DISSERTATION

THE ROLE OF MIDGUT SERINE PROTEASES IN *AEDES AEGYPTI* VECTOR COMPETENCE

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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ABSTRACT OF DISSERTATION

THE ROLE OF MIDGUT SERINE PROTEASES IN *AEDES AEGYPTI* VECTOR COMPETENCE

Numerous gut-associated viruses utilize host proteolytic enzymes to facilitate enhancement of infection. Similarly, arboviruses infecting the invertebrate host (vector) through the alimentary tract may exploit serine proteases in the midgut to enhance vector infection. Recent genetic and biochemical experiments have demonstrated that dengue virus type 2 (DENV-2) may require proteolytic processing by midgut trypsins to efficiently infect *Aedes aegypti*. These results suggest midgut serine proteases may influence *A. aegypti* vector competence. The requirement of serine proteases in DENV-2 infection of the vector provides unique targets for development of novel control strategies through approaches such as transmission blocking vaccines.

Transmission blocking vaccines targeting two midgut serine proteases were developed and assessed for their ability to block DENV-2 infection of *A. aegypti*. This study found that DENV-2 infected the midgut more efficiently when anti-abundant trypsin (AT) immune serum was mixed with an infectious bloodmeal.

To further investigate the importance of proteolytic processing of DENV-2, multiple mutant viruses lacking potential trypsin recognition sites within domain III of the major surface envelope glycoprotein (E) were constructed. It was demonstrated that

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deletion of one of these sites (K305) increased the ability of the virus to infect the mosquito. Furthermore, co-feeding wild-type DENV-2 with trypsin inhibitors increased midgut infection rates. Interestingly, suppression of each serine protease alone by RNA interference had no effect on infection rates. Overall these results suggest that serine proteases within the mosquito midgut do not proteolytically activate, but may limit DENV-2 infectivity of *A. aegypti*.

The RNA interference studies also reveal that one midgut protease, AT, may not be a traditional serine protease. These results were supported by phylogenetic analysis and sequence alignment data of multiple insect serine proteases. It was determined that AT may represent the first mosquito serine collagenase to be identified.

In total, this work highlights the complexity of mosquito digestion and its effects on DENV-2 infectivity and offers new insight into the basic physiology of mosquito digestion.

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Chapter 1

Literature Review

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Background

In the last 30 years there has been a global resurgence of many arthropod-borne viruses (arboviruses) that were once thought to be effectively controlled (Gubler, 1996). Globally these pathogens cause tremendous morbidity, mortality and economic losses especially in under developed and developing nations (Gratz, 1999). These viruses, as the name suggests, utilize hematophagous arthropods to transmit the pathogens between vertebrate hosts. Arboviruses are a diverse group of viruses and have been identified in seven viral families, six of which have RNA genomes (*Flaviviridae, Togaviridae, Bunyaviridae, Reoviridae, Orthomyxoviridae*, and *Rhabdoviridae*) and one having a DNA genome (*Asfariviridae*) (Weaver, 2006). Coincidently, this diversity permits exploitation of many unique ecological niches and nearly every region on Earth has been affected by recent epidemics (Gubler, 2002). Recently, there has been a drastic increase in global arboviral activity, although the majority of these epidemics are found in tropical regions where climate conditions permit year-round transmission (Gubler, 1996, Karabatsos, 1985 (updated 2001)). The reasons for the re-emergence of many of these agents are multi-factorial and include: 1) lack of effective vector control programs, 2) the collapse of public health infrastructure, 3) no effective vaccines or anti-virals, 4) global population growth and uncontrolled urbanization, and 5) the increase and ease of global travel and trade (Gubler, 1998). Efforts to eventually control these pathogens will need to utilize many traditional approaches such as larvicides and source reduction of larval breeding sites and require the development and implementation of novel control strategies such as transmission blocking vaccines or transgenic vectors.

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Dengue Virus Global Importance

Dengue virus (DENV) epidemics have been affecting mankind for centuries. Early descriptions of dengue-like illnesses have been found from antiquity, but it wasn't until the Philadelphia epidemic of 1780 that the first accurate clinical description of dengue fever (DF) was recorded by Benjamin Rush (Kautner et al., 1997, Nobuchi, 1979, Rush, 1789). This 1780 epidemic in Philadelphia not only defined DF, but also confirmed that this disease was distributed worldwide, as epidemics had occurred the year prior in Batavia (Jakarta) and Cairo (Kautner et al., 1997). For the next 150 years DENV epidemics were regularly recorded every 10-30 years on almost every continent. These globally sporadic epidemics were attributed to sailing vessels and the transport of infected mosquitoes to naïve populations (Henchal & Putnak, 1990, Kautner et al., 1997). Despite the duration of these voyages, these transmission cycles were maintained between infected sailors and the continuous supply of mosquitoes which would breed in the large water containers (Mairuhu et al., 2004).

The 1940's marked a drastic change in the epidemiology of DENV. Epidemics became more frequent and patients with hemorrhagic manifestations began to appear. The first dengue hemorrhagic fever (DHF) epidemic was reported in the Philippines in 1953-54 (Hammon et al., 1960). The frequency of DHF epidemics began to increase in Southeast Asia (SEA), but remained confined to SEA through the 1970's. The success of the Pan American Health Organization's *Aedes aegypti* eradication program implemented in the 1950's insulated the America's from serious DENV epidemics. In fact, DENV was not even considered a major public health concern in the America's. The discontinuation of the eradication program in the 1970's allowed for much of the continent to become re-

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infested with *A. aegypti*. This re-population, along with the introduction of new DENV serotypes and genotypes, has facilitated the emergence DHF in the New World. Today, DENV epidemics and DHF are commonplace in the Americas and pose a significant public health threat (Mairuhu et al., 2004).

DENV is the most significant arbovirus afflicting humans today with nearly 2.5 billion people at risk worldwide (Gubler & Clark, 1995, Gubler & Trent, 1993). The global distribution of risk is primarily associated with tropical and subtropical regions, which provide a suitable habitat for the primary vector, *A. aegypti* (Gubler & Clark, 1995, Gubler & Trent, 1993) . There are four distinct serotypes of DENV (DENV-1, DENV-2, DENV-3 and DENV-4) that circulate at any one time in endemic regions and it is common to find two or more serotypes co-circulating, resulting in hyperendemnicity (Gubler, 1998). Annually, there are about 50-100 million cases of DF worldwide along with nearly 500,000 cases of DHF resulting in ~25,000 deaths (Monath, 1994). Furthermore, DHF can have a case fatality rate upwards of 5% during epidemics, although these figures are approximations due to under-reporting and the inability to perform a differential diagnosis in many of the endemic countries (Malavige et al., 2004, Monath, 1994, Ong et al., 2007). DENV not only causes significant morbidity and mortality, but can impose a large economic burden on many under-developed nations (Anderson et al., 2007, Halstead et al., 2007). In fact, DENV cost Puerto Rico an estimated $250 million over a 10 year period (www.cdc.gov/ncidod/dvbid/dengue/facts.htm).

DENV Transmission Cycle

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The DENV transmission cycle is primarily maintained by biological transmission of the virus between vector and vertebrate host, although there have been reports of transovarial and vertical transmission in the vector (Joshi et al., 2002, Khin & Than, 1983, Rosen, 1987, Rosen et al., 1983). The significance of these latter forms of transmission in the maintenance of the virus is thought to be minimal.

Biological transmission can be differentiated into two separate cycles, urban and sylvatic, which occur naturally. The urban cycle, which exists in endemic and epidemic forms, is maintained between humans, the vertebrate host, and peridomestic *Aedes spp.* (Moncayo et al., 2004, Weaver & Barrett, 2004). This cycle is quite unique because unlike many other arboviruses, in which humans are dead-end or incidental hosts, DENV utilizes humans as the primary vertebrate host. Conversely, the sylvatic cycle utilizes non-human primates as the reservoir host and arboreal *Aedes spp.* (*Stegomyia)* as the vector (Diallo et al., 2003, Rudnick, 1965). Interestingly, phylogenetic analysis suggests that, up until 2000 years ago, the urban and sylvatic cycles were one and the same, although this is not surprising considering that urban civilizations large enough to maintain an inter-human transmission cycle did not arise until about 4000 years ago (Gubler, 1997, Holmes, 2003, Rico-Hesse, 1990, Wang et al., 2000). The remainder of this review will focus on the urban cycle.

As previously mentioned, the primary vector for DENV is *A. aegypti*, although depending on the geographic location, other species within the genus *Aedes*, specifically *A. albopictus* and *A. polynesiensis*, can be competent vectors (Ibanez-Bernal et al., 1997, Rosen et al., 1954). The transmission cycle begins when *Aedes spp.* acquire a bloodmeal from a viremic human. The threshold concentration of virus in the blood

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needed to establish a midgut infection is not known, but viremia titers ranging from 10^3 to $10^{8.5}$ 50% mosquito infectious doses (MID_{50}) have been observed (Gubler et al., 1981, Rosen & Gubler, 1974, Vaughn et al., 2000). Once the virus establishes a midgut infection it disseminates to secondary tissues, specifically the salivary glands, for transmission to a new vertebrate host. This stage of the cycle from infection to transmission is termed the extrinsic incubation period (EIP). Typically, the EIP lasts 8-12 days (Gubler & Rosen, 1976, Nishiura & Halstead, 2007, Salazar et al., 2007). Many factors affect the EIP such as environmental temperature, larval nutrition, viral load in the bloodmeal and intra-specific variation of *A. aegypti* vector competence (Baqar et al., 1980, Bates & Rocagarcia, 1946, Chamberlain & Sudia, 1955, Grimstad & Haramis, 1984, Salazar et al., 2007, Takahashi, 1976). Following dissemination to the salivary glands, the vector, during subsequent feedings, will transmit the virus back to a vertebrate host.

The time interval between infection of a vertebrate host and the development of a viremia suitable for transmission of the virus back to a naïve mosquito is termed the intrinsic incubation period. This incubation period typically lasts for 4-7 days, but can range anywhere from 3-14 days (Siler et al., 1926). An infection begins when an infected mosquito probes for small vessels and in doing so expels virus into extravascular tissue. As the virus enters the extravascular space dendritic cells (DC's), specifically Langerhans cells, become infected and subsequently migrate to regional lymph nodes where viral proliferation occurs (Jessie et al., 2004, Wu et al., 2000). While in the lymph nodes, other mononuclear phagocytic cells such as macrophages become infected and virus is released into the bloodstream (King et al., 1991, Neves-Souza et al., 2005, Scott et al.,

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1980). As previously mentioned, high titer viremias of $10^{8.5}$ MID_{50} can occur, but many factors seem to influence the degree of viremia (Gubler et al., 1981). These high titer viremias can lead to infection of secondary tissues such as the liver and endothelial cells (Avirutnan et al., 1998, Jessie et al., 2004, Wei et al., 2003). Subsequent release of viral particles from these tissues can lead to secondary viremias. The secondary viremia marks the end of the intrinsic incubation period and the virus can be acquired by a naïve mosquito.

Dengue Clinical Manifestations

The clinical signs and symptoms of DENV infections are quite broad and range from inapparent infections to hemorrhaging and shock. According to the iceberg concept, most DENV infections are either asymptomatic or fall into the undifferentiated fever category (Gubler, 1998, Guzman & Kouri, 2002). These outcomes are typically associated with primary DENV infections, but can also occur during secondary infections (Malavige et al., 2004). Despite the lack of clinical symptoms these 'silent' infections may play a critical role in maintenance of the transmission cycle (Chen et al., 1996a).

The majority of symptomatic cases are clinically defined as DF, which is also known as breakbone fever. As previously stated, the incubation period is typically 4-7 days followed by viremia which precedes signs of illness (Nishiura & Halstead, 2007, Siler et al., 1926). DF is an acute febrile disease characterized by sudden onset of fever, which is sometimes biphasic and accompanied by other symptoms such as severe headaches, myalgia, arthralgia and rash (Hayes & Gubler, 1992, Malavige et al., 2004, Siler et al., 1926, Waterman & Gubler, 1989). The acute phase of illness generally

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persists for 2-7 days, but weakness and depression can persist for weeks (Gubler, 1998, Malavige et al., 2004).

DHF is a more severe form of DENV infection typically associated with secondary infections, though it sometimes results from primary infections in infants (Halstead et al., 2002, Halstead et al., 1970, Martinez et al., 1993). The febrile phase of disease is marked with many of the same symptoms as DF, but is often accompanied by vomiting, abdominal pain, sore throat, hepatomegaly and splenomegaly (Aggarwal et al., 1998, Anuradha et al., 1998, Richards et al., 1997). This phase lasts for 2-7 days at which point the patient will begin defervescence and either recover or develop hemorrhagic manifestations (Guzman et al., 1999). Tachycardia and hypotension along with hemorrhaging at the gums and gastrointestinal tract are common and can lead to plasma leakage (Gubler, 1998, Malavige et al., 2004). Furthermore, patients typically have pleural effusions and ascites (Kalayanarooj et al., 2002). During the convalescent phase patients regain their appetite and typically experience bradycardia and develop a recovery rash (Malavige et al., 2004).

