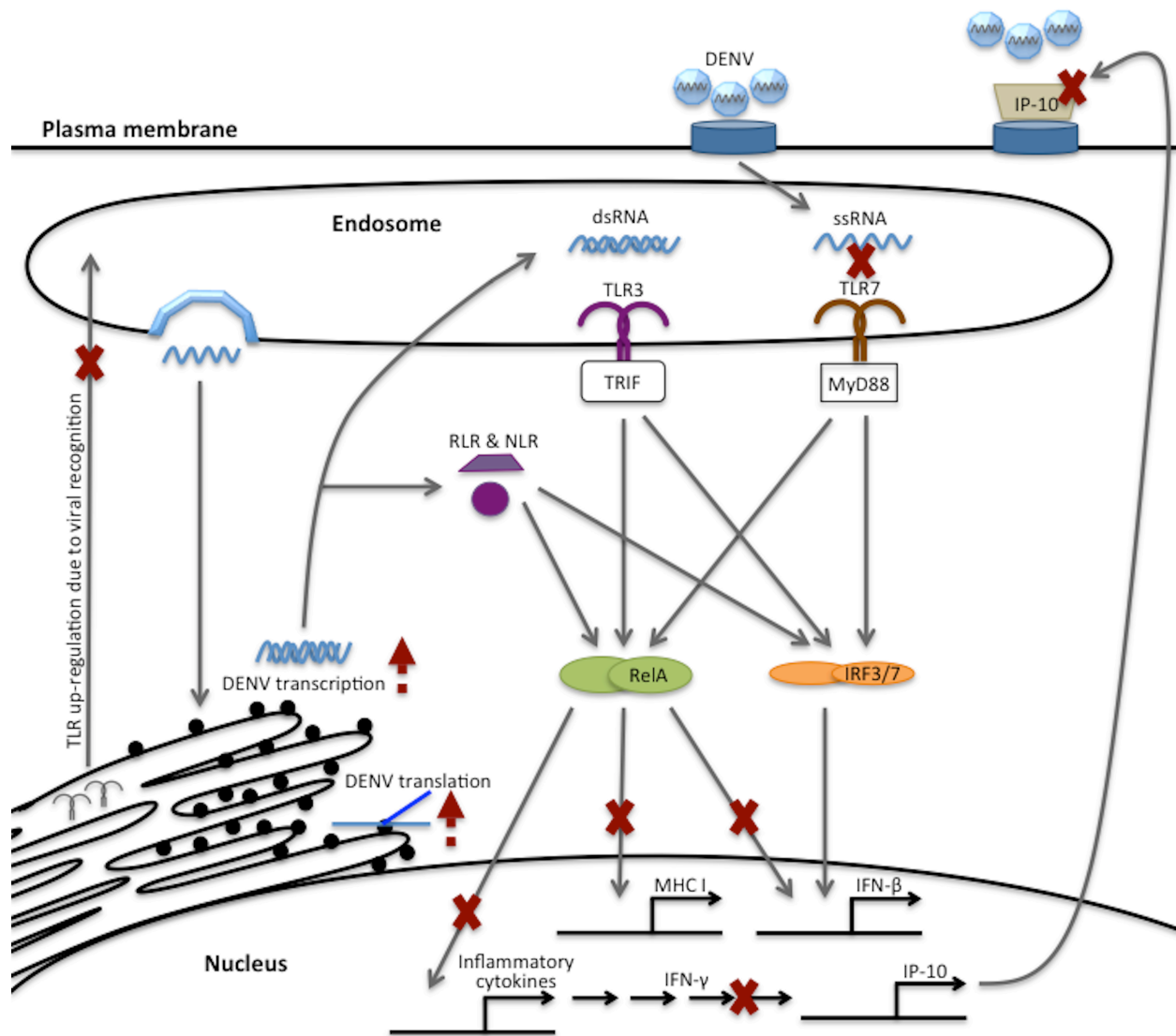


Figure 3.7: Cellular representation of the signaling pathways assayed and their involvement in DENV infection. DENV enters the cell through receptor-mediated endocytosis, where it 1) fuses with the endosomal membrane to release its ssRNA genome into the cytoplasm or 2) is recognized by endosomal TLR7, which signals through RelA to induce production of MHC class I, IFN- β , and inflammatory cytokines that will then induce IFN- γ and IP-10. The DENV dsRNA replication intermediate can be recognized by RLRs, NLRs, and TLR3 to further induce signaling via RelA. IFN- β and IFN- γ can inhibit DENV translation and replication, while IP-10 can competitively inhibit DENV binding to plasma membrane receptors, reducing infection of new cells. Mosquito probing immediately prior to DENV inoculation could inhibit many of these interactions via the observed transcript down-regulation (represented by Xs) and thus allow more successful DENV transcription and translation (represented by dashed arrows).

process indicate important mechanisms by which mosquito probing and salivary protein expectoration serve to enhance the establishment of DENV infections in the vertebrate host.

3.5 Acknowledgements

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CHAPTER 4

IMPACT OF INDIVIDUAL SALIVARY PROTEINS FROM *Aedes Aegypti* ON DENGUE VIRUS PRODUCTION IN A HUMAN HEMATOPOIETIC CELL LINE

4.1 Introduction

Dengue virus (DENV), the etiologic agent of dengue fever, is an enveloped, positive-sense, single-stranded RNA virus of the genus *Flavivirus*. DENV is maintained in a primarily anthroponotic cycle between humans and the *Aedes aegypti* mosquito [1]. Feeding by *Ae. aegypti* on vertebrate hosts involves the deposition of saliva, and thereby virus, into the skin [2-5]. Mosquito saliva contains many proteins that modulate host hemostasis and immune responses, facilitating blood feeding and virus transmission [6, 7]. *Ae. aegypti* saliva also has been shown to contain allergenic proteins [8]. The vertebrate immune response to DENV infection is altered as a result of mosquito probing and the introduction of these immunogenic salivary proteins, thereby altering DENV infection kinetics and the likelihood of viral perpetuation [9].

Several of the individual proteins in saliva have been shown to elicit specific physiological consequences, with many disrupting events in hemostasis (e.g., collagen-binding to platelets or vasoconstriction) or modulating the production of T_H1 and T_H2 associated cytokines [10-19]. Additional experimental investigations of individual mosquito salivary proteins have shown that some serve as a boon to arboviral infection of the vertebrate, while other(s) may prove to be only a detriment [20-22]. Of particular relevance is a recent study demonstrating that DENV serotype 2 (DENV2) infection of *Ae. aegypti* salivary glands significantly lowers the abundance of many expectorated salivary proteins, while leaving the expression of others unaltered [23]. Three of these proteins were chosen for further study herein: an aegyptin (clade I; -14.1-fold), an adenosine deaminase (-2.8-fold), and a C-type lectin (indeterminate).

Aegyptins are a family of GE-rich 30 kDa antigen with two proteins in *Ae. aegypti*, designated as clade I and clade II aegyptin [24]. Clade II aegyptin has been shown to induce positive skin-test reactions and antibody responses in sensitized humans, as well as disrupting hemostasis by binding collagen, inhibiting platelet aggregation and interaction with von Willebrand factor [14, 15, 25]. As the clade I aegyptin noted above was the only aegyptin detected in the previous study, and due to the known characteristics of clade II aegyptin, clade II aegyptin protein was chosen for further study [23].

Adenosine deaminase is an enzyme that hydrolyzes adenosine to inosine and ammonia. The substrate, adenosine, has been associated with pain perception and mast cell degranulation [26, 27]. The degradation product, inosine, was shown to be over 100 times less effective than adenosine at inducing these effects, and was shown to inhibit the production of inflammatory cytokines [27, 28].

C-type lectins are a large family of calcium-dependent, carbohydrate-binding proteins, many of which are involved in cell-to-cell adhesion and innate immune responses [29]. This particular protein from *Ae. aegypti* has not been characterized beyond a putative homology. Instead, it was chosen due to its potential role as a receptor and contrast to the two chosen salivary proteins of known lower abundance.

The DENV2 infection-induced lowered abundance of some salivary proteins as compared to the maintenance of a steady-state expression of others suggested the possibility of a negative pressure by these lowered proteins on DENV perpetuation. In order to determine if some aspect of this pressure exists within the vertebrate segment of the perpetuation cycle, we investigated the influence of the aforementioned individual, recombinant salivary proteins on DENV2 production in the human, hematopoietic cell line, K562.

4.2 Materials and Methods

4.2.1 Viruses

DENV serotype 2 (DENV2) strain 1232 was propagated as described previously, with modification [9]. Briefly, we inoculated T-75 flasks of confluent C6/36 cells with 100 µl of viral stock and incubated them for 30 min. Eight milliliters of M199E medium with 10% fetal bovine serum and 2% penicillin-streptomycin-amphotericin B (Fungizone) (P/S/F) was added. The flask was incubated at 37°C with 5% CO₂ for 5 days, and subsequently the supernatant was collected for virus. The supernatant was found to contain 6×10^6 PFU/ml using a plaque assay as described previously in the supplemental material of reference [30], with modification. M199E medium containing P/S/F was used, and incubations occurred at 37°C.

4.2.2 Mosquito rearing and inoculation

Laboratory strain *Ae. aegypti* (Rockefeller) were maintained in an environmental chamber at 28°C and 75 to 80% humidity and subjected to a 16:8 light-dark photoperiod regimen until the time of use. Mosquitoes were provided with a water and 10% sucrose solution ad libitum, which was removed 24 h prior to blood feeding and replaced thereafter. Approximately one week post emergence, female mosquitoes were allowed to feed on bovine blood in Alsever's anticoagulant via Hemotek feeding device (Discovery Workshops, Lancashire, England) containing DENV2 strain 1232, after which the blood-fed females were sorted. After 14 days, legs were removed and tested as previously performed as a proxy for dissemination [31].

