PROTEIN EXPRESSION IN THE SALIVARY GLANDS OF DENGUE-INFECTED *AEDES AEGYPTI* MOSQUITOES AND BLOOD-FEEDING SUCCESS

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Abstract. Mosquito salivary glands (SG) play an essential role in food digestion and pathogen transmission. The function of the salivary components during infection is poorly understood. In this study, female *Aedes aegypti* mosquitoes were infected with dengue virus serotype 2 (DENV-2) via an artificial membrane feeding apparatus. The mosquito SGs were examined for DENV-2 infection for 14 days post-infection (dpi). The amount of dengue virus increased throughout the 14 dpi. Three different meals were provided for the *Ae. aegypti* mosquitoes. SG protein expression was compared among sugar-fed (SF), blood-fed (BF), and dengue-infected blood-fed (DF) mosquitoes using SDS-PAGE coupled with densitometric analysis. The SG of SF mosquitoes had fewer protein bands than those of BF and DF mosquitoes. The major SG proteins seen among BF and DF mosquitoes had molecular weights of 12-15, 25-30, 35-40, 45-50, 55-60 kDa and 61-67 kDa. We compared the SG protein band expression profiles in BF and DF mosquitoes. Two bands (35-40 and 61-67 kDa) were expressed more by DF mosquitoes and 3 different bands (25-30, 45-50, and 55-60 kDa) were expressed more by BF mosquitoes. These SG proteins may have some role in facilitating blood-feeding and dengue infection. We speculate these specific SG proteins in dengue-infected mosquitoes may increase the chance of blood-feeding and virus transmission by infected mosquitoes. These results may be useful for designing additional tools to investigate the interaction between *Ae. aegypti* SG and the dengue virus.

Keywords: *Aedes*, blood feeding, dengue, mosquito, protein, salivary gland

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INTRODUCTION

Dengue fever is an important mosquito-borne disease. The World Health Organization has estimated the incidence of dengue fever is 50-100 million cases annually (WHO, 2007). The dengue virus

is transmitted to humans by the bite of an infected female *Aedes* mosquito with *Aedes aegypti* being the principle vector for dengue virus (Gubler, 1998). There are no currently available commercial dengue vaccines or antiviral agents effective against dengue virus. Understanding mosquito-pathogen interactions is essential to determining how the pathogen develops in the vector prior to transmission. This may provide information to help develop a vaccine or method to control the spread of the disease.

The mosquito salivary gland (SG) plays an essential role in food ingestion and dengue virus transmission. Mosquito saliva contains various anti-hemostatic agents, which enable the mosquito to feed quickly and efficiently, avoiding the defensive response of the host (Beerntsen *et al*, 2000). These molecules are secreted in the distal lateral and median lobes of the SG (Ribeiro *et al,* 1984; James and Rossignol, 1991; James, 1994). Male mosquitoes are incapable of blood-feeding due to a lack of these specialized regions, consequentially they only feed on nectar. Determination of SG proteins among *Ae. aegypti* (Valenzulela *et al*, 2002; Ribeiro *et al,* 2007; Almeras *et al,* 2010), *Ae. albopictus* (Arca *et al*, 2007), *Anopheles gambiae* (Francischetti *et al*, 2002; Arca *et al*, 2005) and *Culex pipiens quinquefasciatus* (Ribeiro *et al*, 2004) mosquitoes has been conducted. In a study of SG proteins from female *Ae. aegypti* (Wasinpiyamongkol *et al,* 2010) and *Anopheles barbirostris* complex (Jariyapan *et al*, 2010) mosquitoes, the majority of SG proteins were found to be involved in blood feeding. These anti-hemostatic molecules include anti-platelet factors (apyrases), the anticoagulant factor Xa (Ribeiro *et al,* 1984; Champagne *et al,* 1995; Smartt *et al,* 1995; Stark and James, 1998), vasodilators (sialokinins) (Ribeiro, 1992; Champagne and Ribeiro, 1994), D7 proteins (James *et al,* 1991) and α-amylase (Grossman and James, 1993). The saliva of mosquitoes has also been demonstrated to enhance pathogen transmission (Edwards *et al,* 1998; Schneider and Higgs, 2008).