The third and most severe form of DENV infections is dengue shock syndrome (DSS). Common symptoms of DSS are sudden hypotension, persistent vomiting, cold botchy skin, circumoral cyanosis, facial petechiae and circulatory disturbances (Agarwal et al., 1999, Gubler, 1998, Guzman et al., 1999, Malavige et al., 2004). If the shock is prolonged the patient may develop metabolic acidosis leading to massive hemorrhaging. Recently, there has been an increase in the number of complications associated with DHF and DSS such as liver failure, myocarditis and encephalopathy (Pancharoen et al., 2002).

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DEN Disease Risk Factors

There are many risk factors: such as age, immune status, HLA haplotype, virus serotype, virus genotype and secondary DENV infections, which have been linked with severe disease outcomes during DENV epidemics (Balmaseda et al., 2006, Egger & Coleman, 2007, Guzman et al., 2002, Halstead et al., 1970, Marchette et al., 1979, Mongkolsapaya et al., 2006, Rico-Hesse et al., 1997, Thein et al., 1997, Zivna et al., 2002). Specifically, the influences of virus genotype and secondary infection have been the two most frequently cited risk factors.

The most significant risk factor associated with severe disease is secondary infection with a heterologous DENV serotype (Halstead et al., 1970, Thein et al., 1997), although this alone accounts for only 2-4% of DHF patients (Guzman & Kouri, 2002). The antibody-dependent enhancement (ADE) model proposes that primary DENV infection induces the production of serotype specific antibodies, which can be non-neutralizing cross-reactive or subneutralizing. Upon secondary infection with a heterologous serotype, these antibodies facilitate an increased uptake of viral particles, via the Fc receptors, into target cells of the mononuclear phagocytic lineage. Furthermore, these antibodies can be maternally transferred thereby increasing an infant's risk for severe disease during primary infections (Halstead et al., 2002, Martinez et al., 1993).

Viral genotype has also been proposed to have a significant affect on determining disease outcome. The introduction of the Southeast Asian (SEA) genotype of DENV-2 into the Americas has been directly correlated with the introduction of DHF in the New World. Recent epidemiological studies have shown that the SEA genotype is gradually,

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but steadily displacing the American genotype which is primarily associated with DF (Rico-Hesse et al., 1997). Furthermore, the SEA genotypes can out-compete the American genotypes in human DCs as well as in mosquitoes (Cologna et al., 2005). Infection experiments demonstrated the SEA genotypes infected mosquitoes to higher titers and disseminated to the salivary glands seven days earlier than the American genotype. These findings suggest that the SEA genotypes can increase vectorial capacity by 2-to 65-fold (Anderson & Rico-Hesse, 2006).

Dengue Virus

Dengue virus is a member of the family *Flaviviridae* and is single stranded, enveloped, positive sense RNA virus with a genome size of approximately 10.8 kilobases (kb). It contains a 10 gene open reading frame (ORF) sandwiched between a 5' type I methylated cap and untranslated region (UTR) and a 3' UTR that lacks a poly-A tail. The 5' quarter of the ORF encodes the three structural genes, capsid (C), membrane (prM) and envelope (E) and the remaining three quarters encode the nonstructural genes (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5), which are essential for viral replication (Chambers et al., 1990, Lindenbach & Rice, 2001).

The viral life cycle begins when the virus enters the cell through receptor mediated endocytosis by way of the E-glycoprotein homodimers, most likely domain III, which coat the envelope (Crill & Roehrig, 2001, Mukhopadhyay et al., 2005). DENV has been shown to infect numerous human cell types, but primary infections are believed to occur in cells of the mononuclear phagocyte lineage, specifically dendritic cells (DC) (Jessie et al., 2004, Marovich et al., 2001). Many DENV receptors have been reported,

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but only one, DC-specific ICAM 3 grabbing non-integrin (DC-SIGN), has been demonstrated as critical for a productive infection in DCs (Navarro-Sanchez et al., 2003, Tassaneetrithep et al., 2003). To date, a specific receptor required for infection of mosquito cells has not been characterized, although there have been numerous putative receptors identified (Mercado-Curiel et al., 2006).

The virus enters the cell in an endosomal vesicle where the acidic pH causes a conformational change in the planar E homodimer to form a spike-like trimer, thus allowing membrane fusion (Modis et al., 2004). Nucleocapsids escape the endosomal vesicles and un-coat in the cellular cytoplasm. The genome is released and host translational machinery initiates translation from the positive sense genome. Interestingly, the mechanisms that control viral translation seem to be different from those controlling cellular mRNAs (Edgil & Harris, 2006, Edgil et al., 2006). These differences may be accounted for by the absence of a poly-A tail in flavivirus genomes. The genome is translated as a polyprotein and subsequently cleaved by a cellular signal peptidase and the viral serine protease NS2b-NS3 (Cahour et al., 1992). Once the viral nonstructural genes have been translated, viral replication can begin and translation must halt. The input genome serves as a template for negative strand synthesis and must circularize via the 5' and 3' UTR cyclization sequence domains (Hahn et al., 1987, You et al., 2001, You & Padmanabhan, 1999). Subsequently, the negative strand acts as a template for the synthesis of many positive strands. This mechanism of replication in which one strand is produced in excess of the other strand is termed asymmetrical replication and regulation is not well understood. The viral glycoproteins are co-and post-translationally modified as they proceed through the endoplasmic reticulum (ER)

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(Mukhopadhyay et al., 2005). Prior to viral assembly the prM and E-glycoproteins, which accumulate in the lumen of the ER, are closely associated (Konishi & Mason, 1993, Lorenz et al., 2002a). This association is thought to prevent an irreversible conformational change from occurring to E during viral egress through acidic cellular compartments (Heinz et al., 1994). Nucleocapsids are assembled and genomic RNA is packaged on the cytosolic face of the ER. The virion then buds through the ER acquiring an ER-derived lipid bilayer containing prM and E. The immature viral particles proceed to the Golgi apparatus where cellular furin cleaves prM to form M (Elshuber et al., 2003, Stadler et al., 1997). This cleavage event allows a conformational change to occur to the E proteins and thereby producing mature virus particles which are secreted.

Virion Structure

DENV particles can be naturally found in one of two states; the mature virions which are smooth, spherical particles and are ~40-50nm in diameter, and the immature virions that are covered in spikes protruding from the lipid bilayer envelope making them significantly larger (Allison et al., 2003). Immature virions are produced as a result of nucleocapsid passage through the ER envelope. These particles contain clusters of three prM:E heterodimers which form 60 spikes in an ordered lattice with icosahedral symmetry on the surface (Wengler, 1989, Zhang et al., 2003b). In the context of the spikes, prM has two very important functions; it acts to protect the fusion peptide within E from prematurely folding during transport through acidic secretory compartments and acts as a chaperone to assist in proper E folding (Lorenz et al., 2002b, Modis et al., 2004). During transport through a late trans-Golgi compartment prM is cleaved by a cellular

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furin, thereby releasing the 18 kDa amino terminal fragment and leaving the 8 kDa protein M on the viral surface (Stadler et al., 1997, Wengler, 1989). The importance of prM during viral morphogenesis and exocytosis is well characterized, but little is known about the function of M in mature virions. Removal of the ectodomain of prM induces a major structural re-organization of the viral surface. The E-glycoproteins rearrange to form 90 homodimers that lie parallel to the lipid bilayer and result in an icosahedrally symmetric herringbone pattern (Kuhn et al., 2002, Zhang et al., 2004). It has been determined that removal of the 'pr' region of prM is necessary for subsequent infections. Specifically, immature particles still containing the prM are unable to undergo normal fusogenic mechanisms within the acidic endosomal vesicles after receptor mediated endocytosis (Elshuber et al., 2003). The E-glycoprotein on the other hand has very little, if anything, to do with viral morphogenesis, but primarily functions during the initial stages of infection.

The E-glycoprotein is a 495 amino acid (aa) protein divided into three functionally distinct domains (I-III), a 53 aa linker stem and a C-terminal transmembrane anchor (Zhang et al., 2003a). Domain I is the centralized domain which connects domains II and III. Furthermore, at the junction with domain II, it contains the hinge region necessary for low-pH conformational change during fusion. Acidic conditions within endosomal vesicles cause the hinge region to flex, thereby forcing domain II out of the constraints of the dimer contacts and releasing the class II fusion loop, found in domain II, outward (Modis et al., 2004, Rey et al., 1995). Unlike the first two domains, domain III seems to be the critical region for virus attachment and penetration. It contains an immunoglobulin-like structure with the most distal projecting loop on the

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surface of the mature virion and is believed to be the viral attachment peptide (Crill & Roehrig, 2001, Mandl et al., 2000, Roehrig et al., 1998). The majority of DENV neutralizing antibodies are directed against domain III (Roehrig et al., 1998).

DENV Vaccines

Many members of the family *Flaviviridae* including yellow fever virus (YFV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and DENV 1-4 pose a significant public health threat to the world. In the 1930s Max Theiler developed the first arbovirus vaccine by passaging YFV in chicken tissue resulting in the live-attenuated 17D vaccine (Barrett & Higgs, 2007). This vaccine is still used today. A live-attenuated vaccine has also been developed against JEV and inactivated vaccines exist for TBEV and JEV (Whitehead et al., 2007). Unfortunately, the development of a safe and effective DENV vaccine has not been successful.

To this end, numerous laboratories have been actively pursuing the development of a DENV vaccine. Aside from low reactogenicity and induction of life-long immunity, an ideal DENV must also be able to provide sufficient levels of protection against all four serotypes. This is critical to the development of a safe vaccine because the induction of short-lived neutralizing or subneutralizing antibodies could predispose vaccinees to ADE (Whitehead et al., 2007).

Live-attenuated vaccines are traditionally highly immunogenic and can be manufactured economically. For these reasons the production of live-attenuated DENV vaccines are leading this effort. Chimeric viruses in which the structural genes of DENV are placed in the YFV 17D strain backbone and replication deficient viruses are two of

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the front-runners and are currently being tested in clinical trials (Whitehead et al., 2007). Others approaches such as subunit, vectored and DNA vaccines are also being pursued, although these are in the early developmental stages (Whitehead et al., 2007). Despite the increased pace of vaccine development many hurdles still remain. These include formulation of tetravalent vaccines, distribution to endemic countries, storage, vaccination regimens, assessment of long-term antibody titers and safety in an ever increasing population of immuno-compromised patients due to HIV. In light of these facts continued research on novel antivirals and control strategies such as transmission blocking vaccines and transgenic mosquitoes are needed.

DENV Evolution

RNA viruses exist in nature as a diverse population of competing mutants, which vary in their degree of nucleotide divergence from a master sequence (Domingo et al., 1996, Duarte et al., 1994, Eigen et al., 1988). These assorted populations are known as a quasispecies and occur due to their error prone replication machinery (Steinhauer et al., 1992). RNA viruses, in general, have been shown to have very high mutation rates compared to their DNA virus counterparts. The primary reason for this is that the viral RNA dependent RNA polymerases lack proofreading capabilities, thus allowing for an increased rate of genomic substitutions (Steinhauer et al., 1992). Typically, RNA viruses have a mean nucleotide substitution rate of 10^{-3} to 10^{-5} substitutions per site per year (Domingo et al., 1996, Duarte et al., 1994, Eigen et al., 1988). Interestingly, RNA arboviruses have a slightly lower rate of substitutions compared with RNA viruses transmitted by other mechanisms. Most likely, this is a result of the viruses having to

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adapt to two hosts as divergent as insects and mammals (Holland et al., 1991, Jenkins et al., 2002, Novella et al., 1995). For instance, DENV has been shown to have mutation rates ranging from 4.55 x 10^{-4} for DENV-1 to 9.01 x 10^{-4} for DENV-3 (Twiddy et al., 2003), whereas influenza virus A and HIV-1 have rates of 1 x 10^{-3} (Gojobori et al., 1990, Gorman et al., 1990, Leitner & Albert, 1999).

Flaviviruses are transmitted by arthropods and can be categorized into three groups according to their mode of transmission: viruses transmitted by ticks, viruses transmitted by mosquitoes and those with no known vector (Holmes & Twiddy, 2003). These groupings, based on laboratory and field observations, are supported by sequence data. Phylogenetic analysis of 70 flaviviruses, using sequences from the NS5 gene, consistently produced three clades corresponding to their modes of transmission (Holmes & Twiddy, 2003). Further examination of flaviviral phylogeny demonstrated that the four DENV serotypes always group together, and they generally have the same branching order in which DENV-4 was the first to diverge followed by DENV-2 and finally a DENV-1 and DENV-3 divergence (Holmes & Twiddy, 2003). These trends are well supported, although until better resolution can be accomplished with complete genome sequences, identification of DENVs closest relative or its precise origin cannot be determined with any confidence. However, identification of sylvatic cycles of DENV-1, 2 and 4 and the fact that these sylvatic DENV sequences fall basal to human DENVs within their respective serotypes suggests that DENV originated from monkeys (Rodhain, 1991, Rudnick, 1978, Wang et al., 2000, Wolfe et al., 2001). Furthermore, these results suggest that each serotype jumped from monkeys to humans independently.