4.2.3 Cloning and sequencing of target protein cDNA

RNA was extracted from a single, adult female *Ae. aegypti* that tested positive for DENV dissemination above using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). The head and thorax were placed in RLT buffer containing β -mercaptoethanol and extracted as per manufacture's instructions with on-column DNase I treatment. cDNA was reverse transcribed from RNA using the oligo dT protocol of the SuperScript® III First-Strand Synthesis System (Invitrogen/Life Technologies, Carlsbad, CA). A gene-specific primer set was used for full transcript amplification of each target protein (Table 4.1) using the Platinum PCR Supermix (Invitrogen/Life Technologies, Carlsbad, CA). Thermal cycler conditions were as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 2 minutes; 68°C for 5 minutes; 4°C hold. PCR products were cloned into TOPO vectors and transformed into DH5 α -T1 cells for amplification using the TOPO TA Cloning Kit (Invitrogen/Life Technologies, Carlsbad, CA). Plasmids were extracted from amplified broth culture using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen/Life Technologies, Carlsbad, CA) and then sequenced for target verification using Sanger sequencing as follows. Sequencing reactions were performed using 100ng plasmid DNA in a maximum volume of 5uL. A single M13 sequencing primer at 2uM concentration was added to reaction at a volume of 2uL. Big Dye Terminator v3.1 (Applied Biosystems/Life Technologies, Carlsbad, CA) was added at a volume of 4uL. The total sequencing reaction volume is 11uL. Controls were run as supplied with the BDTv3.1 reagent, namely pGEM-3Zf(+) double-stranded DNA control template and M13(-21) Control primer. Controls are set up in the same manner as samples and run on standard thermal cyclers. Sequencing reaction conditions according to ABI protocols were as follows: 96°C for 5 minutes; 34 cycles of 96°C for 10s, 54°C for 5s, 60°C for 4 minutes; 4°C hold. Sequencing products were

cleaned up using Agencourt CleanSeq (Beckman Coulter, Indianapolis, IN) magnetic beads according to manufacturer's protocol. Sequencing runs were performed on Applied Biosystems 3130 Genetic Analyzers, 4-capillary electrophoresis instruments. Previously run sample aliquots were added to sample plate as additional controls.

Table 4.1: Primer sequences for PCR amplification of full length *Ae. aegypti* saliva proteins for cloning and recombinant expression.

NCBI protein / nucleotide	Description	Forward (5'-3')	Reverse (5'-3')
gi 108878609 / XM_001651187.1	Adenosine deaminase	ATG AAA AAT CTA CTT ACA GCA ATT TTG GTC	TTA AAC ACT TCC AGC CAC GAT ATC
	Adenosine deaminase (no stop)		AAC ACT TCC AGC CAC GAT ATC
gi 18568318 / AF466606.1	C-type lectin	ATG GCT CTT TCA TTG TAT CTA ATC GCT GTT	TTA CGC CTG TTC GCA CAC AAA G
	C-type lectin (no stop)		CGC CTG TTC GCA CAC AAA G

4.2.4 Production of ADA and C-lectin

Genes encoding adenosine deaminase (1572 bp; ADA) and C-type lectin (462 bp; C-lectin) were amplified without their stop codons from the previously created TOPO vector using Fail-Safe™ DNA polymerase (Epicentre, Madison, WI) and cloned into pFastBac HBM-TOPO vector (Invitrogen/Life Technologies, Carlsbad, CA). Recombinant baculoviruses producing the respective proteins with 6x His tag at their carboxyl terminus were constructed using Bac-to-Bac Baculovirus Expression System (Invitrogen/Life Technologies, Carlsbad, CA) as described by manufacturer's instructions. Only supernatants from the infected sf9 cells were used for protein purification using Talon metal affinity resin (Clontech Laboratories Inc., Mountain View, CA). Infected supernatants were clarified from the cells debris by centrifugation (4000xg, 10 min) and

NP-40 non-ionic detergent was added to the final concentration of 1%. After one more centrifugation (8000xg, 30 min) clear supernatants were filtered using 0.2 μ m filter and mixed with Talon resin (1v of resin: 50v of supernatant) overnight on a shaker at 4°C. After centrifugation (2000xg, 10 min) resin was washed 4 times with buffer containing 50 mM sodium phosphate, 300 mM sodium chloride and 15 mM of imidazole, pH 7.4. Proteins were eluted in the same buffer only containing 150 mM of imidazole. In order to obtain an imidazole-free solution for downstream experiments, buffer exchange against phosphate-buffered saline (PBS) using Zeba Spin desalting columns (Pierce/Thermo Scientific, Rockford, IL) was performed. The purity of the resulting protein-PBS solution was verified by 1-D gel SDS-PAGE stained with Coomassie Brilliant Blue (Figure 4.1).

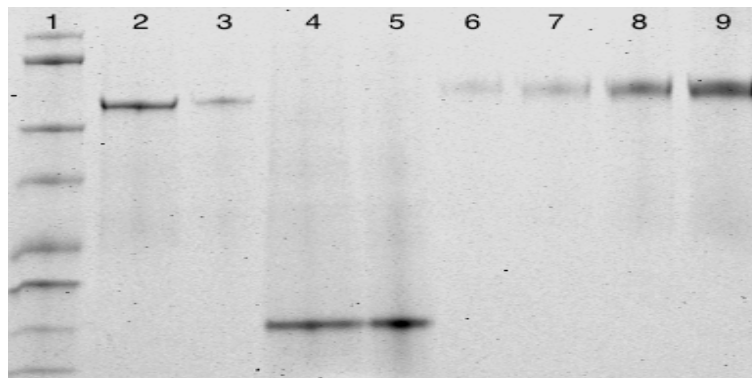


Figure 4.1: Gel Image of recombinant adenosine deaminase (lane 2 in imidazole, lane 3 in PBS) and putative C-type lectin (lane 4 in imidazole, lane 5 in PBS) with ladder (lane 1) and bovine serum albumin protein concentration standards (lanes 6-9; 0.25, 0.5, 1, and 1 μ g).

4.2.5 Cell culture and infection

The human hematopoietic cell line K562 was obtained from ATCC (CCL-243) and maintained at 37°C with 5% CO₂. Complete media utilized was Iscove's Modified Dulbecco's Medium (IMDM) with GlutaMAX™ supplement (Gibco®/Life Technologies, Carlsbad, CA), 10% fetal bovine serum, and 2.85% additional HEPES. Cells were grown to >800,000 cells/ml

and then distributed to 200,000 cells per experimental well of a cell culture plate. Cells were exposed to 0.1 MOI of DENV and 52ng of protein per well and incubated for one hour at 37°C with 5% CO₂ with periodic mixing. After incubation, cells were centrifuged at 900xg for 3 minutes, media decanted, and then washed six times at a 1/10 dilution with 1X PBS to remove unbound virus and protein. Cells were reconstituted in a final volume of 500µl per well and an additional 52ng of protein was added to each well for the duration of the experiment. Each treatment was performed in triplicate wells. After 24 hours, the contents of each well were transferred to 1.5ml microcentrifuge tubes and centrifuged at 900xg for 3 minutes. 400ul of supernatant was transferred to new tubes. The remaining volumes and cell pellets were washed three times in 500µl 1X PBS per wash. The *Ae. aegypti* salivary proteins utilized in this experiment were the recombinant adenosine deaminase and C-type lectin, as well as a recombinant aegyptin (from gi|94468546) generously provided by Dr. Eric Calvo (National Institutes of Health/NIAID). The aegyptin was expressed as described previously in HEK293 cells with a 6x-His tag [14]. For the purpose of comparison to non-salivary proteins, we also examined the effect of three additional proteins: commercially available casein and bovine serum albumin (BSA), as well as a recombinant protein from *Rickettsia conorii*, rOmpB, generously provided by Dr. Juan Martinez (Louisiana State University). rOmpB was expressed as described previously [32].

4.2.6 Nucleic acid extraction and quantification

DENV RNA was extracted from culture supernatants using the MagMax-96 Viral RNA Isolation Kit (Ambion/Life Technologies, Carlsbad, CA) and from cell pellets using the MagMax-96 Total RNA Isolation Kit (Ambion/Life Technologies, Carlsbad, CA) without DNase

I treatment in order to include cellular DNA. DENV RNA was detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay with the Superscripts III® Platinum® One-Step qRT-PCR system (Invitrogen/Life Technologies, Carlsbad, CA) on the LightCycler 480 (Roche Diagnostics Corp., Indianapolis, IN) and DENV concentration standards derived from plaque assays as previously described [20, 31]. Cellular DNA was detected by targeting the nucleotide sequence of endogenous retrovirus ERV-3 using the GoTaq® qPCR Master Mix (Promega, Madison, WI) in a 20µl reaction volume: 10µl of Master Mix, 0.4µl of each 10µM primer, and 4.2µl H₂O. ERV-3 primer sequences were taken from a previous publication and reproduced here as follows: Forward – 5'- CAT GGG AAG CAA GGG AAC TAA TG -3'; Reverse – 5'- CCC AGC GAG CAA TAC AGA ATT T -3' [33]. Cycling conditions were as follows: 95°C for 5 minutes; 40 cycles of 95°C for 10 s, 58°C for 1 minute.

4.2.7 Statistical analysis

An ANOVA with uncorrected p values, comparing all treatments to the control (“Virus only”) was performed. Significance was assessed using an $\alpha=0.05$. Arbitrary ERV-3 values were established by declaring a C_p value of 35 to equal one copy and calculating the corresponding arbitrary values for each treatment using an efficiency of two.

4.3 Results

4.3.1 Uncorrected DENV titers

In this study we investigated the effect of individual salivary proteins on DENV production in the hematopoietic cell line, K562. Virus detection in the supernatant was performed using a fixed volume. Quantification in this manner resulted in no significant

difference in DENV titer between salivary protein or comparison proteins treatments when compared to Virus only control (Figure 4.2, left panel). Virus detection in the cell pellets was performed using the entirety of the pellet processed into a fixed elution volume. Quantification of DENV in these pellets resulted in a significantly lower titers in C-lectin treated wells (mean of 53.7 PFU*/mL) compared to Virus only wells (mean of 147.9 PFU*/mL), with no other significant differences as compared to Virus only wells (Figure 4.2, right panel). Interestingly, however, there was a substantial difference in the quantity of cells per well in each treatment following the 24 hour incubation. With mean values of 263.7 and 254.5 arbitrary units, respectively, the cell quantities in aegyptin and ADA treated wells were significantly greater than in the Virus only wells, with a mean of 135.4. The mean cell quantity of C-lectin treated wells was between that of ADA and Virus only, with the comparison proteins having similar quantities to those of the Virus only wells (Figure 4.2).

4.3.2 Normalized DENV titers

Due to the variability in cell quantity, we normalized the quantity of viral RNA in the supernatant and within the cell pellets to the quantity of cells detected. This normalization resulted in fairly uniform values for DENV in the supernatant of the three comparison proteins, as well as significant decreases within the aegyptin (mean of 0.126) and C-lectin (mean of 0.139) treatments when compared to Virus only control wells (mean of 0.190; Figure 4.3, left panel). The normalization of intracellular DENV did not dramatically alter the relative values for each treatment. However, these subtle changes to the means resulted in all treatment wells, with the exception of ADA, testing as significantly lower than Virus Only control (Figure 4.3, right panel).