Previous studies of *Leishmania major* have shown mice injected with the SG extract of sand flies and *L. major* had a greater parasite burden than mice injected with parasite alone (Titus and Ribeiro, 1988). Mice experimentally infected with West Nile virus through mosquito bite had higher viremia levels than those infected without a mosquito bite (Styer *et al,* 2011). The features of mosquito saliva that might facilitate pathogen transmission include vasodilation, inhibition of platelet activation and suppression of inflammation (Champagne *et al,* 1994, 1995). The sequestration of inflammatory mediators, such as biogenic amines and leukotrienes, is an important function of the abundant D7 protein in *Ae. aegypti* saliva (Calvo *et al,* 2006, 2009). Therefore, saliva not only facilitates blood-feeding, but also plays a vital role in viral infection and transmission. Although, the roles of various mosquito SG components in preventing vertebrate hemostasis have been relatively well-documented, the roles of such compounds in modulating dengue virus infection and promoting transmission have not been clearly described. The aims of this study were to evaluate the expression of mosquito SG proteins in relationship to dengue-infected blood-feeding and the effect of SG proteins in dengue virus infected female mosquitoes on feeding behavior and virus transmission in order to better understand the complex role of SG protein on blood meals and pathogen transmission.

MATERIALS AND METHODS

Mosquitoes and dengue virus

Lab raised *Aedes aegypti* mosquitoes were maintained on 10% sugar solution at 28ºC, 70-80% relative humidity on 12:12 hour light:dark photoperiods at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University. Dengue virus serotype 2 strain 16681(DENV-2-16681) was used for the study, obtained from the TROPMED Dengue Diagnostic Center (TDC), Faculty of Tropical Medicine, Mahidol University, Thailand. The concentration of virus used in this study was approximately $1.7x10^7$ PFU/ml.

Mosquito infection

Five to 7 day-old adult female mosquitoes were orally infected with dengue virus via an artificial membrane feeding apparatus (Ward *et al*, 1978). Mosquitoes were divided into three treatment groups: 1) those fed on 10% sugar solution (SF); 2) those fed on defibrinated human blood (BF); 3) those fed on defibrinated human blood with dengue virus (DF). Engorged mosquitoes were collected from each group 1 day post-infection (dpi), 3 dpi, 5 dpi, 9 dpi and 14 dpi.

Salivary gland dissection and preparation

Mosquito SG tissue samples were dissected in a drop of cold sterile PBS (pH 7.4); the tissue was washed three times with sterile PBS. The SG samples were then homogenized with a pellet pestle and centrifuged at 14,000 rpm for 45 minutes at 4ºC and then the supernatant was collected. The concentrations of SG proteins and RNA supernatant were then determined using a Nanodrop ND-1000, version 3.3.1 (Thermo Scientific, Hamton, NH). The SG supernatant was stored at -80ºC until used.

Dengue viral detection

RNA was extracted from mosquito SG at each time-point using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. The presence of dengue viral RNA was determined using reverse transcriptase-PCR (RT-PCR) as described by Lanciotti *et al* (1992). RT-PCR was carried out on 0.5 µg of SG RNA using a One-step RT-PCR kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The following primers were used: DENV-2_F: 5'-CAATATGCTGAAACGCGCGAGAAACCG-3', DENV-2_R: 5'-CCACAAGGGCCATGAACAG-3' and ribosomal protein S7, rpS7_F: 5' TCAGTGTACAAGAAGCTGACCGGA-3' and rpS7_R: 5'-AACGATGCAGCACAAAGATG-3' was used as an internal control. The RT-PCR products were detected by gel electrophoresis with ethidium bromide staining. The gel was digitized using the Gene Genius Gel documentation system with Gene Snap software (Syngene Laboratories, Cambridge, UK) for ultraviolet visualization.

Characterization of SG protein profiles

The SG proteins were separated using SDS-PAGE with a discontinuous system as described by Laemmli (1970) with some modifications. Determination of SG proteins in BF and DF mosquitoes was conducted in triplicate. Twenty micrograms of SG protein per lane was mixed in a 1:1 ratio (v/v) with 2% sodium dodecyl sulfate (SDS) gel loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% (w/v) SDS, 10% β-mercaptoethanol, 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol]. The mixture was heated to 100ºC for 3 minutes in a hot well and then loaded into wells with 12% acrylamide gel (40 µl/well). The electrophoresis was conducted at 110 V,

Table 1
Aedes aegypti mosquito salivary gland proteins.