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Genetic variability is not only present between the four serotypes, but can also be found within each serotype. Groups of viruses within each serotype that have no more that 6% sequence divergence are called genotypes (Rico-Hesse, 1990). To date, most of the genotyping analyses have been restricted to DENV-2, due to lack of sufficient catalogued sequence data for the other three serotypes. Sequence analysis of DENV-2 shows that there are six distinct genotypes, one of which contains all the sylvatic isolates, and typically align with geographical distribution (Holmes, 2003). The other five genotypes are Asian 1, Asian 2, American, American/ Asian, and Cosmopolitan. The Cosmopolitan genotype can be found throughout much of the tropical world (Twiddy et al., 2002). Despite the obvious clonal manner in which DENVs evolve, recent evidence also suggests that DENV can evolve through recombination (Tolou et al., 2001, Uzcategui et al., 2001). Recombination events between genotypes within a serotype have been reported, although there have been no reports of inter-serotype recombination. Flavivirus recombination is thought to occur through a copy-choice mechanism in which the RNA polymerase switches between parental viral templates in dually infected cells during replication (Lai, 1992). This would be expected considering their extensive genetic divergence.

Aedes aegypti

Peridomestic *Aedes spp.* serve as the vector for epidemic DENV, of which *Aedes aegypti* is the primary vector. The anthropophilic and endophilic nature of these mosquitoes make them an ideal vector for DENV transmission (Nelson, 1986, Tinker, 1964). *A. aegypti* lay their eggs in artificial containers near the home, such as buckets,

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flower vases and automobile tires (Soman, 1977, Tinker, 1964). In addition to the close proximity of these breeding sites, homes in tropical, underdeveloped nations typically lack screens, windows or doors making them easily accessible to newly emerged mosquitoes, which prefer to rest indoors (Nelson, 1986). This domesticated life cycle constantly keeps adult mosquitoes in close contact with their preferred food source, humans (Ponlanwat & Harrington, 2005). The close proximity and finicky feeding behaviors of females promotes the acquisition of multiple bloodmeals in a short period of time. *A. aegypti* have been shown to feed, on average, of 0.63-0.76 times a day (Scott et al., 2000). This greatly increases the likelihood that a naïve mosquito will acquire an infectious DENV bloodmeal and also increases the likelihood of transmission of DENV by an infected mosquito. Adult mosquitoes are day feeders and typically, feed early in the morning for a couple of hours and then again in the late afternoon, although they may feed all day when overcast (Nelson, 1986). These breeding and feeding characteristics are what make *A. aegypti* such an efficient vector for DENV.

Aedes aegypti Control Programs

During the mid-20th century *A. aegypti* had virtually vanished from the Americas as a result of the *Aedes aegypti* eradication program implemented by the Pan American Health Organization (PAHO). Using dichlorodiphenyltrichloroethane (DTT) to target adult mosquitoes and source reduction of larval breeding areas these programs were widely successful. In the 1970s, these programs were stopped and *A. aegypti* quickly re-infested the Americas (Mairuhu et al., 2004).

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Today, aside from personal protection and education of the public, larvicides and source reduction methods are the most successful approaches to minimizing the incidence of dengue (Champakaew et al., 2007). Common larvicides include carbamate, pyrethroids and organophosphates such as temephos (Champakaew et al., 2007). Recently, concerns about the affects of these chemical larvicides on the environment and the hazards to non-target organisms including humans have been raised. Furthermore, mosquitoes have begun to develop resistance to temephos, the primary larviciding agent (Braga et al., 2004). In an effort to circumvent some of these concerns numerous biological larvicides have been investigated such as *Bacillus spp.*, densonucleosis viruses, copepods and larvicidal oils (Lacey, 2007, Ledermann et al., 2004, Morais et al., 2006, Rey et al., 2004). Although, many of these are in the early stages of development and are unproven in the field.

Development of insecticide and larvicide resistance, environmental concerns and ongoing political and economical instability in many of the effected countries has marginalized the efficacy of mosquito control programs. In light of these facts, development and implementation of novel control strategies such as transmission blocking vaccines in conjunction with traditional mosquito control methods may be needed to curb the resurgence of DENV.

Mosquito Digestion

Hematophagous insects require blood from a vertebrate host in order to provide the necessary nutrients for egg production. The process of bloodmeal digestion and subsequent extraction of the vital nutrients and adsorption takes place in the midgut.

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Predictably, this is also the site where by viruses can infect the vector allowing for subsequent transmission to a vertebrate host. Blood is acquired through the proboscis by the pumping actions of the cibarial and pharyngeal pumps (Eldridge, 2005). The blood passes through the esophagus en route to the gut, which is subdivided into three regions the foregut, midgut and hindgut (Figure 1.1). The midgut is the principal site for secretion of the peritrophic matrix, digestive enzymes and digestion. The gut is composed of a single-layer of epithelial cells, with three different cell types, connected to a basolateral membrane. Columnar cells constitute the majority of the cells in the midgut and are the primary cell type involved in absorption of digested products and secretion of digestive enzymes (Pennington & Wells, 2005). These cells have tightly packed microvilli located on the luminal surface, which increases their surface area and allows for maximal absorption and secretory functions (Pennington & Wells, 2005). The midgut also contains regenerative cells, which are found near the basal membrane nestled between mature columnar cells. Their primary function is to repiace cells lost during aging, digestion, apocrine and holocrine secretion (Hecker, 1977). The final cell-type identified in the midgut is the endocrine cell, which synthesize and secrete hormones into the hemolymph, although not all endocrine cells secrete the same hormones (Brown et al., 1985, Houk, 1977, Veenstra et al., 1995). During the course of a bloodmeal, adult female mosquitoes can acquire more than their own weight in blood and for this reason the midgut must respond accordingly in order to quickly digest and also detoxify blood components. Columnar epithelial cells secrete numerous luminal proteolytic enzymes such as endoproteolytic serine proteases, carboxypeptidase and aminopeptidase (Noriega et al., 2002), which immediately begin to break down blood proteins (Noriega & Wells,

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1999, Pennington & Wells, 2005). The subsequent amino acid pool can then be used for energy production and egg maturation (Zhou et al., 2004). Although only a miniscule portion of the blood is composed of carbohydrates, the midgut is equipped with glycosidases that are able to break down the oligosaccharides found in glycoproteins and glycolipids. Specifically, two maltase genes and one alpha-glucosidase gene have been cloned from *Anopheles gambiae* (Billingsley & Hecker, 1991, Zheng L. B. et al., 1995). Lipids, primarily derived from erythrocyte membranes, are also found in the blood. In order to breakdown and utilize these molecules, mosquitoes

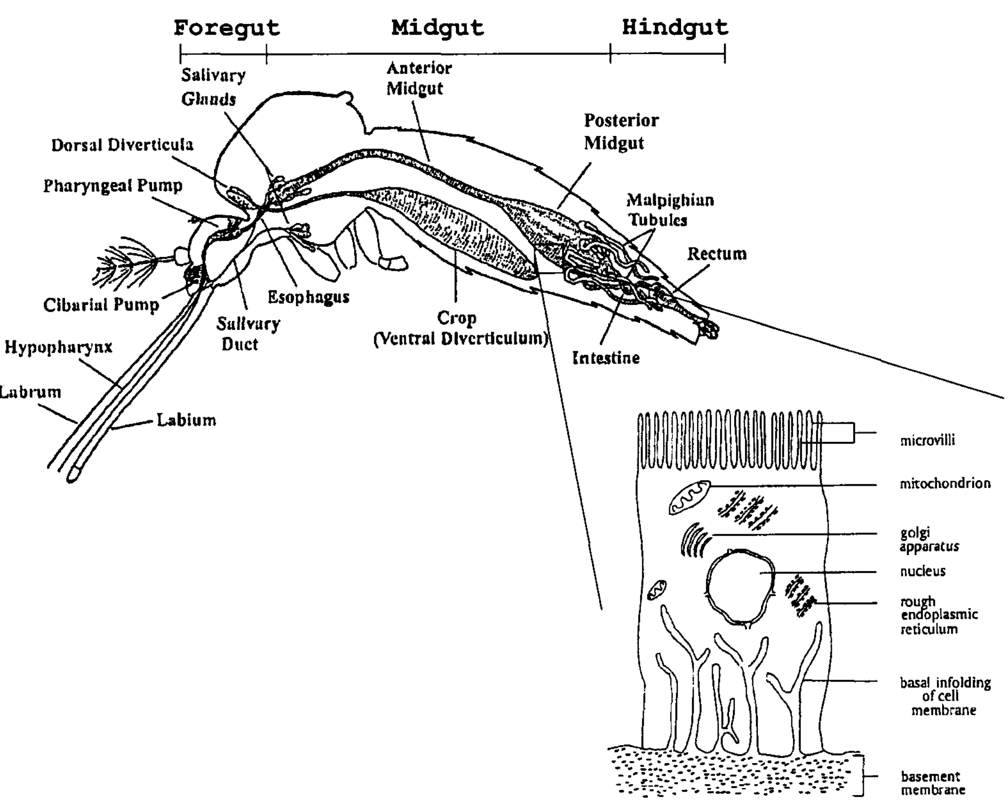

Figure 1.1: Diagram of the adult mosquito alimentary canal. Modified to include foregut, midgut and hindgut demarcations (Snodgras, 1959). The enlarged insert depicts a midgut columnar epithelial cell (Nation, 2002).

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also have triacylglycerol lipase activity (Geering & Freyvogel, 1975).

Another important feature of the midgut that is associated with blood feeding is the secretion and development of an extracellular sheath, which surrounds the blood bolus called the peritrophic matrix 1 (PM1). Shortly after acquiring a bloodmeal, the midgut epithelium begins to secrete chitin, which acts as the framework, along with proteins and proteoglycans, many of which have chitin-binding domains (Moskalyk et al., 1996). The PM1 can be initially detected 5 hours post feeding and is fully developed by 12 hours (Perrone & Spielman, 1988). The specific function of PM1 has not yet been identified, but it is thought to protect against pathogens and abrasion, as well as to act as a detoxifier (Lehane, 1997). Despite the presence of this extracellular sheath the mosquito can digest the contents of the blood bolus well after its full development. The PM1 acts as a sieve whereby molecules larger than 25 kDa are not permitted to escape, but serine proteases in the ectoperitrophic space can still enter (Borovsky, 2003). Despite the sieve-like qualities, the PM1 still impedes digestion as demonstrated by monitoring digestion rates following a bloodmeal with chitinase and anti-PM antibodies (Villalon et al., 2003).

Aedes aegypti Midgut Proteases

As mentioned above, many proteolytic enzymes are secreted by the midgut immediately following a bloodmeal. The primary mediator of blood protein digestion is by the midgut trypsins. The release of bloodmeal-induced trypsins is regulated in a biphasic manner (Felix et al., 1991). Early trypsin (ET)(NCBI accession # X64362) is stored as an intracellular mRNA pool, not a zymogen, in newly emerged adult females.

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Soon after feeding is immediately translated and secreted into the midgut lumen (Figure 1.3)(Noriega et al., 1996a). ET protein levels peak 3 hours post bloodmeal (hpbm) and reach a maximal concentration of 300 ng/midgut (Pennington et al., 1995). The production of the enzyme corresponds to a drop in ET mRNA levels, but mRNA levels are reconstituted at the end of the gonadotrophic cycle, and this cycle is repeated with each subsequent bloodmeal (Noriega et al., 1996a).

The majority of bloodmeal digestion occurs between 12-36 hpbm. During this time many proteolytic enzymes can be found in the midgut, but the predominant species found is that of abundant trypsin (AT), which is secreted by the posterior midgut 8-36 hpbm, but not before (Graf et al., 1986). Immunocytochemical studies demonstrated that AT may be stored as an inactive zymogen in these cells (Graf et al., 1986) and secreted into the lumen at concentrations of 5-6 μg/midgut 24 hpbm (Graf et al., 1988). Utilizing AT monoclonal antibodies the abundant trypsin protein was purified, sequenced and the gene was eventually cloned (NCBI accession #M77814) (Barillas-Mury et al., 1991). AT mRNA begins to be expressed 8 hours post bloodmeal and continue for the next 28 hours, which is subsequently followed by a drastic increase in protein levels (Barillas-Mury et al., 1991).

Coinciding with the secretion of midgut trypsins, a bloodmeal induced chymotrypsin (CHYMO) has also been characterized (NCBI accession #U56423) (Jiang et al., 1997). The deduced amino acid sequence encodes an 18 aa residue signal peptide and a seven aa residue activation peptide indicative of a pre-proenzyme. *A. aegypti* CHYMO is only found in adult females in which mRNA levels can be detected within 24 hours post emergence and attain maximal levels 3-7 days post emergence similar to ET

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(Jiang et al., 1997). CHYMO protein levels and activity are detectable within hours of a bloodmeal and reach maximal levels 20-24 hpbm (Jiang et al., 1997). Unlike ET, CHYMO transcript levels remain high throughout the entirety of bloodmeal digestion.

To date, ET and AT have been the only two trypsins characterized from *A. aegypti* midguts, but there may be many more. There have been seven trypsin genes cloned and characterized from the midgut of *Anopheles gambiae* (Muller et al., 1995). Furthermore, twelve cDNAs were isolated from an *A. aegypti* midgut library using *Drosophila melanogaster* trypsin cross-hybridization, of which three were significantly different from ET and AT (Kalhok et al., 1993). One of these, late trypsin (LT), had a similar expression pattern to AT (NCBI accession #X64363). Probing *Aedes* genomic DNA with the LT clone suggested that it may be encoded by a multigene family (Kalhok et al., 1993). The recent completion and eventual annotation of the *A. aegypti* genome will help elucidate the complexity of the midgut serine protease proteome.