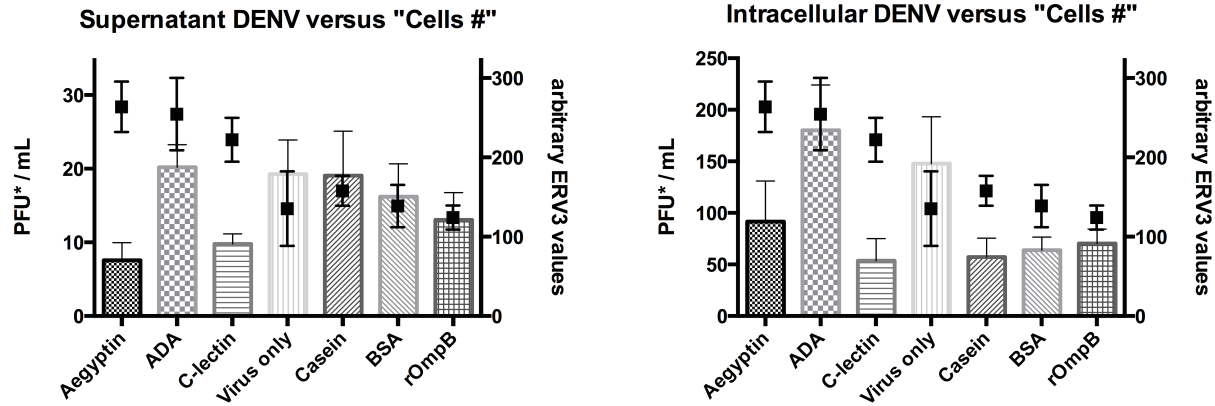


Figure 4.2: Uncorrected DENV titers and arbitrary quantity of K562 cells per well. Left panel represents DENV titers in the supernatant (left axis, bars) overlaid with quantity of K562 cells (right axis, square points) in arbitrary units based upon quantification of the human genome by targeting ERV-3 in the cell pellet. Right panel represents DENV titers measured in the cell pellets; panel data corresponds to axes as in left panel. DENV titers were significantly lower in C-lectin treated wells compared to Virus only wells. K562 cells were significantly greater in number in Aegyptin and ADA treated wells when compared to Virus only wells ($p \leq 0.05$).

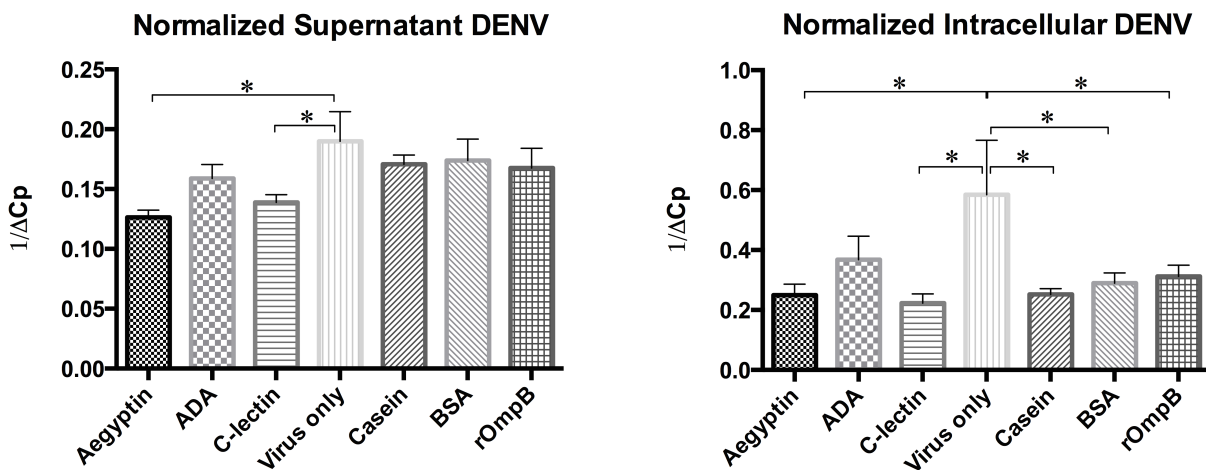


Figure 4.3: DENV titers normalized to quantity of cells per well. Left panel represents DENV RNA in the supernatant normalized to copies of ERV-3 in the cell pellet by subtracting the Cp value of ERV-3 from the Cp value of DENV, and then taking the reciprocal. Right panel represents DENV RNA within the cell pellets normalized to ERV-3 as in left panel. Asterisks represent treatments significantly different from Virus only ($p \leq 0.05$).

4.4 Discussion

This study represents the first investigation of the effect of individual vector salivary proteins on DENV production in a hematopoietic cell line. The differential changes in DENV RNA among *Ae. aegypti* salivary protein treated cells infected with DENV2, strain 1232 occurred in an unexpected pattern. Specifically, the hypothesis upon initiating this study was that proteins previously found in lower abundance due to DENV2, strain 1232 infection of the salivary glands (i.e., aegyptin and ADA) would negatively impact DENV production, whereas other salivary proteins (i.e., C-lectin) would have the opposite effect or otherwise be ineffectual [23]. However, upon normalization, we found that while treatment with aegyptin did lower DENV2 production into the supernatant significantly, as anticipated, treatment with ADA lowered DENV2 production to a degree that was not significant. Further, C-lectin treatment performed in the opposite manner as expected, lowering DENV2 production to a level similar to that of aegyptin treated wells. Importantly, treatment in the present study with the non-mosquito proteins included for the purposes of comparison did not elicit changes to DENV2 production that were significantly different from those of Virus only control, suggesting that the above alterations to DENV2 production are specific to the salivary proteins utilized in this study.

We additionally assessed DENV2 RNA within the cells themselves in order to determine if changes to DENV2 production into the extracellular environment were related to intracellular alterations. Surprisingly, we detected lower mean values of normalized DENV2 RNA in all protein treatments, both salivary and non-salivary, relative to Virus only control, of which all except ADA were significant. In contrast, a previous study has shown that co-inoculation of DENV (unspecified serotype) and individual *Ae. aegypti* salivary proteins onto human keratinocytes *in vitro* induced significantly increased intracellular viral RNA when compared to

virus alone for all proteins assessed, including an adenosine deaminase [21]. Curiously, there does not appear to be a pattern in the means of DENV2 RNA relating the intracellular and extracellular values in the present study.

Although not specifically assessed in this study, the differences between the intracellular and extracellular DENV2 RNA detected could suggest that the various protein stimuli tested herein elicit changes to the mechanism of viral escape into the supernatant. It is reasonable to state that treatment with a protein (of apparently many types) lowers DENV2 RNA within the cell, and for the most part, to a similar degree. Yet, in contrast, only treatment with specific salivary proteins lowered DENV2 RNA detected in the supernatant. This then begs the question of how the non-mosquito protein treated wells presented extracellular DENV2 values approximating that of Virus only control, when aegyptin and C-lectin treated wells remained significantly lower. To answer this question, additional, specifically targeted investigations are required, but there are multiple, potentially overlapping, possibilities. First, increased lysis, via apoptosis or other means, of the cells in the non-mosquito protein treated wells may be occurring, thereby releasing increased DENV2 RNA into the supernatant. However, this is unlikely without a concomitant increase to the number of cells, given that the number of cells detected was similar between these wells and those of Virus only control at this time point. Second, an increase in the budding rate of virions into the supernatant in the non-mosquito protein treated wells could compensate for a slowed intracellular replication. In a tangentially related study, altering intracellular pathways via introduction of a kinase inhibitor resulted in alterations to virion production and secretion of virus particles and viral genomic RNA [34]. Third, the reductions in supernatant titers could be the result of the increased quantity of cells, as newly produced virions would be capable of infiltrating the additional cells, and thereby

reducing the net extracellular measure. Alternatively, the increased quantity of cells could result in an increase to phagocytosis and destruction of extracellular virions and RNA. These scenarios are, of course, purely speculative at this time.

It is evident that co-exposure of K562 cells to DENV2 and recombinant, *Ae. aegypti* salivary proteins elicits alterations in detectable DENV2 RNA as compared to the Virus only control. However, before proceeding further into *in vitro* investigations of the influence of individual salivary proteins on DENV2 virion production in this and additional, human cell types, it will be critical to establish additional controls and assays for the various scenarios outlined above. By doing so we will gain an understanding of the mechanistic impact of these proteins, and thereby a knowledge of novel, potential targets for the disruption of DENV2 pathogenesis.

4.5 Acknowledgements

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CHAPTER 5

***Aedes Aegypti* SALIVARY PROTEIN “AEGYPTIN” CO-INOCULATION MODULATES DENGUE VIRUS INFECTION IN THE VERTEBRATE HOST¹**

5.1 Introduction

Dengue virus (DENV) is maintained in a primarily anthroponotic cycle between humans and the *Aedes aegypti* mosquito [1]. Feeding by *Ae. aegypti* on vertebrate hosts results in the simultaneous introduction of virus and saliva into the skin [2-5]. This saliva contains many pharmacologically important proteins that modulate host hemostasis and immune responses, which in turn facilitate blood feeding and virus transmission [6-8]. Additionally, *Ae. aegypti* saliva has been shown to contain allergenic proteins [9]. The vertebrate immune response to DENV infection may be altered as a result of the immunogenic nature of these salivary proteins, thereby altering DENV infection kinetics.

The effects of salivary proteins on mosquito-borne viral infection have been reviewed previously, and include alterations in cytokine production and potentiation of infection in otherwise non-permissive models [6, 10, 11]. More recently, *Ae. aegypti* saliva has been shown to induce alterations in leukocyte recruitment during West Nile virus infection, and shift the cytokine profile from a Th1 toward a Th2 type immune response during chikungunya virus infection [12, 13]. In the context of DENV infection, the addition of *Ae. aegypti* saliva *in vitro* resulted in the reduced production of antimicrobial peptides and interferons, thereby increasing viral titers [14]. Allowing *Ae. aegypti* to feed on IRF-3/7^{-/-} mice immediately before intradermal injection of DENV resulted in the down-regulation of multiple innate immune

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transcripts and increased DENV viremia titers [15]. Additionally, *Ae. aegypti* saliva has increased the prevalence of disease signs and extended the viremic period in DENV-infected, humanized mice [16].

As an important step in the characterization of mosquito saliva, researchers have examined the composition of *Ae. aegypti* salivary glands at the transcriptional level and protein expression level [7, 17-23]. Work has been done to ascribe function to some of these salivary components and investigate their individual effects on vertebrate hemostasis and immune response outside the context of a viral infection [8, 17, 24-31]. Additionally, *Ae. aegypti* saliva has been shown to elicit specific IgG and IgE responses in humans [23, 32]. Recent studies have described the effect that DENV infection has on protein expression in the salivary glands or expectorated saliva and postulates how these changes could be relevant to virus transmission [33, 34]. Among the proteins that were found in lower abundance in *Ae. aegypti* saliva as a result of DENV infection was a member of the GE-rich 30 kDa antigen family, designated in *Ae. aegypti* as “aegyptins” [8, 35]. The decreased expression of this protein during DENV infection could suggest that it supplies negative pressure on viral perpetuation. This pressure may be inherent to the aegyptin protein family and perhaps impacts transmission or establishment of infection.