Proteins	Molecular weight	References
Apyrase (Aed a 1)	62-68 kDa	Ribeiro *et al*, 1984; Smartt *et al*, 1995; Champagne *et al*, 1995; Peng *et al*, 2001
Anticoagulant-factor Xa	54 kDa	Stark and James, 1998
Salivary serpin putative anticoagulant	47-50 kDa	Orlandi-Pradines *et al*, 2007; Almeras *et al*, 2010
Aed a X1, Aed a X2	37-44 kDa	Peng *et al*, 1998, 1999
Female-specific protein, D7 (Aed a 2)	36-39 kDa	James *et al*, 1991; Peng *et al*, 1998; Almeras *et al*, 2010
Aed a 3, Putative 30 kDa allergen-like protein	23-30 kDa	Xu *et al*, 1998; Simons and Peng, 2001; Ribeiro *et al*, 2007; Orlandi-Pradines *et al*, 2007; Almeras *et al*, 2010
Adenosine deaminase	53-59 kDa	Ribeiro *et al*, 2001; Almeras *et al*, 2010
Angiopoietins-like protein	23-30 kDa	Hackett *et al*, 2000; Valenzuela *et al*, 2002
Antigen-5 protein family	23-30 kDa	Schreiber *et al*, 1997
Vasodilator sialokinins	1.4 kDa	Beerntsen *et al*, 1999; Ribeiro, 1992

300 mA for 45 minutes. After electrophoresis, the SG proteins were detected with 0.2% Coomassie® brilliant blue stain. The molecular weights of the SG proteins were determined by comparing the relative electrophoretic mobility of an unknown component with standard protein markers (Bio-Rad, Hercules, CA). Digital images of protein bands were captured at 600 dpi using a color scanner (SPEC). A linear relationship was obtained by plotting the relative mobility of the protein markers against the logarithmic values for their molecular weight. The density of band expression was determined using Genetools version 4.0 (Syngene Laboratories, Cambridge, UK).

Statistical analysis

SPSS version 13.0 (SPSS, Chicago, IL) was used to compare the expressions of the DENV bands at each time-point and to compare the SG protein bands between BF and DF mosquitoes. Differences between BF and DF mosquito SG protein bands were analyzed using a standard *t*-test to determine statistical significance. Statistical significance was set at $p \leq 0.01$.

RESULTS

DENV-2 replication among the female *Ae. aegypti* salivary glands (SG)

The DENV-2 replication in the mosquito SG increased slowly during the

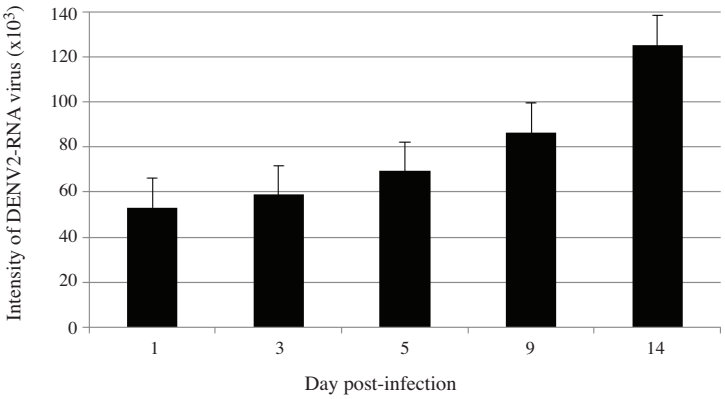

Fig 1–Number of DENV-2 viruses in salivary glands of *Ae. aegypti* by day post-infection.