To date, the nomenclature applied in the literature to each of the four identified *A. aegypti* midgut serine proteases is quite confusing. For clarification the names, accession numbers and abbreviations that will be used throughout the remainder of this dissertation are shown in Table 1.1.

Name	NCBI Accession #	Abbreviation	Previous Name
Early Trypsin	X64362	ET	Early Trypsin
Abundant Trypsin	M77814	AT	Late Trypsin
Late Trypsin	X64363	LT	5G1 a.k.a. Kalhok Late Trypsin
Chymotrypsin	U56423	CHYMO	Chymotrypsin

Table 1.1: Current *Aedes aegypti* midgut serine protease nomenclature.

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Regulation of Midgut Trypsins

ET is transcriptionally controlled by juvenile hormone (JH), which is integral in the post-emergence maturation of the midgut (Figure 1.2)(Noriega et al., 1997). Translation of ET is dependent on the size of the free amino acid pool ingested and not stretch receptors (Noriega et al., 1999). JH has been shown to regulate ET transcription in a stage- and dose-dependent manner, but whether JH directly effects gene regulation of ET or whether some downstream factor controls ET transcription is unknown (Noriega & Wells, 1999). mRNA pools of ET can last for up to a month post-emergence without translation or degradation. *In vitro* translation experiments demonstrated that there are no secondary structures inhibiting translation and that a small intron, with an in-frame stop codon, is not located in ET pre-mRNA. Furthermore, the transcripts had no recognizable difference in poly-A lengths before and after bloodmeals (Noriega & Wells, 1999). It has been suggested that activation of ET translation may be associated with whorls located in the endoplasmic reticulum (ER) of columnar cells in unfed females, which may sequester the mRNA away from the translational machinery (Staubli et al., 1966). Subsequently, unfolding of the ER following a bloodmeal may release the mRNA, thereby allowing for translation (Hecker et al., 1971). Despite the appearance of ET immediately following a bloodmeal, little protein degradation occurs during these early stages of digestion and therefore its function is not well understood (Briegel & Lea, 1975, Felix et al., 1991).

As compared to ET regulation, AT seems to be regulated at the transcriptional level (Barillas-Mury et al., 1991, Barillas-Mury & Wells, 1993). Experimental evidence has demonstrated that highly concentrated protein meals and the factors associated with

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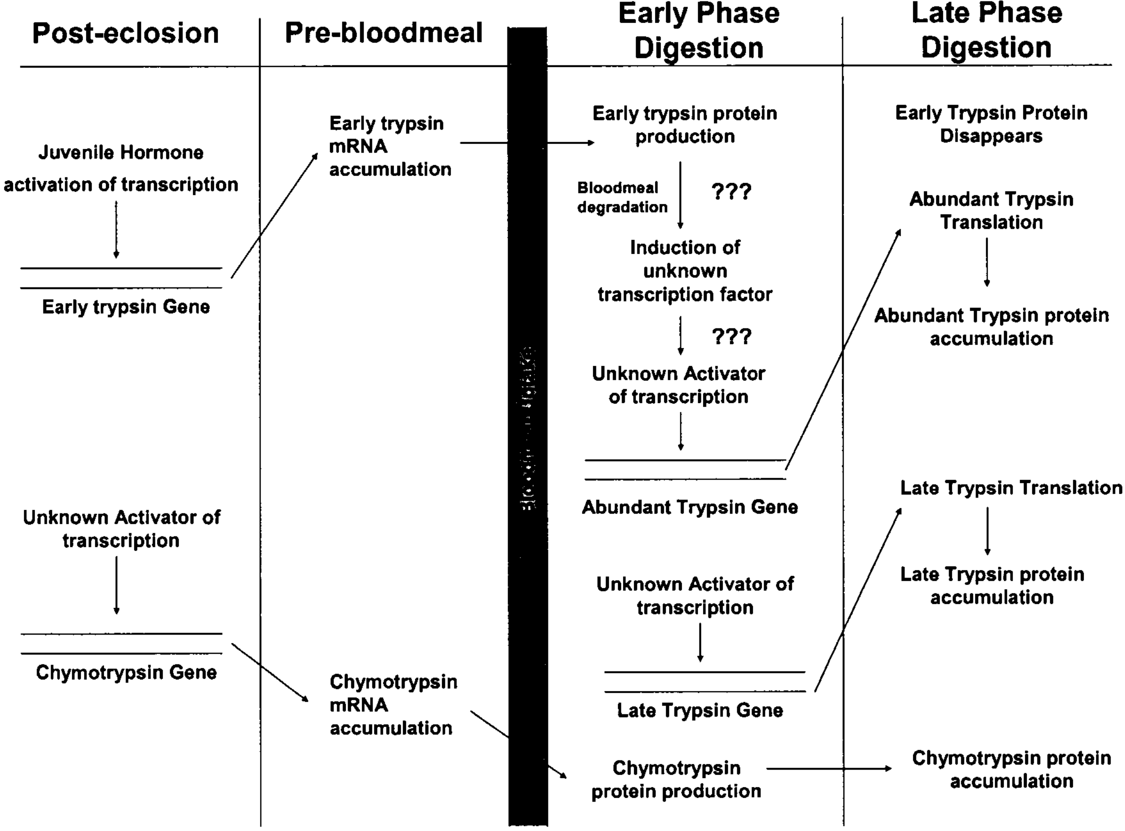

Figure 1.2: Schematic of *A. aegypti* midgut serine protease regulation and expression. The model represents the original paradigm of abundant trypsin transcriptional regulation, but ??? mark steps that have been recently questioned (Lu et al., 2006, Noriega & Wells, 1999). Early phase digestion corresponds to the first 8 hpbm and late phase digestion includes everything after 8 hpbm.

early digestion are potent inducers of AT transcription. Amino acids alone are very poor inducers of AT transcription (Briegel & Lea, 1975, Noriega et al., 1994). The mechanisms involved in AT transcription and subsequent translation were investigated by feeding mosquitoes a bloodmeal in the presence of soybean trypsin inhibitor (STI). The researchers found that inhibiting early phase tryptic activity associated with ET greatly diminished AT transcription and translation (Barillas-Mury et al., 1995). It was concluded that ET activity along with another factor(s) is necessary for induction of AT transcription (Figure 1.2)(Barillas-Mury et al., 1995). Recently, the validity of this

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paradigm has been questioned. New evidence suggests that ET has no effect on the downstream transcriptional control of AT. Through the use of dsRNA suppression of ET, along with a panel of trypsin inhibitors, researchers were able to demonstrate that a significant reduction in ET and early phase tryptic activity had no effect on AT transcription, translation or late phase tryptic activity (Lu et al., 2006). Furthermore, the authors demonstrated that the STI had global physiological effects on the midgut such as reduction in oviposition rates, PM1 formation, AT transcription and expulsion of the bloodmeal. These effects were not observed when egg white trypsin inhibitor (EWTI) and a synthetic serine protease inhibitor, AEBSF, were fed to the mosquitoes. It was proposed that the STI used in the previous experiments may have had a contaminant that was responsible for these physiological changes (Lu et al., 2006). In light of these recent findings, the mechanisms controlling AT transcription are unknown.

Serine Proteases

Serine proteases (SPs) are a diverse class of proteolytic enzymes that constitute approximately one third of all proteases and are appropriately named for their hallmark nucleophilic Ser residue at the active site. Originally, this class of enzymes was characterized by the presence of an Asp-His-Ser catalytic triad that is fundamental to their function (Blow, 1997). Despite the highly conserved triad sequence, phylogenetic analysis and structural modeling suggests that this class arose along four distinct evolutionary lineages, which segregate into four distinct families; chymotrypsin, subtilisin, carboxypeptidase Y and Clp protease (Dodson & Wlodawer, 1998). The chymotrypsin family contains the majority of SPs, with over 300 known members that

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are further divided into 10 subfamilies (Barrett & Rawlings, 1995). These proteases have been identified in all kingdoms, as well as in viruses, and are involved in many physiological processes, including digestion, the immune response, reproduction, apoptosis, signal transduction and homeostasis (Barros et al., 1996, Coughlin, 2000, Johnson, 2000, Joseph et al., 2001, Neurath, 1984).

The chymotrypsin subfamily of SPs have the same basic three dimensional structure, which is arranged in two six-stranded beta barrels, and contain catalytic, substrate recognition and zymogen activation domains (Figure 1.3)(Blow, 1971). Amino acid numbering from this point forward will be presented in the context of bovine chymotrypsinogen (NCBI Accession # P00767). The catalytic triad, Ser195 – Asp102 – His57, spans the active site cleft, which is located between the two beta barrels. Ser195 lies on one side of the cleft, whereas His57 and Asp102 lie on the other side. The substrate recognition sites of SPs are divided into two regions; the polypeptide binding site (S1) and the binding pockets for the side chains of the peptide substrate (Hedstrom, 2002). Typically, chymotrypsin-like SPs specificities are characterized by the interactions between S1 and the scissile bond of the peptide substrate denoted P1-P1', where P1 is the acyl peptide residue recognized by the substrate recognition site. The S1 site is made up of amino acids 189-192, 214-216 and 224-228, where 189, 216 and 226 determine the specificity of the polypeptide binding site (Czapinska & Otlewski, 1999, Perona & Craik, 1995, Perona et al., 1995). The zymogen activation domain of chymotrypsin-like SPs is made up of four segments that are deformed following translation thereby producing the inactive zymogen. The N-terminus to residue 19, residues 142-152, 184-193 and 216-223 collectively make-up the activation domain and

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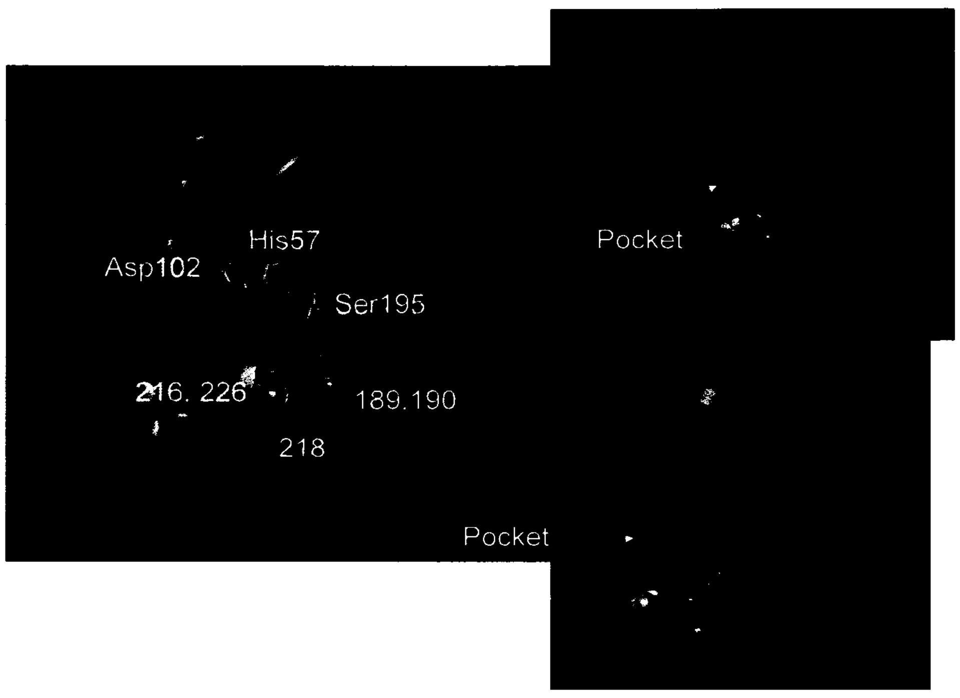

Figure 1.3: Bovine chymotrypsin three dimensional structure. This structure was rendered using PYMOL (PDB #2CHA) in collaboration with Dr. Pamela Hall (University of New Mexico).contain the S1 site (Huber & Bode, 1978).

upon proteolytic processing and release of the N-terminal Ile16, the new N-terminus forms a buried salt-bridge with Asp194 inducing a conformational change exposing the activation domain and allowing the S1 pocket to form, thus creating an active protease.

The chymotrypsin subfamily of serine proteases within the chymotrypsin family is predominantly composed of three enzymatically distinct types; trypsin-like, chymotrypsin-like and elastase-like. Trypsin activity is characterized by the S1/P1 interaction where residue 189 is an Asp in the S1 pocket and preferentially cleaves basic residues Lys/Arg. Chymotrypsins are identified by the presence of a Ser at 189 and recognize large aromatic residues such as Phe/Tyr/Trp. Finally, elastases cleave Ala/Val

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and have an S1 binding pocket that is determined by Val216 and Thr226 (Hedstrom, 2002). Experimental evidence supports each of these rules; for example, the specificity of chymotrypsin for large aromatic residues at P1 correlates with a 50,000 fold increase in activity when a Phe is positioned at P1 compared to an Ala (Dorovska et al., 1972, Knowles, 1965).

In addition, a novel group of chymotrypsin-like serine proteases, the serine collagenases, have been recently characterized. These enzymes have been shown to have collagenolytic activity, although the sites targeted for cleavage differed among these enzymes (Grant & Eisen, 1980, Lecroisey & Keil, 1985). Furthermore, one of these enzymes the *Uca pugilator* (fiddler crab) serine collagenase has been shown to have tryptic, chymotryptic and elastolytic activity (Grant & Eisen, 1980). Interestingly, the *Hypoderma lineatum* (botfly) serine collagenase has no activity towards synthetic peptide substrates of trypsin or chymotrypsin (Lecroisey & Keil, 1985). Due to the small number of identified serine collagenases and the discrepancies in substrate specificity, little is known about the amino acid sequence determinants influencing the S1/ P1 interactions.