The archetypal protein of the 30 kDa antigen family in *Ae. aegypti* has been shown to perform two distinct roles within the vertebrate. First, as an allergen, aegyptin has been shown to induce positive skin-test reactions and antibody responses in sensitized humans [36]. Second, aegyptin has demonstrated the ability to bind to collagen, inhibiting platelet aggregation and interaction with von Willebrand factor, which could facilitate blood feeding by reducing the formation of blood clots [8, 28].

Due to the involvement of aegyptin with both the vertebrate immune response and the clotting cascade, we explored the impact of aegyptin within the context of a DENV infection. Recombinant aegyptin was used for *in vivo* evaluation of the effects of this protein on the murine immune response to DENV and the resulting infection in the inoculation site, draining lymph nodes, and in circulation.

5.2 Materials and Methods

All experiments met the approval and conditions of the LSU Institutional Animal Care and Use Committee (protocol #12-079). LSU IACUC procedures and policies adhere to and comply with the guidelines stated in the NIH Guide for the Care and Use of Laboratory Animals.

5.2.1 Mice

Mice were the generous gift of Dr. M. Diamond (Washington University, St. Louis, MO) with permission from Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). These IRF-3/7^{-/-} mice are on a C57Bl/6 background and lack functional IRF 3 and 7, and as such have a deficient, but not abrogated, type I IFN response [37].

5.2.2 Virus

Dengue serotype 2 (DENV2), strain 1232 was propagated as described previously, with modification [38]. Briefly, we inoculated a T-75 flask of confluent Vero cells with 100 µl of viral stock and incubated for 30 minutes. Eight mL of Medium 199 with Earle's salts (M199E) with 10% fetal bovine serum and 2% penicillin / streptomycin / amphotericin B was added. The flask was incubated at 37°C with 5% CO₂ for 5 days and subsequently the supernatant was

collected for virus at peak titer. The viral titer of the supernatant was determined using a plaque assay as described previously in supplemental material, with modification [39]. The complete medium stated above was used and incubations occurred at 37°C. This strain was originally isolated from a patient in Indonesia in 1978 (personal communication, R. Tesh). As of this study, it has been passaged four times in Vero cells, and then alternatingly passaged between C6/36 (*Aedes albopictus* cell line) and Vero twice.

5.2.3 Mouse Exposure and Sample Collection

Recombinant aegyptin was generated at a concentration of 9 uM in 25mM Tris 150mM NaCl, pH 7.4. It was expressed as described previously in HEK293 cells and has a 6x-His tag [8]. Aegyptin was diluted 1/10 in 0.2µm-filtered 1X phosphate-buffered saline (PBS) for experimentation.

5.2.3.a Inoculation site mice

A total of 17 male and female mice, age fifteen weeks, were divided into four treatment groups and inoculated in the pinnae of both ears via 25µL intradermal injection. These groups were DENV (n=5), DENV + aegyptin (n=5), aegyptin (n=3), and a mock inoculation (n=4). The compositions of the inocula were as follows: DENV – 10µL DENV (1×10^5 PFU total in cell culture supernatant) + 15µL 1X PBS; DENV + aegyptin – 10µL DENV + 2µL aegyptin 1/10 + 13µL 1X PBS; aegyptin only – 10µL age-matched cell culture supernatant + 2µL aegyptin 1/10 + 13µL 1X PBS; mock – 10µL age-matched cell culture supernatant + 15µL 1X PBS. Forty-eight hours later, mice were euthanatized according to our IACUC protocol, and blood was collected via cardiac puncture using ethylenediaminetetraacetic acid (EDTA)-coated syringes and then

placed into EDTA-coated BD Microtainer®™ tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ). This blood was used for the creation of blood films for quantification of circulating leukocytes and then centrifuged at 1000 relative centrifugal force (rcf) for 10 minutes for plasma separation for use in cytokine protein and viral RNA analysis. Inoculated ears and draining submandibular lymph nodes were also collected and processed for cytokine protein and viral RNA analysis. Individual mouse ears and lymph nodes were disrupted and homogenized in 100µL and 20µL, respectively, of 1X PBS using the TissueLyser (QIAGEN, Valencia, CA) for two cycles of 2 minutes at 25Hz. For cytokine analysis, 60µL and 10µL of the homogenized ear and lymph node solutions were placed in 440µL and 40µL, respectively, of radioimmunoprecipitation assay (RIPA) lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) diluted 1/5 in 1X PBS containing protease inhibitor (cOmplete, Mini, EDTA-free protease inhibitor cocktail tablets, Roche, Indianapolis, IN). For viral RNA detection, 20µL and 5µL of the homogenized ear and lymph node solutions were placed into 600µL and 350µL, respectively, of QIAGEN's RLT buffer with β-mercaptoethanol and vortexed. RNA was extracted using the RNeasy Tissue Mini Kit (QIAGEN, Valencia, CA).

5.2.3.b Serially bled mice

A total of 19 female mice, age ten±two weeks, were divided into two treatment groups and inoculated in the pinnae of one ear or rear footpad via 25µL intradermal injection. These groups were DENV (n=9) and DENV + aegyptin (n=10). The compositions of the inocula were as above, with a DENV titer determined to be 6.7×10^4 PFU total. Mice were bled via submandibular vein puncture immediately prior to inoculation and then daily for the next 6 days

[40]. This blood was collected in microcentrifuge tubes, allowed to clot for thirty minutes at room temperature, and then centrifuged at 3300 rcf for four minutes. Clarified serum was collected and placed into clean microcentrifuge tubes for nucleic acid extraction using the MagMax-96 Total Nucleic Acid isolation kit (Ambion/Life Technologies, Carlsbad, CA) and subsequent quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) measurement of viremia.

5.2.4 Hematology

Differential leukocyte counts were performed in a blinded manner by a board-certified veterinary clinical pathologist, as previously described, in order to examine circulating leukocyte populations during the inoculation site and serially bled mouse studies [41]. Briefly, blood films were made within one hour of collection. Blood films were Wright Giemsa stained and counted in duplicate for differentiation of lymphocytes, monocytes, eosinophils, and basophils. Blood in EDTA was diluted 1:100 in 2% acetic acid and incubated for 10 minutes at room temperature. Total nuclei were counted in duplicate for each mouse using this blood-acetic acid solution on a hemocytometer in order to derive absolute leukocyte concentrations. Absolute leukocyte concentrations and differential counts were then used to calculate the absolute differential leukocyte concentrations.

5.2.5 Viral Detection

Before inoculation, DENV was titered via plaque assay and experimental titers from cell culture supernatant were confirmed by qRT-PCR as previously described [38]. DENV RNA was extracted using the MagMax-96 Total Nucleic Acid isolation kit (Ambion/Life Technologies,

Carlsbad, CA) or RNeasy Tissue Mini Kit (QIAGEN, Valencia, CA). Serum samples were brought to volume where necessary with BA-1 diluent (M199E, 10% bovine serum albumin, 0.1 g/L L-glutamine, 2.2 g/L sodium bicarbonate, 25mM HEPES, 2% penicillin / streptomycin / amphotericin B, titrated to 7.4 pH with Tris and HCl) and kits were run per manufacturers' instructions [42]. Detection of DENV RNA in mice was performed using the Superscript® III Platinum® One-Step qRT-PCR system (Life Technologies, Carlsbad, CA). Serial dilutions of DENV culture supernatant (from above) were quantified via plaque assay, extracted alongside samples, and used to generate the qRT-PCR standard curve for estimation of DENV titer. Thus, viral concentrations are expressed as PFU-equivalents/mL where appropriate, symbolized as PFU*/mL.

5.2.6 Cytokine Measurement

Granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , IFN- γ -inducible protein-10 (IP-10), tumor necrosis factor (TNF)- α , interleukin (IL)-1a, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were measured in the inoculation sites, draining lymph nodes, and serum at 48 hours post inoculation in the inoculation site cohort of mice using the Milliplex® MAP Mouse Cytokine/Chemokine kit (EMD Millipore, Billerica, MA) as per manufacturer's instructions.

5.2.7 Statistical Analysis

Tissue viral titers and cytokine levels were statistically analyzed using analysis of variance (ANOVA). Leukocyte values were analyzed using odds ratios. These analyses were performed in SAS 9.13 (Carey, NC). Viremia titers were statistically analyzed using grouped t-

test by day in GraphPad Prism version 6.0b for Mac OS X (San Diego, CA). Significance is reported at the $\alpha=0.05$ level.

5.3 Results

5.3.1 Inoculation site mice

5.3.1.a Viral titers

DENV titers were measured at 48 hours post inoculation in the inoculated ears and draining submandibular lymph nodes of mice inoculated with DENV + aegyptin or DENV only. DENV titers in the ears of DENV + aegyptin inoculated mice had a mean of 1.65×10^6 PFU*/mL, while the inoculated ears of mice that received DENV alone had a significantly greater mean titer of 3.38×10^6 PFU*/mL ($p=0.0057$). The lymph nodes of DENV + aegyptin inoculated mice did not differ significantly from mice inoculated with DENV alone ($p=0.0678$) with mean titers of 7.21×10^5 and 1.42×10^6 PFU*/mL, respectively. (Figure 5.1)

5.3.1.b Cytokine responses

The cytokines GM-CSF, IFN- γ , IP-10, TNF- α , IL-1a, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p70 were assessed in the inoculated ears, draining submandibular lymph nodes, and the serum of mice inoculated with DENV + aegyptin, DENV alone, aegyptin alone, and those that received a mock inoculation as outlined above. All differences stated are significant ($p \leq 0.05$). In the ears (Figure 5.2A), the concentrations of IFN- γ and IL-2 were found to differ between all treatment groups except DENV + aegyptin compared to DENV alone. IL-10 differed among all comparisons except DENV + aegyptin compared to DENV alone and aegyptin alone compared to mock. IP-10 differed when comparing DENV + aegyptin to aegyptin alone or mock, and