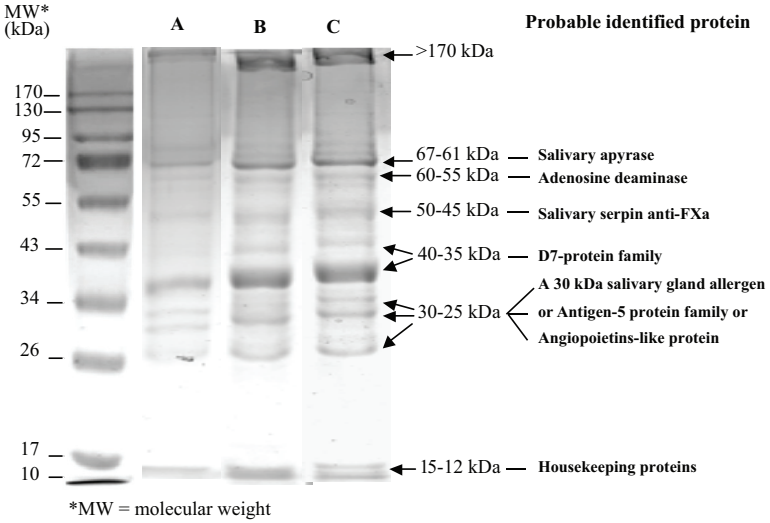

Fig 2–Electrophoresis of salivary gland proteins among A) sugar-fed, B) blood-fed, and C) DENV-2 infected blood-fed female *Ae. aegypti* mosquitoes 14 days post-infection.

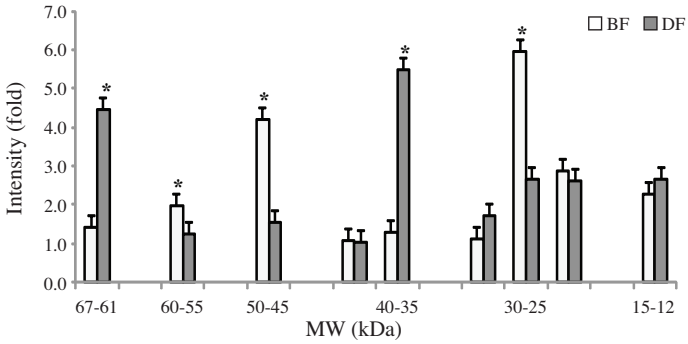

Fig 3–Intensity of female *Ae. aegypti* salivary gland proteins 14 days post-infection.

first 3 dpi, then increased rapidly until it reached a peak 14 dpi (Fig 1).

Salivary gland protein profiles

We observed considerable variation in protein profiles by meal type; the bands ranged in molecular weight from 12 kDa to >170 kDa (Fig 2). At 14 dpi 9 SG protein bands were observed with molecular weights of 12-15, 25-30, 35-40, 45-50, 55-60, and 61-67 kDa. Most of the bands were found in the BF and DF groups, with fewer protein bands seen in the SF group (Fig 2).

The densities of band expression of 5 of the 9 SG protein bands (25-30, 35-40, 45-50, 55-60 kDa and 61-67 kDa) were significantly different between DF and BF mosquitoes (Fig 3), *ie*, two protein bands (35-40 and 61-67 kDa) were expressed in a significantly higher amount ($p \leq 0.01$) among DF mosquitoes, while three protein bands (25-30, 45-50 and 55-60 kDa) were expressed in a significantly higher amount ($p \leq 0.01$) among BF mosquitoes. The other 4 SG protein bands (12-15, 25-30, and 35-40 kDa) exhibited similar expressions in both DF and BF mosquitoes ($p > 0.01$). Some proteins were

expressed to a greater extent among DF mosquitoes and some were expressed to a greater extent among BF mosquitoes and some by both groups (Fig 3).

DISCUSSION

Once a mosquito is infected with an arbovirus, it is capable of transmitting that virus for the rest of its life. The concentration of dengue virus in the SG gradually increased throughout the study. These findings suggest *Ae. aegypti* is a remarkably competent vector (Armstrong and Rico-Hesse, 2003; Salazar *et al*, 2007). A previous study using fluorescence staining demonstrated DENV-2 reached a maximum concentration by 14 dpi (Watt *et al,* 1987). The time from when the mosquito becomes infected with dengue virus until transmission is 7-14 dpi, called the extrinsic incubation period (EPI) (Salazar *et al*, 2007). Thus, the first 14 dpi are a good time to investigate changes in mosquito SG proteins during infection (Salazar *et al,* 2007; Zhang *et al,* 2010). The present study supports previous reports that *Aedes* mosquito SG are susceptible to dengue infection and transmission (Salazar *et al*, 2007). Understanding SG infection with dengue virus and how replication facilitates dissemination can aid in a greater understanding of vector-virus interactions on dengue transmission.