Vector Competence

Vector competence is defined as the ability of a specific vector to become infected with a pathogen, permit replication and eventually transmit that pathogen (Black & Severson, 2005, Hardy, 1988). In the context of the mosquito/ arbovirus transmission cycle, arboviruses must overcome multiple barriers within the mosquito for transmission to occur (Figure 1.4). The virus enters the midgut following acquisition of a bloodmeal and must first infect the midgut epithelial cells. Virus attachment, penetration, uncoating,

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transcription, and translation mark the early stage of infection and the inability of the virus to complete one of these processes terminates the infection. This early barrier to infection is termed the midgut infection barrier (MIB). Virus maturation, dissemination into the hemocoel, and infection of secondary tissues defines the next stage in infection and blockage of any of these processes constitutes the midgut escape barrier (MEB). If the virus has made it past these first two barriers, the salivary gland infection barrier (SGIB), characterized as the inability to infect and replicate within the salivary glands, must be overcome. Finally, in order to be transmitted to a new host during a subsequent

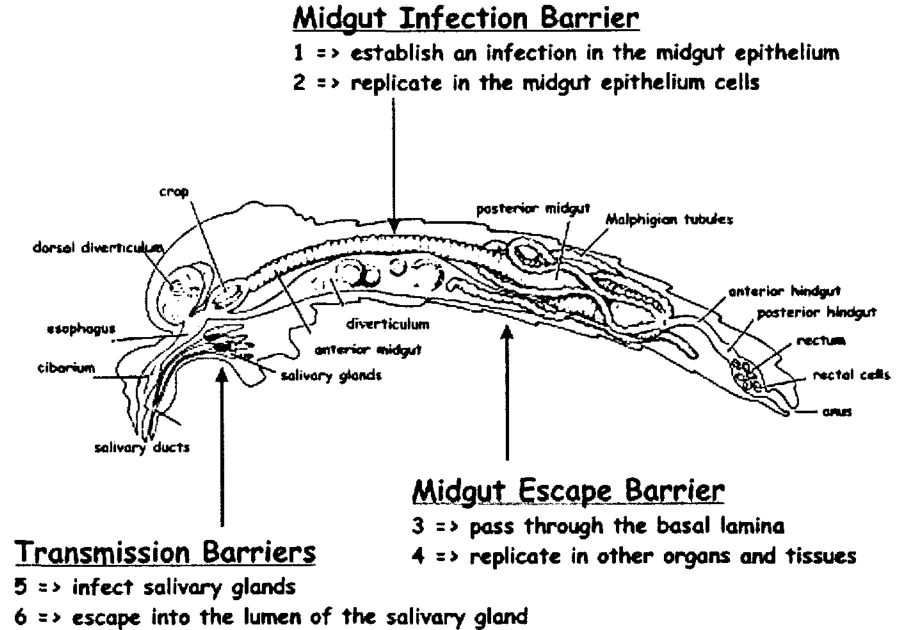

Figure 1.4: Mosquito transmission barriers. A general diagram of the potential transmission barriers an arbovirus encounters during infection of a mosquito (Black et al., 2002).

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bloodmeal, the virus must move from the salivary lobes to the salivary lumen for secretion into the saliva. This final barrier is coined the salivary gland escape barrier (SGEB) (Figure 1.4)(Black et al., 2002).

The specific factors determining vector competence are not well understood, but a number of genes involved with arthropod innate immunity, digestion and signal transduction have been associated with vector competence. Recent advances with mosquito genome projects have enabled researchers to more precisely identify genes within loci that may be involved with vector competence. For instance, natural populations of *A. gambiae* were used to produce isofemale pedigrees in order to map allelic variants that have major effects on malarial infections (Riehle et al., 2006). Using information from the complete genome, the researchers were able narrow down the 976 Ensembl-predicted transcripts from a 15-Mb interval and identify a handful of probable genes. Eventually, they were able to determine that APL1 (*Anopheles Plasmodium*-responsive leucine-rich repeat 1) is the primary determinant for *A. gambiae* refractoriness to *Plasmodium falciparum* (Riehle et al., 2006). Other vector/ pathogen systems are less well understood, although with the completion of more vector genomes, similar techniques may be applied to these systems in the future.

As stated previously, genes involved with digestion have been linked with vector competence in numerous studies. Biochemical analysis of the role proteolytic enzymes play in vector competence was initially described in the *Phlebotomus papatasi*/ *Leishmania* system. Experimental evidence suggests that in order for *Leishmania* to efficiently infect the gut of the sand fly, it must limit its exposure to the proteolytic enzymes found in this environment. *L. major* is able to decrease gut tryptic activity in

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order to successfully infect the vector, whereas *L. donovani* is unable to do so and thus cannot infect the vector (Borovsky & Schlein, 1987).

The role of trypsin activity has also been associated with *Plasmodium gallinaceum* infection of *A. aegypti*. Unlike the *P. papatasi*/ *Leishmania* system, midgut tryptic activity is actually necessary for the effective transversal of the malarial ookinete through the PM1 (Shahabuddin et al., 1993). Inhibition of tryptic activity using multiple trypsin inhibitors or anti-trypsin antibodies blocked *P. gallinaceum* infection (Shahabuddin et al., 1995, Shahabuddin et al., 1996). Furthermore, co-feeding an exogenous chitinase with the anti-trypsin antibodies was able to reverse the inhibitory effects (Shahabuddin et al., 1996). The authors concluded that trypsin activity was necessary for cleavage of the malarial pro-chitinase, which is required for ookinete migration, and integral in vector competence.

The importance of proteolytic enzymes in viral enhancement of infectivity is well described for many gut-associated viruses. Gut-associated viruses infect their host through the alimentary canal and are subject to a battery of digestive enzymes. To this end, these viruses have adapted to utilize host proteases for their own benefit. For example when rotaviruses, a member of the family *Reoviridae*, are subjected to trypsin the VP4 surface protein is cleaved into two smaller proteins VP8 and VP5. This cleavage event proceeds with a concomitant enhancement of viral infectivity (Espejo et al., 1981, Estes et al., 1981). Similarly, arboviruses infect the invertebrate vector host through the alimentary canal. Evidence suggests that arboviruses may utilize a similar mechanism for enhanced infectivity of the midgut.

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The role of midgut proteolytic enzymes in vector competence has been described in multiple viral systems. La Crosse virus (LACV), a member of the family *Bunyaviridae*, has an increased affinity towards insect cells and vector midgut tissue after exposure to pronase, a proteolytic enzyme cocktail. LACV has a lipid envelope coated with two viral glycoproteins G1 and G2 in equimolar concentrations. G1 appeared to have a role in attachment of the virus to mammalian cells, but the role of G2 had been undetermined (Ludwig et al., 1989). However, when LACV was treated with pronase, thereby removing G1 and preserving the pronase-resistant G2, the virus had a higher affinity to mosquito cells and tissue (Ludwig et al., 1989). Furthermore, LACV infection of mosquito cells and midguts could be greatly reduced when inhibited by pre-treating the cells with purified G2 (Ludwig et al., 1991). The researchers concluded that G2 may act as the viral attachment protein under highly proteolytic conditions in the mosquito midgut in which G1 may be removed (Ludwig et al., 1991).

A similar mode of action was described with bluetongue virus (BTV), a member of the family *Reoviridae*, and its vector *Culicoides variipensis*. Infectious subviral particles (ISVP) can be produced by treating BTV with trypsin and chymotrypsin and that these ISVPs infect mammalian cells with the same efficiency as disaggregated virus particles. However, these same ISVPs demonstrated a 100-fold increase in infectivity of two insect cell lines and two species of *Culicoides* compared to disaggregated virus particles (Mertens et al., 1996). These results suggest that degradation of VP2 on the outer capsid layer by midgut enzymes, thereby producing ISVPs, enhances the ability of the virus to infect its vector.

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Recently, investigators have described a comparable event with DENV-2 and its interactions with the mosquito midgut. Mosquitoes were fed an infectious bloodmeal containing STI and analyzed for DENV-2 infection everyday for 14 days. They found a drastic reduction in DENV-2 transcripts and envelope glycoproteins in the midgut between 4-7 days post-infection, as well as delayed dissemination kinetics when compared to controls. Furthermore, when virus was pre-treated with bovine trypsin and then fed to mosquitoes in the presence of STI, normal infection rates were reconstituted (Molina-Cruz et al., 2005). The authors concluded that midgut tryptic activity was required for DENV-2 to reach optimal levels of infection and dissemination. Additionally, suppression of ET, by RNA interference, using a double subgenomic Sindbis virus (dsSIN) construct resulted in a reduction DENV-2 dissemination rates (personal communication Dr. Irma Sanchez-Vargas).

Genetic analysis of vector/ pathogen interactions has also implicated proteolytic enzymes as being involved with vector competence. In order to study the genetics of vector competence linkage maps had to be constructed. By crossing susceptible and refractory strains of mosquitoes, researchers are able to use the linkage maps in order to identify specific regions within the genome associated with vector competence. These regions are known as quantitative trait loci (QTL) (Black et al., 2002). Using these technologies researchers were able to identify two independently segregating loci that determine the MIB and one locus that was weakly linked to the MEB of DENV-2 within *A. aegypti*. One of the loci identified in this study was located at the 22 cM position on chromosome II and the representative marker at this position was ET (Bosio et al., 2000). It was concluded that DENV-2 transmission was a quantitative genetic trait regulated by

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at least three loci. Another study found an association between two other QTLs influencing the MIB, along with another locus linked to the MEB (Gomez-Machorro et al., 2004). Most recently, it was determined that there were three more QTLs associated with the MIB, one of which mapped to the AT marker on chromosome II (Bennett et al., 2005b). Furthermore, this same group also associated AT with disseminated infections. To date, there have been six loci associated with the DENV-2 MIB and three linked to the MEB. It has been suggested that virus exploitation of multiple host proteins and physiological processes can account for the large number of QTLs that condition DENV-2 transmission by *A. aegypti* (Bennett et al., 2005b).

Proteolytic Processing of Virions

As previously stated numerous viruses have been implicated in utilizing host proteolytic enzymes to enhance infectivity. Structural studies of virions have assisted in our understanding of this process. Rotaviruses are composed of three concentric layers of structural proteins. The outermost layer has two predominant viral proteins VP7 which forms the smooth external surface from which VP4 extends forming the spike-like structures. VP4 is present on the outer capsid as 60 spikes and protrudes approximately 10-12 nm from the outer capsid (Kapikian et al., 2001). The orientation of these spikes makes them highly accessible to gut proteases. *In vitro* analysis of VP4 has revealed that it is proteolytically cleaved to form the two fragments VP5 and VP8 which mediate cell binding (Espejo et al., 1981, Estes et al., 1981). Furthermore, this cleavage event is required for the production of infectious virions and penetration of the cell membrane (Konno et al., 1993).

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Similarly, BTV, a member of the genus orbivirus, has spike-like structures protruding from its outer capsid. The outer capsid is composed of two major proteins VP2 and VP5. There are 120 globular-shaped VP5 proteins that sit neatly into the grooves formed by the outer core protein VP7. On the other hand VP2 forms sail-shaped spikes that cover VP5 and VP7 and protrude approximately 4 nm beyond VP5 globular domains thereby exposing VP2 to environmental proteases (Roy, 2001). Complete removal of VP2 from virions results in a significant reduction in hemagglutinin, infectivity and binding to vertebrate cells (Cowley & Gorman, 1990, French et al., 1990, Oldfield et al., 1991). Interestingly, removal of VP2 and VP5 by enzymatic treatment increases infectivity of invertebrate cells (Mertens et al., 1996). It is believed that this treatment exposes the RGD motif of VP7 thought to be involved in *Culicoides* cell entry (Nason, 1998).

LACV, family *Bunyaviridae* genus bunyavirus, is spherical in shape and has heterodimeric viral glycoproteins protruding from the lipid bilayer envelope. These spikes that extend 5-10 nm from the lipid bilayer are formed by glycoproteins G1 and G2 and are highly accessible to proteolytic enzymes (Schmaljohn & Hopper, 2001). Cell binding assays demonstrated that G1 was used for attachment to mammalian cells whereas G2 was the viral attachment protein for vertebrate cells (Ludwig et al., 1989, Ludwig et al., 1991). However, other experiments have found that *in vitro* proteolytic treatment of virions degraded G1 resulting in diminished infectivity of mammalian and insect cells as well as mosquitoes (Hacker et al., 1995). The importance of G1 in cellular fusion has also been described. Using a G1 specific monoclonal antibody, researchers developed a mutant LACV that had reduced ability to undergo low pH conformational

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changes and induce cell-to-cell fusion (Gonzalez-Scarano et al., 1985). However, recombinant G1 expressed in a vaccinia virus was unable to induce cell-to-cell fusion alone (Jacoby et al., 1993). Combined these results suggest that both G1 and G2 may be required for attachment and fusion with host cells. These results do not eliminate the possibility that limited proteolysis of G1 within the mosquito midgut may enhance infections. Perhaps minor changes as a result of proteolytic digestion may expose viral attachment epitopes without destroying the fusogenic properties of G1. Nevertheless further investigation is warranted.