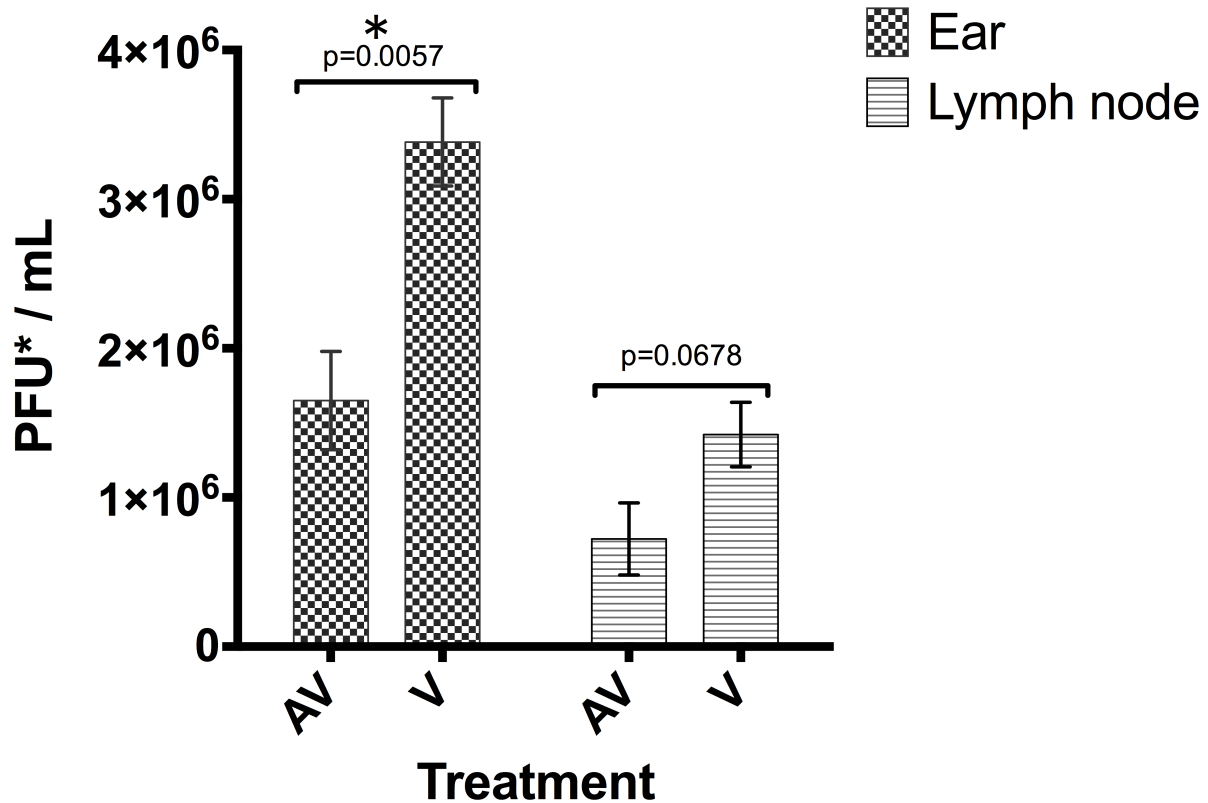


Figure 5.1: DENV titers in inoculated ears and draining submandibular lymph nodes at 48 hours post inoculation. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL) were significantly lower in the ears of mice that received co-inoculation of DENV + aegyptin (AV) as compared to the cohort that received DENV only (V, $p=0.0057$), as indicated by an asterisk. DENV titers were not found to be significantly different in the lymph nodes ($p=0.0678$), although they followed a similar trend as those of the ears. Associated bars represent standard error of the means.

mock differed from DENV alone. IL-6 differed only in ears inoculated with aegyptin alone compared to DENV alone. IL-4 and IL-12p70 were below the limit of detection in all treatment groups. All other cytokines displayed no significant differences between treatment groups, and no cytokines were found to differ in the ear between DENV + aegyptin and DENV alone ($p>0.05$). In the draining submandibular lymph nodes (Figure 5.2B), GM-CSF, IFN- γ , and IL-6 were found to differ significantly between DENV + aegyptin compared to DENV alone, aegyptin

alone, and mock treatment mice. IL-5 in DENV + aegyptin inoculated mice differed from mice inoculated with DENV alone. TNF- α differed among all comparisons except DENV + aegyptin compared to DENV alone and aegyptin alone compared to mock inoculated mice. IP-10 differed only between aegyptin alone compared to DENV + aegyptin or DENV alone. Again, IL-4 and IL-12p70 were below the limit of detection and all other cytokines displayed no significance between treatment groups ($p>0.05$). Cytokine concentrations in the serum were not found to be significantly different between any treatment groups ($p>0.05$, data not shown).

5.3.1.c Hematology

The percentages (counts) and concentration (per μL) of circulating leukocytes were determined as described above for all four treatment groups. All stated differences are significant ($p\leq 0.05$) as determined using odds ratios. The eosinophil counts in the DENV + aegyptin inoculated mice were more likely to be elevated when compared to mice inoculated with DENV alone, as well as in mock inoculation compared to DENV alone. The monocyte counts were correspondingly more likely to be lower in these comparisons as well as in mice inoculated with aegyptin alone compared to DENV alone. Neutrophil counts were found more likely to be lower between mice inoculated with aegyptin alone as compared to all other treatments. Lymphocyte counts were correspondingly more likely to be higher in the aegyptin alone group compared to all other treatment groups, as well as in mock inoculated mice compared to those inoculated with DENV alone. All other comparisons of counts, the leukocyte concentrations (data not shown), and total nucleated cell concentrations (data not shown) were not found to differ significantly ($p>0.05$). The comparison of leukocyte counts from the aegyptin + virus inoculation cohort as compared to the virus only inoculation cohort is displayed

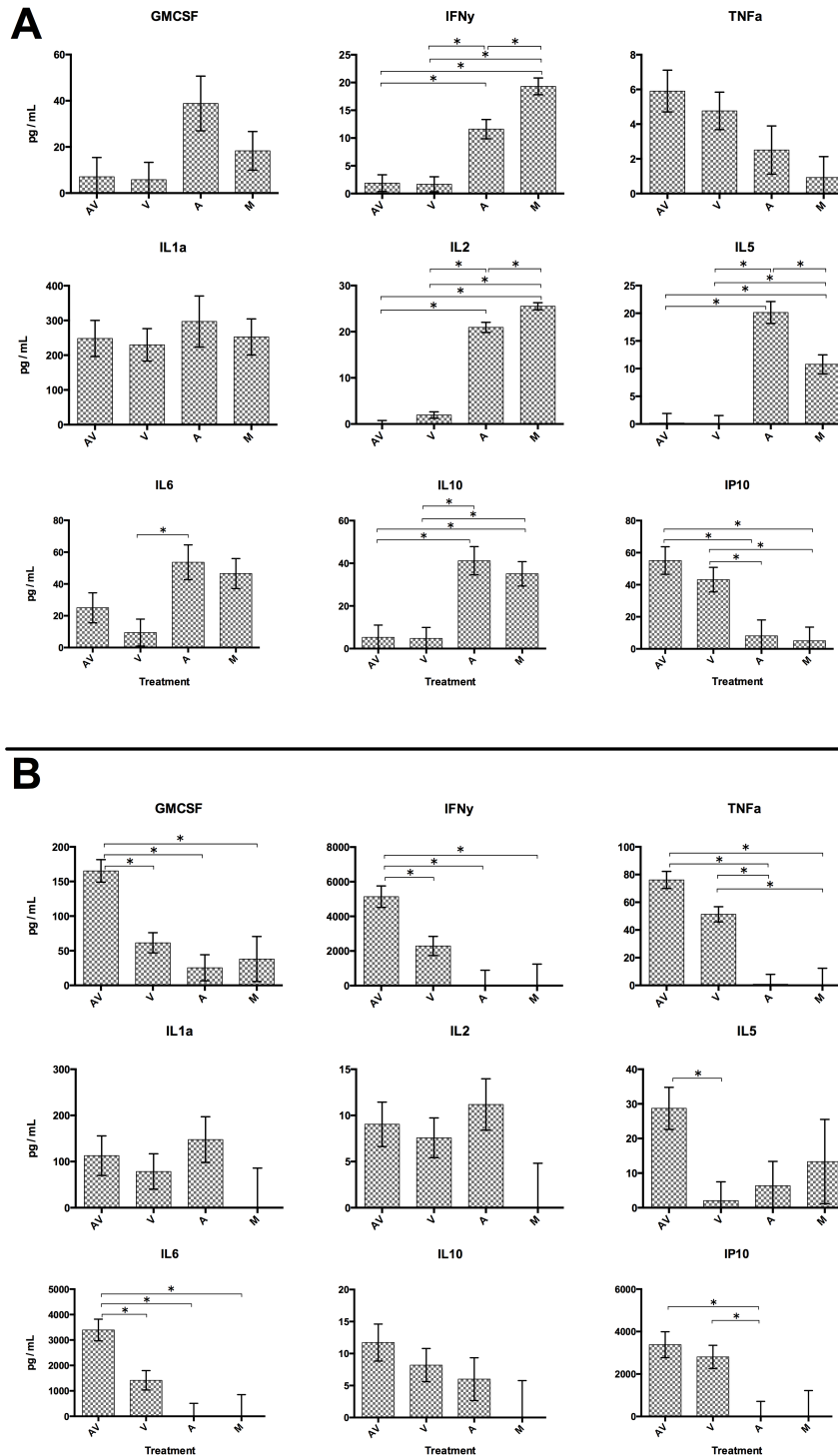


Figure 5.2: Cytokine concentrations in the inoculated ears (A) and draining submandibular lymph nodes (B) at 48 hours post inoculation. The y-axis displays concentration in pg/mL and the x-axis denotes treatment groups (AV = DENV + aegyptin, V = DENV only, A = aegyptin only, M = mock inoculation). Significant comparisons are indicated by an asterisk (p \leq 0.05). Associated bars represent standard error of the means.

in Figure 5.3. The odds ratios with associated 95% confidence intervals and p values for this and all other comparisons of counts are in Appendix 4.