Salivary gland proteins by meal type

A previous study showed mosquito SG are comprised of fewer than 20 protein bands, as demonstrated by a SDS PAGE (Racioppi and Spielman, 1987). In the present study, SF mosquitoes had fewer protein bands than BF and DF mosquitoes. The major SG proteins seen in BF and DF mosquitoes had molecular weights in the ranges of 12-15, 25-30, 35-40, 45-50, 55-60 and 61-67 kDa (Fig 2). Two bands (35-40 and 61-67 kDa) were expressed more by DF mosquitoes and three bands (25-30, 45-50, and 55-60 kDa) were expressed more by BF mosquitoes (Fig 3). Comparing the effects of the blood meal on SG protein expression revealed the protein band density among SF mosquitoes was different than that in BF mosquitoes. Our observations are similar to a previous study comparing differences in SG protein expression between sugar- and blood-fed female *Ae. aegypti* mosquitoes (Wasinpiyamongkol *et al,* 2010) who found those SG proteins up-regulated in SF mosquitoes tended to be housekeeping proteins, those related to energy metabolism (Das *et al,* 2010); whereas, the SG proteins up-regulated in BF mosquitoes were proteins that facilitated the blood-feeding process. Our study found the SG of BF and DF mosquitoes expressed different levels of some proteins after blood feeding than SF mosquitoes.

Several authors have studied the proteins from the SG of *Ae. aegypti;* Valenzuela *et al* (2002) identified 10 amino-terminal sequences obtained by Edman degradation with 1D SDS-PAGE from SG proteins. Ribeiro *et al* (2007) succeeded in identifying 24 SG proteins using 2D gel electrophoresis. The majority of SG proteins related to blood feeding in *Ae. aegypti* have been classified according to their known or predicted cellular location, biological function or allergenicity using proteomic approaches (Valenzulela *et al,* 2002; Ribeiro *et al,* 2007; Orlandi-Pradines *et al,* 2007; Almeras *et al,* 2010; Wasinpiyamongkol *et al,* 2010). These include the D7 family, salivary serpin anticoagulant, salivary apyrase, angiopoietin, antigen-5 protein, adenosine deaminase and certain salivary allergens (Table 1). However, there has been little work on the role of SG proteins in *Ae. aegypti* mos-

quitoes infected with dengue virus.

In our study expression of some SG proteins in DF and BF mosquitoes was different. This suggests dengue infection induces expression of specific proteins and normal blood-feeding induces expression of different proteins. A previous study suggested this difference in protein expression may be an extensive process and there may be post-translational modifications of protein among infected mosquitoes (Choumet *et al*, 2007). Many studies support the hypothesis the presence of pathogens in mosquito SG not only induces modifications in saliva composition but also induces modifications in insect behavior (Rossignal *et al*, 1984; Garcia *et al*, 1994; Mourya *et al*, 2003; Luz *et al*, 2011).

The behavior of insect vectors may be affected by infection with viruses, parasites (Grimstad *et al*, 1980; Rowland and Lindsay, 1986) or symbionts (Evans *et al*, 2009; Moreira *et al*, 2009). There is evidence the dengue virus can alter the behavior of *Ae. aegypti* mosquitoes. For example, dengue-infected mosquitoes spend a longer time feeding than non-infected mosquitoes (Platt *et al*, 1997). *Ae. aegypti* female mosquitoes experimentally infected with dengue virus have increased locomotor activity, which can increase their chances of finding a suitable host and their biting rate (Lima-Camara *et al*, 2011). Previous studies found viral infection may only affect behavior if certain organs are infected (Platt *et al*, 1997). Pathogens must travel from the insect's midgut epithelial cells into the lymph, and then to other organs to ultimately reach the SG. *Ae. aegypti* SG may play a major role in dengue virus transmission and express a variety of bioactive components in saliva that facilitate blood-feeding by inhibiting hemostatic activity. We hypothesized SG proteins expressed among infected female mosquitoes may affect blood-feeding behavior and enhance virus transmission. We investigated some of these SG proteins by looking at how they influence mosquito feeding and probing behavior. Our observations were performed 14 dpi, a time corresponding to a late stage in SG infection when the dengue virus should be at a high concentration (Fig 1).

To identify SG proteins with significantly altered expression in DF mosquitoes, we made predictions based on previously identified SG proteins isolated with a SDS-PAGE that yielded amino-terminal sequencing with Edman's degradation (Valenzuela *et al,* 2002). Our predictions were based on previously described proteins identified by proteomic approaches (Valenzuela *et al,* 2002; Orlandi-Pradines *et al,* 2007; Ribeiro *et al,* 2007; Almeras *et al,* 2010).