Structural analysis of DENV reveals that there are 90 E-glycoprotein homodimers that coat the virion. These homodimers lie parallel to the lipid bilayer and have carbohydrate side chains which extend outward (Rey et al., 1995). There are three domains within E (domains I, II and III) that are critical for attachment, penetration and fusion with host cells (Chin et al., 2007, Modis et al., 2004). Domains III is the most distal projecting of these domains and acts as the viral attachment peptide (Chin et al., 2007). Furthermore, domain III acts to stabilize domain II as E trimerizes during formation of the fusion peptide (Modis et al., 2004). Despite the smooth spherical context of these virions *in vitro* tryptic and chymotryptic digestion reveals that E-glycoprotein is cleaved into six peptide fragments, one of which is located near amino acid 300 in domain III (Roehrig et al., 1998). These experiments provide a rough estimate of the location of these cleavage events, although without further analysis by mass spectrometry based mapping the exact location of these sites remains unknown. Furthermore, the significance of these cleavage events in viral infectivity of mammalian

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or mosquito cells has not been determined. Studies evaluating the importance of E-glycoprotein proteolytic processing during viral infectivity in *A. aegypti* are needed.

Arbovirus Kinetics in the Mosquito Midgut

In order for an arbovirus to eventually be transmitted back to a vertebrate host it must overcome multiple barriers of infection within the mosquito. MIB is the first barrier encountered by the virus and many factors influence its effect on the virus/ mosquito interaction (Black et al., 2002). The ability of a virus to infect, replicate and escape the midgut is dependent upon such variables as type and dose of virus, mosquito species and temperature during the EIP. These factors along with PM1 formation and the release of digestive enzymes may also influence the rate at which a competent vector becomes infected (Hardy et al., 1983).

As mosquitoes imbibe an infectious bloodmeal the blood and virus particles are pumped to the posterior midgut for digestion. This is the region of the midgut where proteolytic enzymes are secreted (Graf et al., 1986). In *A. aegypti*, two serine protease ET and CHYMO can be detected within the first hour following a bloodmeal (Jiang et al., 1997, Noriega et al., 1996b). Subsequently, by 8 hpbm a second phase of proteolytic enzymes that include AT and LT begin to be produced (Barillas-Mury et al., 1991, Kalhok et al., 1993). In addition to the proteolytic enzymes, the midgut begins to secrete the PM1 by 5 hpbm and fully surrounds the blood bolus by 12 hpbm (Perrone & Spielman, 1988).

Due the hostile environment of the midgut, arboviruses must be able to quickly and efficiently infect the midgut epithelium. For example, one study showed that western

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equine encephalitis virus (WEEV) (a member of the family Togaviridae genus alphavirus) could rapidly infect the midgut of *Culex tarsalis* as nucleocapsids could be visualized by electron microscopy within the cytoplasm by 3 hpbm (Houk et al., 1985). This demonstrates that WEEV escapes the hostile environment of the lumen quite rapidly and suggests that PM1 formation and digestive enzymes do not act as barriers to infection. However, this fast rate of midgut infectivity does not seem to be conserved in all virus/ mosquito systems. For instance, St. Louis encephalitis virus (SLEV) (a flavivirus) virions were detected in only one midgut epithelial cell of *C. pipiens* by 8 hpbm (Whitfield et al., 1973). These data suggest that unlike WEEV, SLEV remains in the lumen for extended periods of time and must persist in the presence of proteolytic enzymes and PM1 formation. The mechanisms by which SLEV is able to bypass the PM1 are unknown considering that the mean pore size of the fully developed matrix is only 20-30 nm in diameter, much smaller than any arbovirus that are 40-50 nm in diameter (Hardy et al., 1983). Furthermore, numerous arboviruses have been shown to be sensitive to *in vitro* proteolytic digestion by chymotrypsins and trypsins, yet SLEV is able to persist in the midgut during early phase and early late phase accumulation of proteolytic enzymes (Cheng, 1958, Gorman & Goss, 1972).

Overall these studies demonstrate that the differences in the rate of mosquito midgut infectivity are virus and vector dependent. Understanding the kinetics of arbovirus infectivity of midguts is paramount to identifying suitable targets for transmission blocking vaccines.

Anti-vector Vaccines

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The worldwide burden of arthropod-borne diseases and the lack of effective vaccines have led scientists to develop novel approaches to combat these diseases. In light of the hurdles facing traditional vaccines, novel vaccine approaches targeting the vector are now being considered.

The hallmark experiments that demonstrated the potential of this approach were performed in 1939 by William Trager. Vaccination of the vertebrate host with tick (*Dermacentor variabilis*) extracts induced an immune response lethal to subsequently fed ticks (Trager, 1939a, Trager, 1939b). This approach for vector control has since been applied to many other medically and agriculturally significant species such as *Boophilus microplus, Anopheles quadrimaculatus, Aedes aegypti, Anopheles stephensi, Stromoxys calcitrans, Glossinia moristans, Pediculus humanus* and *Lucilia cuprina* (Alger & Cabrera, 1972, Bowles et al., 1987, Dubin et al., 1948, Hatfield, 1988, Mumcuoglu et al., 1997, Nogge, 1978, Schlein & Lewis, 1976). To date, only acaricidal vaccines have been approved for use by the Food and Drug Administration. The effectiveness achieved by these acaricidal vaccines has been unparalleled in hematophagous insects. The discrepancy between the efficacy in ticks and insects can be attributed to two factors a) proximity and longevity of feeding and b) the mode of digestion. Ticks remain in close contact with their host for their entire lifetime and take multiple meals thereby constantly exposing the gut to host immune factors. Furthermore, they digest the blood intracellularly, hence protecting host immune components from degradation by gut proteases (Kay & Kemp, 1994). Insects, on the other hand, take multiple short blood meals and quickly respond with a robust release of proteolytic enzymes and the formation of the PM1. These digestive enzymes and PM1 formation may make it difficult for

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antibodies, cytotoxic T-cells and complement factors to effectively bind to and act upon the vector (Kay & Kemp, 1994).

Traditionally, anti-vector vaccines have been directed towards the midgut because the luminal contents and midgut epithelium are in direct contact with the blood meal. Interestingly, researchers have found that antibodies directed against other mosquito tissues such as salivary glands and hemolymph are able to transverse the midgut and reach target tissues (Brennan et al., 2000, Ramasamy et al., 1988). Typically, hemolymph antibody titers are 10^5 lower than those of the original blood meal, although the degree of antibody crossing is species dependent and in some species, such as *Aedes aegypti*, it does not occur (Lackie & Gavin, 1989, Vaughan & Azad, 1988).

Anti-vector vaccines have also been designed to target molecules that affect vector competence. These are classified as transmission blocking vaccines (TBV). There are two modes by which a TBV can disrupt infection of a vector with a specific pathogen. First, researchers have been able to immunize the vertebrate host against specific mosquito tissues and elicit immune responses that invariably reduced infection by a specific virus or parasite (Brennan et al., 2000, Lal et al., 2001, Ramasamy & Ramasamy, 1990). Furthermore, immunization with specific target molecules, such as midgut trypsin, can also significantly reduce parasite development in the midgut (Shahabuddin et al., 1996). The second approach has been to target antigens found on mosquito specific life stages of parasites, specifically the malarial ookinete. Vaccination with antigens found on the ookinete can effectively block ookinete midgut transversal, oocyst formation and subsequent transmission (Arakawa et al., 2003, Coban et al., 2004, Kaslow et al., 1988). These studies demonstrate the utility of TBV as potential control methods

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for many vector-borne diseases and highlight the importance of investigating these further in the context of arboviruses.

RNA Silencing

RNA silencing is a biological mechanism by which target double stranded RNA (dsRNA) species can be identified, degraded and eventually serve as a guide for subsequent degradation of the cognate RNA sequence. This process was first identified in plants and fungi and termed post-transcriptional gene silencing and quelling, respectively (Cogoni et al., 1996, Napoli et al., 1990). In each case attempts were made to increase a desired phenotypic trait through the introduction of transgenic copies of endogenous genes, but instead of an over-expression, the genes were suppressed. Subsequently, a similar mechanism has been identified in animals. Utilizing antisense RNA, researchers attempted to silence a specific gene in *Caenorhabditis elegans*, but upon silencing of the cognate gene it was observed that the effects identified were not only produced by the antisense RNA, but also the mRNA (Guo & Kemphues, 1995). It was later demonstrated that this mechanism, eventually dubbed RNA interference (RNAi), was triggered by the formation of dsRNA, which was identified as the trigger molecule (Fire et al., 1998).

Originally, RNA silencing was thought to be an ancient intracellular defense mechanism by which foreign dsRNA species such as viral replication intermediates or transposons could be identified by the cell as being "foreign" and appropriately degraded (Ketting et al., 1999, Li et al., 2002). Further investigation has shown that RNA silencing is far more important to cells beyond cellular defenses. RNA silencing has been shown

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to be essential for normal cellular functions through the use of endogenous dsRNA species that regulate endogenous gene expression (Lee et al., 1993, Pasquinelli et al., 2000). The biogenesis of exogenous and endogenous dsRNA molecules are distinct, but in both cases they are cleaved into 21-27 nt. effector fragments termed small interfering RNAs (siRNA) and micro RNAs (miRNA), respectively (Hutvagner & Zamore, 2002). The remainder of this review will focus on the exogenous dsRNA pathway. siRNAs are generated when dsRNA is cleaved by the cellular enzyme Dicer which has ribonuclease III activity (Bernstein et al., 2001, Knight & Bass, 2001). Dicer-2 mediates this event in Diptera. The siRNAs are eventually loaded into a multi-protein RNA-induced silencing complex (RISC) and is unwound by Argonaut 2 (Okamura et al., 2004, Tomari et al., 2004). Upon unwinding of the siRNA, the RISC acts as a docking station in which complementary single stranded RNA molecules are loaded and subsequently cleaved by the endoribonucleolytic activity of Argonaut 2. Once the target RNA is cleaved, the siRNA is released from RISC and cleavage of more target RNAs may commence.

Recently, researchers have begun studying the importance and possible applications of the RNAi pathway in medically relevant vectors, specifically mosquitoes. Initially, the utility of RNAi was demonstrated in mosquito cell culture. Double subgenomic Sindbis viruses (dsSINV) expressing the DENV-2 prM gene in the sense and antisense orientations were shown to be able to inhibit DENV-2 infection upon challenge (Gaines et al., 1996). Subsequently, these same constructs were applied *in vivo* and, as before, prior infection with the prM antisense dsSINV completely inhibited DENV-2 dissemination to the salivary glands (Olson et al., 1996). These initial studies demonstrated that the RNAi pathway in mosquitoes is intact, fully functional and has the

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potential to be utilized for the development of transgenic mosquitoes. Researchers have since exploited this pathway to study endogenous genes involved in vector competence and basic insect physiology. Furthermore, it has been applied as a tool by which to develop transgenic mosquitoes that are refractory to DENV-2 infection.

In mosquitoes endogenous gene silencing was first demonstrated when dsRNA specific for *A. gambiae* defensin was intrathoracically injected in order to suppress defensin expression. Suppression of defensin allowed the researchers to analyze the importance of this gene product in insect innate immunity (Blandin et al., 2002). This same technique was used to demonstrate that dsRNA could be used to suppress components of the RNAi pathway, itself. Specifically, it was demonstrated that Argonaut 2 could be suppressed and that suppression of RNAi pathway genes could weaken the natural antiviral state in mosquitoes (Keene et al., 2004). Multiple other studies have since utilized this approach.

These findings have, in fact, led to the development of transgenic mosquitoes that are refractory to DENV-2 infection. Franz *et. al.* transformed the mosquito with a nonautonomous *mariner* element containing a carboxypeptidase A promoter and a DENV-2 inverted repeat effector gene (Franz et al., 2006). Upon feeding, the carboxypeptidase A promoter produces a 578 bp dsRNA inverted repeat specific for DENV-2 and activates the RNAi pathway. This system effectively reduced DENV-2 infection of the mosquito midgut and salivary glands (Franz et al., 2006). Furthermore, dsRNA suppression of Argonaut 2 resulted in normal DENV-2 infection levels, demonstrating that the effects observed were indeed a result of DENV-2 RNA degradation by the RNAi pathway.

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Research Project

Arboviruses are a diverse group of viruses that cause significant morbidity and mortality worldwide; DENV has the greatest human health impact. To date, there are no vaccines or antivirals for DENV, and the current mosquito control programs are less than desirable. Novel approaches for disease transmission are needed in order to curb the continuing resurgence of DENV.

The overall research objective of this dissertation is to analyze the complex interplay between DENV-2 and the mosquito vector, *A. aegypti*. Specifically, the role of mosquito midgut serine proteases in vector competence and their utility as targets for novel control strategies were studied. Preliminary evidence had suggested that DENV-2 may require proteolytic processing, probably of domain III within the E-glycoprotein, in order to efficiently infect the mosquito midgut. In order to fully examine these interactions, multiple genetic and biochemical approaches were implemented allowing for a comprehensive analysis of the virus/ vector relationship. In light of the previous studies outlined in the Vector Competence section, I initially pursued a more translational research based approach to analyzing this system.