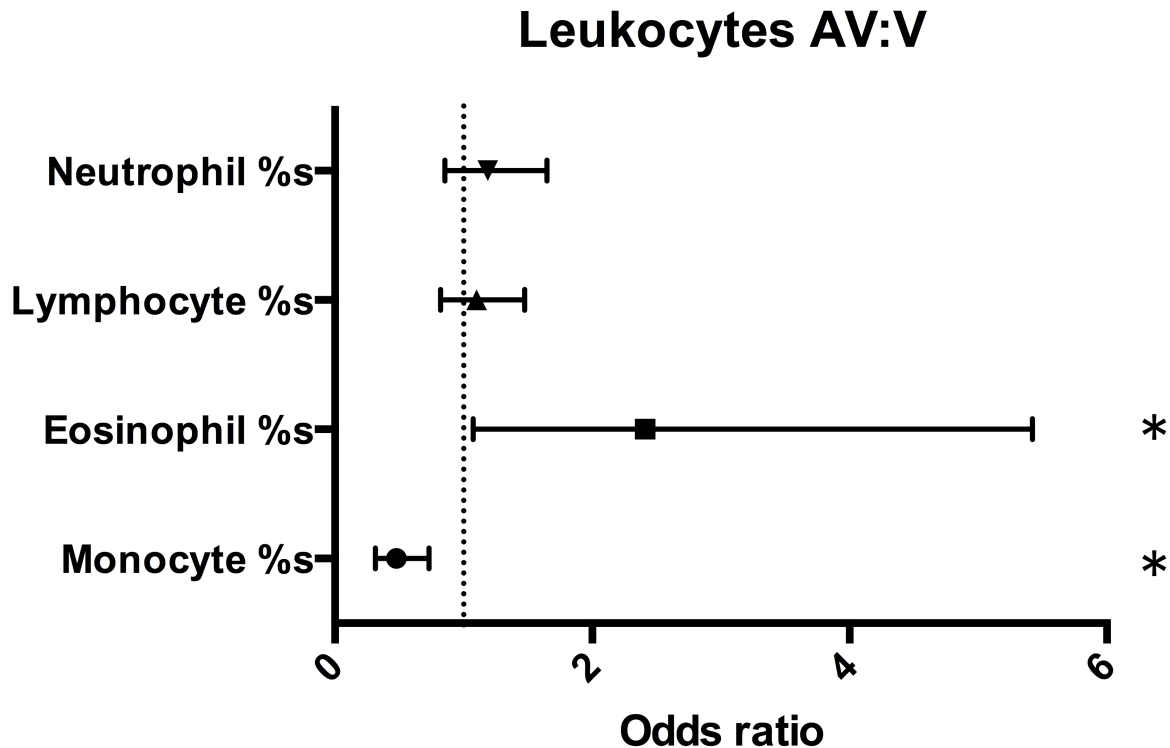


Figure 5.3: Comparison of circulating leukocyte percents (counts) at 48 hours post inoculation for the DENV + aegyptin inoculated cohort (AV) compared to the virus only cohort (V). Comparisons of each leukocyte were performed using odds ratios and significance is indicated by an asterisk ($p \leq 0.05$). Ninety-five percent confidence intervals are displayed.

5.3.2 Serially bled mice

5.3.2.a Viremia

DENV viremia titers were measured each day for six days following inoculation in both the DENV + aegyptin and DENV alone treatment groups. These titers were found to differ significantly between the two treatment groups on day two post inoculation, which corresponds

to the difference observed in the inoculation sites at 48 hours post inoculation, and on day five post inoculation ($p \leq 0.05$). (Figure 5.4)

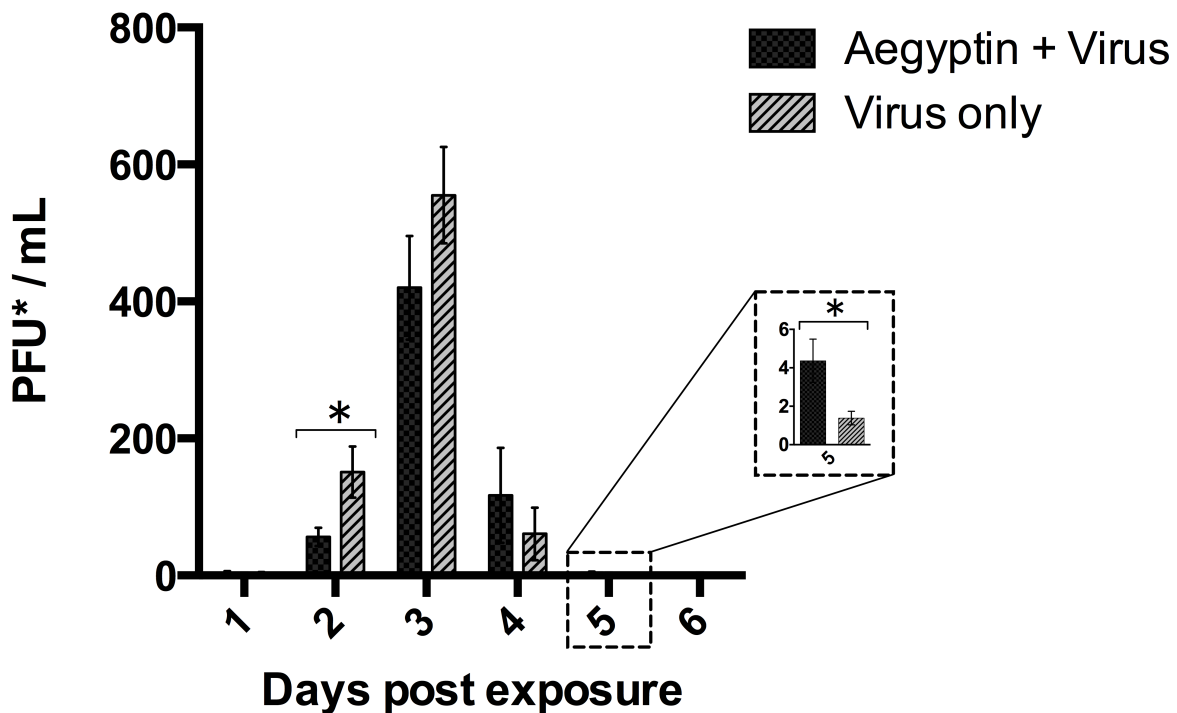


Figure 5.4: DENV viremia titers in serum samples on the first six days post inoculation. DENV titers, expressed as PFU-equivalents/mL (PFU*/mL) were significantly lower in mice inoculated with DENV + aegyptin as compared to those inoculated with virus alone on day 2 post inoculation. DENV titers were significantly higher in mice inoculated with DENV + aegyptin as compared to those inoculated with virus alone on day 5 post inoculation. Significance is indicated by an asterisk ($p \leq 0.05$). Associated bars represent standard error of the means.

5.4 Discussion

The 30 kDa antigen family of proteins contains multiple alleles and splice variants that are compositionally similar, consisting of two domains and possessing similar molecular weights. Prior analysis has revealed that these aegyptins represent two subclades within *Aedes* (designated I and II)[35]. Analysis of expectorated saliva from DENV2 infected *Ae. aegypti* using 2D gel

electrophoresis and LC-MS/MS has previously identified a subclade I aegyptin (gi|18568322) that was reduced 14.1-fold when compared to saliva from uninfected mosquitoes [34]. This aegyptin has also been referred to as “SAAG-4,” which has demonstrated the capacity to suppress IFN- γ expression while elevating IL-4 expression by CD4⁺ T cells outside the context of a viral infection [26]. The recombinant aegyptin used in the current study *in vivo* is a member of subclade II (gi|94468546) and is the archetypal aegyptin utilized in the biopharmaceutical characterization of the capacity to inhibit clotting [8, 28]. This aegyptin is also identical in sequence to that of *Ae. aegypti* salivary allergen “Aed a 3” (gi|205525920), a protein shown to induce allergic responses in both mice and humans [43]. Additionally, other researchers have found an association between serum reactivity to Aed a 3 and the dengue fever (mild) disease state in clinical patients in Thailand [44]. While the two aegyptin groups represent distinct subclades, the acidic (glycine-, aspartic acid-, and glutamic acid-rich) aminoterminal domain and the more complex carboxyterminal domain characteristic of this protein family remain conserved [35].

The goal of this study was to determine the impact of the *Ae. aegypti* salivary protein aegyptin on DENV infection kinetics and the corresponding vertebrate immune response, in light of the allergenic and anti-clotting effects already ascribed to this protein. We designed two *in vivo* murine experiments examining the two vertebrate facets critical to mosquito-borne viral transmission: establishment of DENV infection within the bite site of the vertebrate host, and systemic infection (circulation) that enables transmission from the vertebrate to naïve mosquitoes. The time point of the first experiment, 48 hours post inoculation, is the day most often observed for the onset of DENV viremia in these mice [45], and as such potential differences seen in the bite site and draining lymph nodes were assumed to be near their peaks. Mice for the second

experiment were examined daily through the end of viremia for differences that have the potential to lead to differential acquisition by the vector, such as differences in the magnitude of viremia titers or day of viremia onset [46].

The addition of aegyptin to the DENV inoculum resulted in significantly decreased viral titers in the inoculated ears 48 hours post inoculation, which could provide a rationale for the DENV2-induced reduction of aegyptin in the saliva of infected mosquitoes. It may be that this reduction in expectorated aegyptin decreases the likelihood of an allergen-related or otherwise inflammatory immune response at the bite site. As an extreme example, humans bitten by mosquitoes lacking the ability to expectorate saliva failed to manifest wheals [47]. Mosquito saliva also has been shown previously to induce numerous immunological reactions at the site of inoculation including T-cell-mediated hypersensitivities, IgE-independent mast cell degranulation, and immune cell recruitment [9]. Lymph node GM-CSF, IFN- γ , IL-5, and IL-6, as well as the percentage of eosinophils in circulation, were observed to be greater in the DENV + aegyptin mice than in mice inoculated with DENV alone at this time point. GM-CSF was demonstrated previously to promote growth, maturation, and survival of eosinophils, a pro-inflammatory granulocyte typically associated with immune responses to allergens [48, 49]. IL-6 is known to be an important mediator of the acute phase response to infection and other antigenic stimuli, and its influences have been reviewed previously [50]. IFN- γ and IL-5 promote the activation and survival of phagocytes and eosinophils, respectively, influence B cell isotype switching events, and are involved in allergic immune responses [51-54]. A reduction or alteration in these immune responses via a reduction in expectorated aegyptin could benefit the establishment of DENV within the vertebrate host. As a consequence of lessened aegyptin in the site of viral establishment, there may be less non-specific destruction of virions or infected cells

by local inflammation, cytokine-mediated cellular activation, and eosinophil-derived degranulation.

Importantly, the addition of aegyptin to the DENV inoculum of the serially bled cohort of mice resulted in an approximately three-fold reduction in viremia titer on day two post inoculation, which corresponds to the decrease seen in the inoculation site at 48 hours post inoculation. Analogously, a reduction in expectorated aegyptin, as was observed in DENV infected *Ae. aegypti* mosquitoes, may result in greater viremia titers at this time point, which has been shown to lead to increased acquisition rates by mosquitoes from human clinical patients [34, 46]. The increase in DENV + aegyptin viremia titer seen on day 5 post inoculation (mean = 4.36 PFU*/mL) may not result in meaningful differences in transmission when compared to the DENV alone cohort (mean = 1.39 PFU*/mL) given the relatively minimal magnitude of these titers. Alternatively, the significance of changes at these two time points may have a more epidemiological importance. Mosquitoes have been shown to successfully acquire DENV up to two days prior to the onset of illness in human cases, at which time these individuals may seek medical attention or otherwise sequester themselves from access by mosquitoes [55]. As such, the minimization of contact between clinically ill individuals and mosquitoes might select for a viral phenotype that induces decreased expression of aegyptin, thereby resulting in greater early (potentially prodromal) viremia rather than late viremia enhancement.