Five SG protein bands were selected based on their predicted involvement in the anti-hemostatic process, being major *Ae. aegypti* SG secretory proteins. Of the three SG protein bands with less expression among DF than BF mosquitoes, the first SG protein band (25-30 kDa) was believed to be a salivary allergen (Simons and Peng, 2001), a member of the aegyptin family (Ribeiro *et al*, 2007), part of the antigen-5 family (Schreiber *et al*, 1997), or an angiopoietin (Hackett *et al*, 2000; Valenzuela *et al,* 2002). The function of these SG proteins is unclear, thus we cannot anticipate the consequences of altering their expression during dengue infection and the blood-feeding process. The second band (45-50 kDa) is similar to salivary serpin putatively associated with the salivary factor Xa-directed anticlotting of *Ae. aegypti* (Stark and James, 1998). The last band (55-60 kDa) may be the previously described salivary ad-

enosine deaminase (ADA) (Ribeiro *et al*, 2001). Adenosine deaminase hydrolyzes adenosine to make inosine and ammonia; removal of adenosine may inhibit platelet aggregation and mast cell degranulation. Inosine interferes with host coagulation at the bite site to inhibit the production of inflammatory cytokines. These processes may facilitate blood feeding (Hasko *et al*, 2000; Ribeiro *et al*, 2001; Valenzuela *et al*, 2002). The SG protein bands expressed in smaller amounts among DF mosquitoes (25-30, 45-50 and 55-60 kDa) may be four proteins. These include the 30 kDa salivary protein allergen, the antigen-5 family, the salivary factor Xa-directed anticlotting and adenosine deaminase (Table 1). The present study suggests the expressed SG proteins are likely to play a role, either directly or indirectly, in the feeding process. These proteins have been found to be significantly regulated by blood feeding by *Ae. aegypti* mosquitoes (Das *et al*, 2010; Wasinpiyamongkol *et al*, 2010). It is possible that the expression of these SG proteins is related to blood feeding.

Two SG protein bands were highly expressed among DF mosquitoes. A 35-40 kDa band had a strong similarity to the D7 protein family (James *et al*, 1991). D7-protein, a major SG secretory protein identified in *Ae. aegypti* mosquitoes, exists in two forms. The long form (~30-35 kDa) is found exclusively in mosquitoes (James *et al*, 1991). The D7 protein is expressed predominantly in the SG of adult female mosquitoes, especially in the distal-lateral and median lobes (Suwan *et al*, 2002). The D7 protein has been shown to bind to biogenic amines, which may antagonize vasoconstriction, platelet aggregation and pain during feeding (Calvo *et al*, 2006). This protein may act as an anti-hemostatic factor (Arca *et al*, 2007). Its sequestration by D7 proteins could antagonize these host defense functions, thus promoting blood acquisition and pathogen transmission (Calvo *et al*, 2006). Another 61-67 kDa band could be a salivary apyrase (Champagne *et al*, 1995). Salivary apyrases in *Ae. aegypti* mosquitoes are members of a protein family that includes 5′ nucleotidases (Champagne *et al*, 1995), which are expressed specifically in the distal-lateral and median lobes of SG mosquitoes (Ribeiro *et al*, 1984; Smartt *et al*, 1995). Salivary apyrase is known to facilitate blood-feeding by inhibiting platelet aggregation (Champagne *et al*, 1995). The level of apyrase activity is correlated with the length of probing time during feeding, suggesting the enzyme facilitates the speed of locating blood (Ribeiro, 1987). Salivary apyrase is known to have anti-inflammatory and vasodilatory activity while the D7 family proteins show anti-platelet activity (James *et al*, 1991; Champagne *et al*, 1995). Higher levels of these proteins may result in an increase in mosquito blood-feeding capacity. A previous study revealed that aberrant feeding behavior manifested after increased probing resulting in reduced feeding. Reduced feeding was reported for *Ae. triseriatus* mosquitoes orally-infected with Lacrosse virus (Grimstad *et al*, 1980) and in *Ae. aegypti* mosquitoes parenterally-infected with Semliki Forest virus (Mims *et al*, 1966). The dengue virus may stimulate over-expression of certain SG proteins among *Aedes* mosquitoes, modifying their behavior to enhance virus transmission. Our study provides a better understanding of the relationship between SG proteins expressed in dengue infected mosquitoes and changes in blood-feeding behavior. We speculate the specific SG proteins found in dengue-infected female mosquitoes may increase blood-feeding and virus transmission by

infected mosquitoes.

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