Specific Aim 1: The objective of this aim was to determine the transmission blocking potential of immune sera developed against ET and AT. I hypothesized that interference of normal ET and AT activity with immune sera would decrease the ability of DENV-2 to infect and/ or disseminate in *A. aegypti*. An anti-AT immune response was induced as detected by anti-AT specific antibodies, but I was unable to detect anti-ET antibodies. Interestingly, sera from the AT-immunized mice resulted in approximately a two and half-fold increase in midgut infection rates (MIR). The anti-ET

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immune sera had no effect on MIR. These results demonstrate that AT is not a suitable TBV candidate and further complicates our understanding of the role of midgut serine protease in vector competence.

Specific Aim 2: The objective of this aim was to determine the importance of potential trypsin cleavage sites within domain III of the E-glycoprotein during the course of a DENV-2 infection of the mosquito. Despite the result from specific aim 1, I hypothesized that disruption of one or more of the solvent exposed potential trypsin recognition sites in domain III would reduce the ability of DENV-2 to infect the midgut and/or disseminate to the salivary glands of *A. aegypti*. Using infectious clone technologies, I selectively mutagenized the four most solvent exposed trypsin sites within domain III. Mutant virions were generated and growth kinetics were then determined in mammalian and insect cell culture. In addition, MIR and dissemination rates (DR) were determined for each of the four mutant viruses. I determined that abolishment of one of the potential trypsin sites, K305, significantly increased MIR. These results are consistent with those from specific aim 1 and suggest that midgut proteases do not enhance, but rather limit viral infectivity.

Specific Aim 3: The objective of this aim was to determine which, if any, of the four known midgut serine proteases is responsible for limiting DENV-2 infectivity. I suppressed each of the target genes with dsRNA and then challenged the mosquitoes with an infectious bloodmeal. In light of the results from the two prior specific aims, I hypothesized that suppression of one or more of these midgut serine proteases would increase MIR and/ or DR of DENV-2. Suppression of each of the genes was measured by Q-RT-PCR and protease activity assays. I determined that suppression

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of AT, as determined by Q-RT-PCR, did not correlate with a reduction in trypsin activity. I also found that suppression of LT resulted in a minor, not significant, increase in MIR. In addition, infection experiments in the presence of trypsin inhibitors, STI and egg white trypsin inhibitor, significantly increased DENV-2 MIR. These results supported the hypothesis that proteolytic activity within the midgut limits DENV-2 infectivity, although this effect could not be attributed to one specific serine protease.

Specific Aim 4: The objective of this aim was to determine the functional activity and genomic relationship of the four identified midgut serine proteases. Based on the aforementioned results I hypothesized that ET and LT were traditional trypsins, CHYMO was a traditional chymotrypsin and that AT, despite its name, was not a trypsin. Using an expanded set of proteolytic substrates I determined that AT was not a trypsin, chymotrypsin or an elastase. ET, LT and CHYMO had functional specificities as hypothesized. Furthermore, phylogenetic analyses and sequence alignments revealed that AT clustered with *Hypoderma linateum*, botfly, and *Uca pugilator*, fiddler crab, serine collagenases. From these results I concluded that AT may be a serine collagenase, the first identified from a mosquito. ET and LT clustered with other trypsins and CHYMO, interestingly, grouped with *Glossinia moristans* chymotrypsin, but not *A. gambiae* chymotrypsin.

Overall, this dissertation, initially aimed at developing a TBV targeting midgut trypsins, resulted in a more complete understanding of the virus/ vector interactions and *A. aegypti* midgut serine proteases.

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Chapter 2

The Potential of a DENV-2 Transmission Blocking Vaccine with *Aedes aegypti* Midgut Serine Proteases as Targets

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Hypothesis

The objective of this research aim was to investigate the feasibility of using mosquito midgut serine proteases as targets for development of a DENV-2 transmission blocking vaccine. I hypothesized that midgut trypsin specific antisera would interfere with the proposed proteolytic processing of DENV-2 and reduce the ability of the virus to infect *Aedes aegypti*.

1. Introduction

Arboviruses have re-emerged in the past 30 years and there have been recent epidemics in nearly every region on Earth (Gubler, 1996, Gubler, 2002). In particular, DENV has become a significant public health threat in most of the tropical regions. Historically, DENV epidemics were managed with *Aedes aegypti* eradication programs, but increases in urbanization and global populations, as well as deterioration of public health infrastructures, has marginalized their effectiveness (Gubler, 1998). Furthermore, safe vaccines that can provide protection against all four serotypes are currently not available. As epidemics continue to expand in size and numbers the need for an effective vaccine or antivirals has never been so important. In light of these facts, development of novel control strategies may be necessary to eventually manage this disease.

A. aegypti is the primary vector transmitting dengue and yellow fever viruses to humans. This species is highly suited for maintenance of these transmission cycles due to its endophilic nature and preference for human blood (Nelson, 1986, Tinker, 1964). Transmission of these pathogens begins when the mosquito imbibes a bloodmeal, which is used for energy metabolism and egg production (Zhou et al., 2004). The virus and

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blood are pumped into the midgut for digestion by a battery of proteolytic enzymes. To date, four endoproteolytic enzymes have been characterized in the midgut, two of which are of particular interest; early trypsin (ET) and abundant trypsin (AT). QTL mapping revealed that each of these genes is associated with *A. aegypti* susceptibility to DENV-2 (Bennett et al., 2005b, Bosio et al., 2000). These results were confirmed by experiments using a double subgenomic Sindbis virus (dsSIN) construct. This dsSIN virus expressing ET in the antisense orientation was fed to mosquitoes 8 days prior to DENV challenges. Suppression of ET via this method decreased DENV dissemination rates (DR) 11 days post infection (dpi) (personal communication Dr. Irma Sanchez-Vargas). Furthermore, an *in vivo* inhibition assay, in which soy bean trypsin inhibitor (STI) was co-fed with an infectious bloodmeal, also resulted in decreased DR and midgut infection rates (MIR). This showed that suppression of early phase tryptic digestion directly affected the ability of DENV-2 to infect the midgut and disseminate to secondary tissues (Molina-Cruz et al., 2005). Similar examples of protease enhancement of viral infectivity have been documented in other virus/ vector systems such as La Crosse virus (LACV) in *A. triseriatus* and blue tongue virus (BTV) in *Culicoides variipensis* (Ludwig et al., 1989, Mertens et al., 1996).

The idea of vaccines that target the vector rather than the pathogen was first conceptualized in 1939. William Trager found that vaccination with tick extracts could induce potent immune responses that could kill ticks when they fed upon the vaccinated animal (Trager, 1939a, Trager, 1939b). Researchers have since applied these techniques to many medically relevant vectors, including mosquitoes (Alger & Cabrera, 1972, Bowles et al., 1987, Dubin et al., 1948, Hatfield, 1988, Mumcuoglu et al., 1997, Nogge,

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1978, Schlein & Lewis, 1976). To date, development of a mosquitocidal vaccine has been complicated with problems such as immuno-dominance and masking, high variability between replicates and identification of sufficient targets (Foy et al., 2002).

A derivation of this vaccine approach termed transmission blocking vaccines (TBV) aims to target molecules that affect vector competence. There are two modes by which TBV can function. First, invertebrate stage specific molecules found on the pathogen, such as Pfs25 on malarial ookinetes, can be targeted (Coban et al., 2004, Kaslow et al., 1988). Second, vector molecules that are necessary for pathogen infectivity of the vector can be targeted. Multiple studies have demonstrated that vaccination against specific mosquito tissues could induce immune responses capable of reducing infection of the vector by a specific parasite (Brennan et al., 2000, Lal et al., 2001, Ramasamy & Ramasamy, 1990). In fact, one study showed that immunization with a midgut trypsin could significantly reduce plasmodium development in the vector (Shahabuddin et al., 1996). Little is known about the feasibility of using TBV to disrupt arboviral transmission cycles. One study has been published on the topic and it demonstrated that vaccination against *A. aegypti* extracts could reduce alphavirus and flavivirus infection rates by 50% (Ramasamy et al., 1990). Thus far, the utility of TBV have primarily been investigated in the context of vector-borne parasitic diseases and the applicability of this approach for controlling arboviruses requires more attention.

Based on evidence suggesting that DENV-2 utilizes midgut trypsins in order to effectively infect *A. aegypti*, I tested the applicability of a DENV-2 TBV specific for ET and AT to interfere with DENV-2 infection of *A. aegypti*. To my knowledge, this is the first attempt to develop a protein-specific TBV aimed at disrupting the transmission cycle

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of an arbovirus. Initially, a DNA vaccination approach was tested and this was followed by a DNA: alphavirus prime-boost regimen. These approaches were chosen because of their ability to induce strong humoral immune responses, which are thought to be important for TBV, and cellular immune responses which may be important in mosquitocidal vaccines (Foy et al., 2003, Gurunathan et al., 2000, Penttila et al., 2004, Pugachev et al., 1995, Rayner et al., 2002). Each regimen was evaluated for its ability to induce a gene specific immune response and its efficacy as a mosquitocidal and transmission blocking vaccine.

2. Materials and Methods

Mosquito Rearing

The Rexville (RxD) strain of *Aedes aegypti*, originally colonized from Puerto Rico, was used for these experiments. The mosquitoes were maintained at a constant temperature of 28°C and a relative humidity of 80%. The photoperiod was set to a 14:10 light: dark cycle.

DNA Vaccine Preparations

The cDNA pools used for gene amplification were produced from total RNA extracted from either unfed or fed adult female *A. aegypti* using TRIzol according to manufacturer's instructions (Invitrogen, Carlsbad, CA). ET transcripts accumulate in adult females prior to feeding, so the unfed cDNA pool was used for whole gene amplification. The ET sequence was amplified using forward primer, 5'-ATT TAT **AAG CTT** CAG CCA TGA ACC AAT TTC TCT TTG-3' and reverse primer, 5'-ATT TAT

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TCT AGA TTA AAC CTC GGA AAC CTC TCG G-3' (bold font indicates *Hind*III and *Xba*I sites, respectively). Conversely, AT transcripts are induced by blood feeding, so the fed cDNA pool was used for its amplification. The AT sequence was amplified using forward primer, 5'-ATT TAT **GGT ACC** CAA AAT GTT CAC TTC AAC GGT GG-3' and reverse primer, 5'-ATT TAT **TCT AGA** TCA CAG TCC AGT CTT CTG CTT GAT C-3' (bold font indicates *Kpn*I and *Xba*I sites, respectively). All primer sets included a Kozak translation initiation sequence, as well as start and stop codons.

All PCR products for these experiments were amplified using the following settings; 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds followed by a final 10 minute extension step at 72°C using a 2XMasterMix containing Taq polymerase (Promega, Madison, WI).

The DNA vector used was pcDNA3.1^{+}, which contains a CMV promoter that enables high-level transient expression in mammalian cells (Invitrogen). The genes were cloned downstream of the CMV promoter and transformed into electrocompetent cells. Plasmids were isolated and sequenced. Upon confirmation of proper gene insertion, high concentration stocks were produced using the Qiagen Endotoxin-Free Giga-Prep Kit (Qiagen, Valencia, CA).

Alphavirus Vaccine Production

The alphavirus vector chosen for these experiments was a 5' double subgenomic Sindbis virus (5'dsSIN), specifically 5'dsMRE16. The MRE16 infectious clone was originally produced by Myles *et. al.* and was later modified to contain a double subgenomic promoter (Foy et al., 2004, Myles et al., 2003). The ET sequence was

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amplified using forward primer, 5'-ATT ATA **GCG GCC GC**T ATG AAC CAA TTT CTC TTT GTC AG-3' and reverse primer, 5'-ATT ATA **GCG GCC GC**T TAA ACC TCG GAA ACC TCT CGG-3' (bold font indicates *Not*I site). The AT sequence was amplified using forward primer, 5'-ATT ATA **GCG GCC GC**A AAA TGT TCA CTT CAA CGG TGG-3' and reverse primer, 5'-ATT ATA **GCG GCC GCT** CAT TAC CCT GCT CAC AGT CCA GTC-3' (bold font indicates *Not*I site). Start and stop codons were included in the amplification of each of the genes. The amplification products were inserted into the infectious clone and resultant plasmids were sequenced.

Recombinant Virus Production

Recombinant virus particles were produced as previously described (Olson et al., 2000). Briefly, 5 μg of 5'dsME16ic DNA was linearized with Asc I and treated with proteinase K. The reactions were phenol: chloroform extracted and ethanol precipitated. Subsequently, 1 μg of linear DNA was incubated in the presence of rNTP's, m^7-GpppG cap analog, 5X transcription buffer and SP6 RNA polymerase at 39°C for 1 hour. Upon completion of the transcription reaction, 12 μl of positive sense ssRNA was electroporated into 4 x 10^6 BHK-21 cells which were pulsed twice using the following settings: 400 V, 1200 Ω and 150 μF. Cells were placed in flasks at 37°C for 4 days at which point the supernatant was collected. The virus was subsequently passaged in C6/36 cells for amplification, titrated, aliquoted and stored at -80°C as working stocks.

Transient Expression

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Prior to vaccinations each of the plasmids was transfected into HEK 293T cells in order to confirm that each of the proteins was being properly produced. Briefly, once the HEK 293T cells had grown to 70% confluency, 3 μg of plasmid DNA were mixed with TransIT-293 transfection reagent and DNA:lipid complexes were allowed to form (Mirus Bio Corp, Madison, WI). Subsequently, the DNA:lipid complexes were added dropwise to the cells and the cells were incubated for 72 hours at 37°C. Cellular lysates and supernatant were collected and prepared for western blotting analysis.