In conclusion, the influence of aegyptin on DENV infections of mice was exemplified by decreased viral titers early in the infection in inoculation sites and in circulation, and by a day with increased viremia titer late in infection. These modulations of DENV infection corresponded to increases in cytokines with known functions in pro-inflammatory and allergen-mediated immune responses, as well as an increased likelihood of eosinophil production.

Together, these data support a role for aegyptin in the modification of the host immune response during DENV infection. The role of aegyptin in a naturally introduced DENV infection of humans may be additionally influenced by other, co-expectorated saliva proteins, as well as prior immunity to aegyptin or the same. Future studies should seek to further characterize pro- and anti-viral aspects of mosquito saliva-DENV interactions in the context of human infection and immunity.

5.5 Acknowledgements

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CHAPTER 6

DISCUSSIONS AND CONCLUSIONS

6.1 Introduction

Presented in this dissertation are four experimental studies investigating the influence of *Aedes aegypti* saliva on the vertebrate host response to dengue virus (DENV) infection, of which chapters 3 and 5 are currently published in peer-reviewed scholarly journals. Rather than the chronological order in which each study was performed, the chapters presented herein are organized by their relative position in the transmission cycle of DENV and then by the deconstruction and analysis of factors expected to impact the perpetuation of DENV. Particular emphasis in each study was given to factors and time points relevant to transmission of DENV to and from the vector and to establishment of DENV within the vertebrate host. This dissertation makes no attempt to investigate factors associated with the onset of disease symptoms or progression of disease severity, as our murine model is established as one of transmission, rather than disease recapitulation. Summarized below are the results of each chapter, a discussion of their interrelatedness and relevance to previous works (of which I am a contributing author), and an indication of the future direction additional works should follow.

6.2 Summary of results and comparison between works

In chapter 2, I conducted a high-throughput RNA-sequencing analysis of the effect that infection with different strains of DENV within serotype 2 has on gene expression within the salivary gland. These proteins are highly relevant to the immunological context of DENV2 establishment within the vertebrate and have been shown previously to be altered in an investigation of a single strain [1-3]. Similar to the previous protein studies, but on a grander

scale, we found differential expression at the transcript level of many thousands of different genes involved in a variety of cellular activities. Notably relevant changes were found between the expression of known and putatively excreted salivary proteins that differed based upon the strain of DENV2 (1232 versus 16803) infecting the salivary glands. Importantly, both clade I and clade II aegyptins were identified in this study, whereas previous protein work missed the clade II aegyptin [2]. Both of these aegyptins were found to be expressed at different levels between the two virus strains tested, and importantly, only strain 1232 expressed these genes at levels different from control. These results indicate the potential importance of aegyptins in the larger dynamics of DENV perpetuation and provide a greater rationale for their investigation in chapters 4 and 5.

In chapter 3, I conducted an *in vivo* study examining the impact of *Ae. aegypti* probing (i.e., salivation and mechanical damage) on establishment of DENV2, strain 1232 infection in a murine model of transmission. This study utilized naïve (i.e., non-infectious) mosquitoes to probe mouse ears, and then the immediate intradermal inoculation of DENV into the probed locations. By utilizing naïve mosquitoes, the results stem from the influence of steady-state, rather than virally-modified, saliva protein levels on the vertebrate response to DENV introduction. This study provided evidence of global reductions in the immune response, with emphasis on the reduction of inflammatory and anti-viral gene transcript expression. These immunological changes then associated with significantly enhanced magnitude and duration of viremia. Higher concentrations of viremia have been associated empirically with increased acquisition rates and downstream prevalence of infectious mosquitoes, and a longer duration of viremia would provide additional opportunities for acquisition events by naïve, feeding

mosquitoes [4]. These results indicate for the first time that vector saliva can impact DENV establishment in the vertebrate in meaningful ways relevant to the perpetuation of the virus.

At this stage, our *a priori* knowledge of DENV-induced alterations to the salivary protein and now transcript profile, and the above observations on the impact of a steady-state salivary protein profile on DENV infection of the vertebrate required reconciliation. In this dissertation, I provide and execute a two-stage experimental plan (*in vitro* and *in vivo*), consisting of chapters 4 and 5, to account for the observations of salivary proteins in lower abundance upon DENV infection in the context of the observed benefits to DENV perpetuation caused by a steady-state salivary protein profile.

In chapter 4, I conducted an *in vitro* experiment testing the effects of individual, recombinant, *Ae. aegypti* salivary proteins on DENV2, strain 1232 infection in the human hematopoietic cell line, K562. This study utilized three salivary proteins, of which two were known to belong to families found in lower abundance in the previous protein examination (an adenosine deaminase and an aegyptin), and one for which the protein relative abundance value is unknown (a C-type lectin). Comparing to the transcript results from chapter 2, the adenosine deaminase and the aegyptin were again found to differ from control, whereas the C-type lectin of closest BLAST alignment was not found to differ ($p > 0.05$) in transcript quantity from that of control, though other C-type lectins were. Upon co-exposure of K562 cells to DENV and a salivary protein, DENV concentration in the supernatant (measured targeting RNA) was altered in interesting ways when compared to cells exposed to DENV alone. DENV + aegyptin resulted in a significant decrease in the quantity of virus produced, suggesting a possible rationale for the lowering of aegyptin protein expectorated by DENV2-infected *Ae. aegypti*. However, this alteration in DENV production was not seen in supernatants whose cells were exposed to

adenosine deaminase (the other lower-abundance salivary protein tested). Interestingly, DENV + C-type lectin resulted in a similar decrease to that of aegyptin co-exposed cells, which is important given that the protein abundance level of this C-type lectin in DENV-infected mosquitoes is currently unknown. These results do not provide an immediate pattern for the postulation of an overarching statement about the nature of altered protein abundance and DENV infection of or perpetuation through the vertebrate. Vertebrate immune responses are a larger network of interactions that far exceeds the scope of a single cell line. Therefore, I moved into the more complex *in vivo* system, conducted in the next chapter.

In chapter 5, I chose the recombinant aegyptin protein to evaluate the impact of a lower-abundance *Ae. aegypti* salivary protein on DENV2, strain 1232 infection of the vertebrate. DENV titers were examined in the inoculation sites and over time in circulation. Intradermal co-inoculation of DENV and aegyptin resulted in significantly decreased DENV titers in the inoculation sites at 48 hours post exposure when compared to inoculation of DENV alone. This trend continued in the draining lymph nodes, and was observed (as significant) in circulation at the same time point. The decreases in viral titers observed in the present study occurred contemporaneously with an increase to allergen-associated and anti-viral cytokines, including IFN γ , GM-CSF, IL5, and IL6, as well as the increased likelihood of elevated eosinophils in circulation. These immunological results are not surprising given the previous characterization of aegyptin (aka Aed a 3) as an allergen [5]. Looking at the present study from a reciprocal perspective, if a reduction of aegyptin in the salivary expectorate were to raise the viremia at the observed early time point above a threshold required for successful acquisition of DENV by naïve mosquitoes, this could increase the duration of human infectiousness. Accordingly, high early DENV viremia titers have been shown to be a marker of the duration of infectiousness to

mosquitoes, and, as stated above, higher viremia titers are associated with higher acquisition rates and prevalence of infectious mosquitoes [4]. Additionally, it has been shown using mathematical models that an increased duration of human infectiousness of the index case upon entering a naïve human population results in an increase in the probability of DENV emergence within said population [6]. Therefore, these data could indicate the existence of a negative pressure on DENV perpetuation by at least one of the *Ae. aegypti* salivary proteins found in lower abundance upon DENV2 infection of the salivary glands, and thereby account for its lowered abundance.

6.3 Conclusions and future direction

Together, the data presented in this dissertation inform on a complex and dynamic relationship between DENV, the vector, and the vertebrate host in which *Ae. aegypti* saliva plays an integral role. Most studies of DENV transmission, infection, or disease pathogenesis do not involve or account for the contribution of mosquito saliva. Even subtle changes due to mosquito saliva in the immunological response of the vertebrate, upon introduction of DENV or further along in the infection process, might have as yet uncharacterized consequences. One consequence could be a differential progression to or risk of disease severity, as has been suggested by a correlative study of secondary DENV-infection, clinical patients in Thailand. This study found that seroreactivity to the protein Aed a 3 (i.e., aegyptin) associated with a mild clinical illness, whereas seroreactivity to the salivary protein apyrase associated with dengue hemorrhagic fever, and seroreactivity to a salivary D7 family member protein associated with clinical dengue disease regardless of severity [7]. Another consequence of not accounting for mosquito saliva during experimental investigations could arise during the development and

testing of DENV vaccine candidates. For example, the Sanofi Pasteur vaccine candidate, currently the furthest along in clinical trial proceedings, is a promising tetravalent formulation, and yet generates only 60.8% overall efficacy and comparatively low protection against DENV serotype 2 [8]. While there are a great many factors that could impact the success of a vaccine, such as viral diversity and variation in individual, human immune responses, it is possible that earlier testing of vaccine formulations with vector saliva or salivary components could generate more efficacious results in the long term, especially when considering that mosquito saliva is present in all naturally acquired DENV infections.

Therefore, I propose that all future research into the establishment of arboviral infections and disease pathogenesis within the vertebrate include, or at least account for, some aspect of vector saliva. Additional work in line with that of the present dissertation would include: 1) expanding upon chapter 2 with multiple specimen, individual target verification at the transcript and protein level, as well as including the additional three serotypes of DENV and other mosquito-transmitted viruses; 2) expanding chapter 3 through the characterization of additional time points, infiltrating cell types, and progression of virus from the site of inoculation to circulation, as well as the utilization of infectious mosquitoes and animals sensitized to mosquito saliva; 3) repetition of chapter 5 using other, individual salivary proteins; and 4) reconstructing the experimental setup of chapter 5 to utilize mosquitoes with a genetic- or RNAi-mediated depletion of individual proteins in a spot-feeding method of exposure, so that testing the impact of lowered protein abundance can be performed in a direct rather than reciprocal manner. Performing future experimentation in this manner would undeniably improve the body of knowledge regarding DENV perpetuation.

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APPENDIX 1

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APPENDIX 3

DATA SUPPLEMENT FROM CHAPTER 3

Supplemental Table 3.1: SABiosciences RT² Profiler PCR Arrays - TLR, RLR, and NLR transcript results.

<u>Transcription Factors</u>	<u>Fold-regulation</u>	<u>Gene name</u>
Elk1	-2.96	ELK1, member of ETS oncogene family
Fos	-1.67	FBJ osteosarcoma oncogene
Irf1	1.60	Interferon regulatory factor 1
Irf3	-1.59	Interferon regulatory factor 3
Irf5	1.84	Interferon regulatory factor 5
Irf7	1.45	Interferon regulatory factor 7
Jun	2.00	Jun oncogene
Nfkb1	1.42	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1, p105
Nfkb2	-1.77	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
Nfrkb	1.29	Nuclear factor related to kappa B binding protein
Nr2c2	2.31	Nuclear receptor subfamily 2, group C, member 2
Ppara	-1.79	Peroxisome proliferator activated receptor alpha
Rel	3.81	Reticuloendotheliosis oncogene
Rela	3.40	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
Stat1	-1.57	Signal transducer and activator of transcription 1
<u>Receptors</u>		
Aim2	-1.57	Absent in melanoma 2
Cd14	1.73	CD14 antigen
Clec4e	-3.65	C-type lectin domain family 4, member e
Ddx58	2.19	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
Dhx58	-4.20	DEXH (Asp-Glu-X-His) box polypeptide 58
Ifih1	-12.23	Interferon induced with helicase C domain 1
Ifnar1	-2.25	Interferon (alpha and beta) receptor 1
Il1r1	1.26	Interleukin 1 receptor, type I
Il6ra	1.19	Interleukin 6 receptor, alpha
Muc13	-3.63	Mucin 13, epithelial transmembrane
Nlrp3	-2.70	NLR family, pyrin domain containing 3
Nod2	-3.79	Nucleotide-binding oligomerization domain containing 2
Tlr1	-4.53	Toll-like receptor 1
Tlr2	1.22	Toll-like receptor 2
Tlr3	-1.51	Toll-like receptor 3
Tlr4	1.24	Toll-like receptor 4

Tlr5	1.52	Toll-like receptor 5
Tlr6	-2.45	Toll-like receptor 6
Tlr7	-3.88	Toll-like receptor 7
Tlr8	-1.59	Toll-like receptor 8
Tlr9	-3.16	Toll-like receptor 9
Tnfrsf1a	1.56	Tumor necrosis factor receptor superfamily, member 1a
<u>MAPKs</u>		
Mapk1	-4.60	Mitogen-activated protein kinase 1
Mapk3	-2.48	Mitogen-activated protein kinase 3
Mapk8	-1.65	Mitogen-activated protein kinase 8
Mapk9	-2.46	Mitogen-activated protein kinase 9
Mapk14	-2.50	Mitogen-activated protein kinase 14
Map2k1	-1.74	Mitogen-activated protein kinase kinase 1
Map2k3	1.91	Mitogen-activated protein kinase kinase 3
Map2k4	3.62	Mitogen-activated protein kinase kinase 4
Map3k1	-1.36	Mitogen-activated protein kinase kinase kinase 1
Map3k7	1.21	Mitogen-activated protein kinase kinase kinase 7
<u>Enzymes</u>		
Btk	-1.19	Bruton agammaglobulinemia tyrosine kinase
Casp1	-4.60	Caspase 1
Casp8	1.26	Caspase 8
Chuk	1.72	Conserved helix-loop-helix ubiquitous kinase
Ctsb	-2.02	Cathepsin B
Ctsl	-2.21	Cathepsin L
Ctss	-1.64	Cathepsin S
Cyld	-2.85	Cylindromatosis (turban tumor syndrome)
Dak	-1.25	Dihydroxyacetone kinase 2 homolog (yeast)
Ddx3x	-1.35	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3, X-linked
Eif2ak2	1.20	Eukaryotic translation initiation factor 2-alpha kinase 2
Irak1	-1.75	Interleukin-1 receptor-associated kinase 1
Irak2	1.36	Interleukin-1 receptor-associated kinase 2
Pin1	-2.57	Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1
Ptgs2	1.92	Prostaglandin-endoperoxide synthase 2
Ripk1	1.31	Receptor (TNFRSF)-interacting serine-threonine kinase 1
Ripk2	-1.09	Receptor (TNFRSF)-interacting serine-threonine kinase 2
Tbk1	-1.56	TANK-binding kinase 1
Trim25	-2.28	Tripartite motif-containing 25
Ube2n	-1.72	Ubiquitin-conjugating enzyme E2N
Ube2v1	1.00	Ubiquitin-conjugating enzyme E2 variant 1
<u>Cytokines and chemokines</u>		

Ccl2	1.17	Chemokine (C-C motif) ligand 2
Ccl3	-3.84	Chemokine (C-C motif) ligand 3
Ccl4	-2.68	Chemokine (C-C motif) ligand 4
Ccl5	-3.39	Chemokine (C-C motif) ligand 5
Csf2	-1.33	Colony stimulating factor 2 (granulocyte-macrophage)
Csf3	-1.41	Colony stimulating factor 3 (granulocyte)
Cxcl10	-2.86	Chemokine (C-X-C motif) ligand 10
Ifnb1	-4.07	Interferon beta 1, fibroblast
Ifng	-6.13	Interferon gamma
Il1a	-1.16	Interleukin 1 alpha
Il1b	3.26	Interleukin 1 beta
Il2	-4.83	Interleukin 2
Il6	1.33	Interleukin 6
Il10	-1.09	Interleukin 10
Il12a	-3.73	Interleukin 12A
Il15	-1.99	Interleukin 15
Il18	-1.10	Interleukin 18
Lta	2.01	Lymphotoxin A
Tnf	-2.08	Tumor necrosis factor
<u>Activators, Inhibitors, & Adaptors</u>		
Agfg1	1.96	ArfGAP with FG repeats 1
Atg12	-1.36	Autophagy-related 12 (yeast)
Atg5	1.22	Autophagy-related 5 (yeast)
Azi2	-2.09	5-azacytidine induced gene 2
Card9	-1.48	Caspase recruitment domain family, member 9
Cd40	-4.51	CD40 antigen
Cd80	-1.14	CD80 antigen
Cd86	-1.23	CD86 antigen
Cebpb	-1.08	CCAAT/enhancer binding protein (C/EBP), beta
Cnpy3	-1.43	Canopy 3 homolog (zebrafish)
Fadd	-2.01	Fas (TNFRSF6)-associated via death domain
Hmgb1	1.12	High mobility group box 1
Hras1	-1.03	Harvey rat sarcoma virus oncogene 1
Hsp90aa1	-1.08	Heat shock protein 90, alpha (cytosolic), class A member 1
Hspa1a	3.79	Heat shock protein 1A
Hspd1	-1.05	Heat shock protein 1 (chaperonin)
Ikbkb	-1.72	Inhibitor of kappaB kinase beta
Isg15	-4.26	ISG15 ubiquitin-like modifier
Ly86	1.28	Lymphocyte antigen 86
Ly96	-1.32	Lymphocyte antigen 96
Mapk8ip3	-1.13	Mitogen-activated protein kinase 8 interacting protein 3

Mavs	-2.10	Mitochondrial antiviral signaling protein
Mefv	-3.10	Mediterranean fever
Myd88	-1.62	Myeloid differentiation primary response gene 88
Nfkbia	2.78	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
Nfkbib	1.49	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
Nfkbil1	-1.45	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1
Peli1	2.46	Pellino 1
Pglyrp1	-1.80	Peptidoglycan recognition protein 1
Pstpip1	-1.08	Proline-serine-threonine phosphatase-interacting protein 1
Pycard	-2.24	PYD and CARD domain containing
Spp1	1.52	Secreted phosphoprotein 1
Sugt1	-1.16	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)
Tank	-2.77	TRAF family member-associated Nf-kappa B activator
Tbkbp1	-2.55	TBK1 binding protein 1
Ticam1	-2.24	Toll-like receptor adaptor molecule 1
Ticam2	1.63	Toll-like receptor adaptor molecule 2
Tirap	-1.27	Toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
Tnfaip3	1.40	Tumor necrosis factor, alpha-induced protein 3
Tollip	-1.36	Toll interacting protein
Tradd	-1.71	TNFRSF1A-associated via death domain
Traf3	1.56	Tnf receptor-associated factor 3
Traf6	1.49	Tnf receptor-associated factor 6

Red values indicate a greater than 2-fold up-regulation of expression.
Blue values indicate a greater than 2-fold down-regulation of expression.

APPENDIX 4 **DATA SUPPLEMENT FROM CHAPTER 5**

Supplemental Table 5.1: Additional comparisons of circulating leukocyte percents (counts) at 48 hours post inoculation in percents (counts). Comparisons of each leukocyte were performed using odds ratios and significance is indicated by an asterisk ($p \leq 0.05$).

Lymphocyte	Estimate	95% CI		Pr > t	
		Lower	Upper		
A vs AV	2.019	1.488	2.74	0.0006	*
A vs M	1.581	1.148	2.178	0.0148	*
A vs V	2.223	1.639	3.016	0.0002	*
AV vs M	0.783	0.6	1.023	0.0963	
AV vs V	1.101	0.859	1.412	0.4603	
M vs V	1.406	1.077	1.836	0.0263	*

Neutrophil	Estimate	95% CI		Pr > t	
		Lower	Upper		
A vs AV	0.369	0.251	0.545	0.0002	*
A vs M	0.486	0.323	0.732	0.0042	*
A vs V	0.439	0.297	0.65	0.0012	*
AV vs M	1.317	0.974	1.78	0.0969	
AV vs V	1.188	0.898	1.571	0.2482	
M vs V	0.902	0.664	1.226	0.5221	

Monocyte	Estimate	95% CI		Pr > t	
		Lower	Upper		
A vs AV	1.15	0.733	1.803	0.5528	
A vs M	1.468	0.895	2.407	0.1523	
A vs V	0.552	0.366	0.834	0.0143	*
AV vs M	1.276	0.812	2.006	0.3096	
AV vs V	0.48	0.335	0.688	0.0015	*
M vs V	0.376	0.249	0.57	0.0005	*

Eosinophil	Estimate	95% CI		Pr > t	
		Lower	Upper		
A vs AV	0.702	0.352	1.403	0.3352	
A vs M	0.533	0.267	1.063	0.0973	
A vs V	1.694	0.751	3.821	0.226	
AV vs M	0.759	0.444	1.298	0.3322	
AV vs V	2.412	1.212	4.799	0.0262	*
M vs V	3.178	1.6	6.312	0.0057	*

VITA

Michael K. McCracken was born in Louisiana and moved across the state until settling in Lake Charles. He is the son of Melba L. McCracken and Thomas C. McCracken, and the younger brother of Thomas S. McCracken. Michael graduated with his bachelor of sciences in Biochemistry with minors in Chemistry and Anthropology from Louisiana State University in 2010. Later that year, he entered graduate school in the Department of Pathobiological Sciences in the School of Veterinary Medicine. Michael joined the laboratory of Christopher N. Mores where he researched the interaction of vector, virus, and vertebrate that led to the writing of this dissertation. Michael will graduate in December 2014. Upon acceptance of his National Research Council Research Associateship Programs application in early 2015, Michael will pursue his research interests as part of a larger, non-human primate investigation in a post-doctoral researcher position at the Walter Reed Army Institute of Research in Silver Spring, Maryland.