Confirmation of expression of each of the genes in the dsMRE16 constructs was carried out on C6/36 cells. Briefly, cells were infected with each of the virus stocks at a multiplicity of infection (MOI) of 0.1 and allowed to incubate for 72 hours. Cellular lysates and supernatant were collected and prepared for western blotting analysis.

Recombinant Protein Expression

Recombinant proteins were expressed using the Insect Select System (Invitrogen). The expression vector used for these experiments was pIZ/V5-His, which has a baculovirus immediate early promoter (OpIE2) that allows for constitutive expression of recombinant proteins in insect cells. Furthermore, it contains a C-terminal His tag and V5 epitope allowing for easy purification and detection. The ET sequence was amplified using forward primer, 5'-ATT ATA **AAG CTT** AGC CAT GAA CCA ATT TCT C-3' and reverse primer, 5'-ATT ATA **CCG CGG** AAC CTC GGA AAC CTC TCG G-3' (bold font indicates *Hind*III and *Sac*II sites, respectively). The AT sequence was amplified using forward primer, 5'-ATT ATA **GGA TCC** AAA ATG TTC ACT TCA ACG G-3' and reverse primer, 5'-ATT ATA **CCG CGG** CAG TCC AGT CTT CTG

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CTT G-3' (bold font indicates *BamHI* and *Sac*II sites, respectively). A Kozak translation initiation sequence and start codon were included. Plasmids were sequenced to verify sequence, orientation and alignment with the V5 and His tags. Proteins were transiently expressed in High Five (*Trichoplusia ni*) cells and purified on a Ni^{+2} column according to the manufacturer's protocols (Invitrogen).

Western Blots

Western blotting analysis to test for transient expression of the target proteins was performed according to the manufacturer's protocols (Invitrogen). Briefly, cellular pellets were directly resuspended in 300 µl of 1X NuPAGE LDS sample buffer, while supernatants were mixed 3:1 with 4X NuPAGE LDS sample buffer. No reducing agent was used. Samples were heated to 70°C for 10 minutes and 10 µl were loaded onto a NuPAGE Novex 10% Bis-Tris gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% non-fat powdered milk in phosphate buffered saline with 0.1% Tween-20 (PBST) for 2 hours at room temperature (RT). The membranes were washed twice with PBST for 10 minutes and incubated with the primary antibodies. Rabbit α-ET and rabbit α-AT antibodies were generously provided by Dr. Irma Sanchez-Vargas. The α-ET antibody was produced by Dr. Fernando Noriega in 1996 and the α-AT antibody was produced in 1995 by Dr. Carolina Barillas-Mury (Barillas-Mury et al., 1995, Noriega et al., 1996a). These primary antibodies were diluted 1:500 in blocking buffer and membranes were probed overnight (ON) at 4°C. The mouse α-V5 antibody was diluted 1:5000 (Invitrogen). Membranes were subsequently washed twice with PBST and probed with a horseradish peroxidase (HRP) conjugated goat α-rabbit

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secondary antibody (ZyMax, San Francisco, CA) diluted 1:5000 in blocking buffer, incubated at RT for 2 hours, washed, developed with the ECL Plus Western Blotting Detection Kit (Amersham, Arlington Heights, IL) and visualized with a StormImager (GMI Inc., Ramsey, MN). Using recombinant protein purified from the pIZ expression system sera from vaccinated mice were assessed. Sera was diluted 1:300 in blocking buffer and a HRP conjugated rabbit α-mouse antibody was used as the secondary antibody (ZyMax, San Francisco, CA)

Vaccination Regimen

Groups of four Balb/c mice were immunized intramuscularly with 50 μl of DNA in each of the hind quadriceps. A total of 200 μg of DNA was injected into each mouse per vaccination. The DNA mixtures contained 100 μg of target DNA and 100 μg of pcDNA3.1 containing IL-12 which was co-injected as an immuno-stimulator. Control groups were vaccinated with empty vector pcDNA3.1 and pcDNA3.1 containing IL-12. Mice were vaccinated three times at two week intervals. Two weeks after the final booster mice were immobilized and offered as a bloodmeal to mosquitoes in order to test the mosquitocidal potential of each of the targets. Mice were subsequently euthanized and serum was collected for use in western blotting analysis and transmission blocking assays.

The DNA: alphavirus prime-boost regimen consisted of two rounds of DNA vaccination as described above and one round of vaccination with the recombinant 5'dsMRE16 viruses. A 5'dsMRE16 virus containing enhanced green fluorescence (EGFP) was used as the control. The virus stocks were diluted to a final concentration of

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1 x 10^6 plaque forming units (pfu)/ ml in 100 µl of PBS and injected subcutaneously. Mosquitocidal and transmission blocking potential were evaluated and the sera were tested for ET and AT specific antibodies by western blotting analysis.

Mosquitocidal Assays

Two weeks after the final vaccination mice were offered as a bloodmeal source for uninfected mosquitoes. Mosquitoes were counted, sorted and mortality was recorded for 7 days.

Transmission Blocking Assays

Aedes aegypti were provided a bloodmeal that contained 250 µl of pelleted defibrinated sheep red blood cells, 400 µl of pooled sera from either the experimental or control groups of mice, 400 µl of cell culture medium from C6/36 cells infected with DENV-2 (13 days post infection (dpi)) and 100 µl of 10mM ATP. Mosquitoes were allowed to feed for one hour at which point they were sorted and maintained in the insectaries for 7 days. The infectious bloodmeal titers were: empty control 8 x 10^5 pfu/ ml, anti-ET 6.67 x 10^5 pfu/ ml and anti-AT 7.63 x 10^5 pfu/ ml. Individual midguts were dissected from mosquitoes and tested for the presence of DENV antigen by indirect immunofluorescence assay (IFA). The remaining mosquitoes were placed back in the insectary for analysis of dissemination rates (DR) on day 14, but the majority of them died. Due to low quantities of sera this was only completed once.

Analysis of sera collected from the DNA: alphavirus prime-boost vaccination regimen was performed slightly differently from that of the DNA vaccination alone.

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Briefly, 250μl of pooled sera were mixed with 250 μl of DENV-2 13 dpi and 500 μl of defibrinated sheep blood. The infectious bloodmeal titers are an average from the two replicates: empty control 4.4 x 10^5 pfu/ ml, anti-ET 6.3 x 10^5 pfu/ ml and anti-AT 5.33 x 10^5 pfu/ ml. Midgut infection rates (MIR) were determined 7 dpi, but due to low feeding numbers DR were not determined. This experiment was repeated twice.

Indirect Immunofluorescence Assay

IFA's were performed according to the protocol outlined in Bennett *et. al.* (Bennett et al., 2002). Briefly, dissected midguts and heads were fixed in either 4% paraformaldehyde or acetone, respectively. Midguts were washed and stained in PBS+0.1% Triton X100, while PBS was used for the heads. Initial staining was carried out with the E-glycoprotein specific mouse derived primary monoclonal antibody, 3H5. The samples were washed and the secondary stain applied which included biotinylated sheep anti-mouse antibody (Amersham, Arlington Heights, IL) and 0.005% Evans blue counter stain. Finally, samples were developed with streptavidin-fluorescein (Amersham) and visualized for fluorescence. MIR is reported as a percentage and is determined by dividing the number of positive midguts by the total number of midguts tested. Relative infection intensities (RII) were determined for midguts 7 dpi. This was determined by scoring positive midguts on a scale of 1-4 then totaling these values and dividing by the number of positive midguts.

Statistical Analysis

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A log rank test was performed on the Kaplan Meier survival curves in order to determine if there was a significant difference in mosquito survivorship between mice within groups. This same test was then applied to test for significant differences between groups. A standard two-tailed T-test was used to analyze midgut infection rates.

Results

Sequencing data revealed that each of the genes, ET and AT, was correctly inserted into pcDNA3.1^{+}. Prior to vaccinating mice with these constructs, I first determined if they were capable of expressing the recombinant proteins in cell culture. Each plasmid was transfected into HEK 293T cells along with the control plasmid containing EGFP. The EGFP plasmid acted as the positive control in order to monitor protein expression and transfection efficiency. I determined that the transfection efficiency was about 15% and that optimal expression occurred 72 hours post transfection. Western blotting analysis demonstrated that each of the target proteins was expressed and secreted into the supernatant (Figure 2.1).

I then verified that each the target genes was correctly inserted into 5'dsMRE16ic. Viral particles were tested for expression of ET and AT by western blot. I determined that AT was produced and secreted into the supernatant, but I was unable to detect ET expression (Figure 2.2).

Finally, expression of the recombinant proteins from the transiently transfected High Five cells was analyzed by western blot. The α-V5 antibody revealed that each of the proteins was expressed and that they were secreted into the supernatant (Figure 2.3). Attempts were made to quantify the proteins after purification using the Micro BCA

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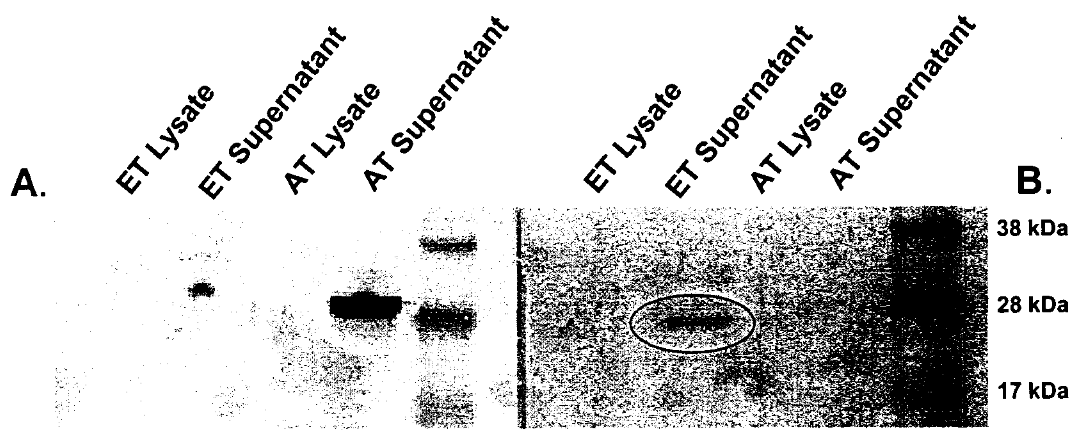

Figure 2.1: Western blotting analysis of transiently expressed recombinant proteins from the pcDNA3.1 constructs prior to vaccination. Cellular supernatant and lysate were collected from pcDNA3.1+AT and pcDNA3.1+ET transfected HEK293T cells. Equal volumes were load on a 10% Bis-Tris gel and probed with either (A) rabbit α-AT antibodies or (B) rabbit α-ET antibodies.

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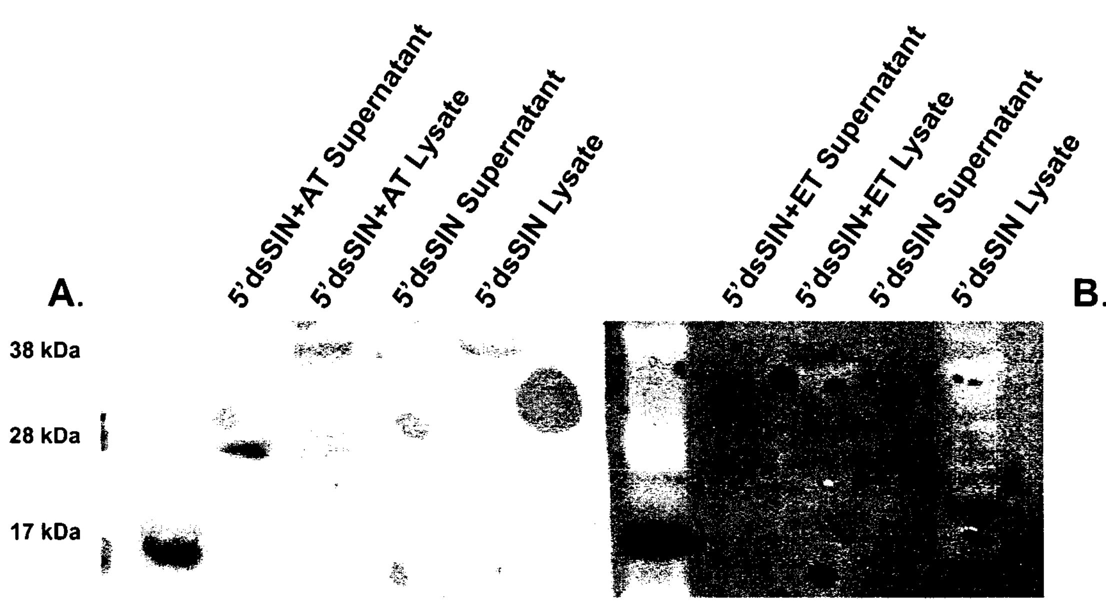

Figure 2.2: Western blotting analysis of transiently expressed recombinant protein from the 5'dsMRE16 constructs prior to vaccination. Cellular supernatant and lysate were collected from 5'dsMRE16+AT and 5'dsMRE16+ET infected C6/36 cells. Equal volumes were loaded on a 10% Bis-Tris gel and probed with either (A) rabbit α-AT antibodies or (B) rabbit α-ET antibodies.

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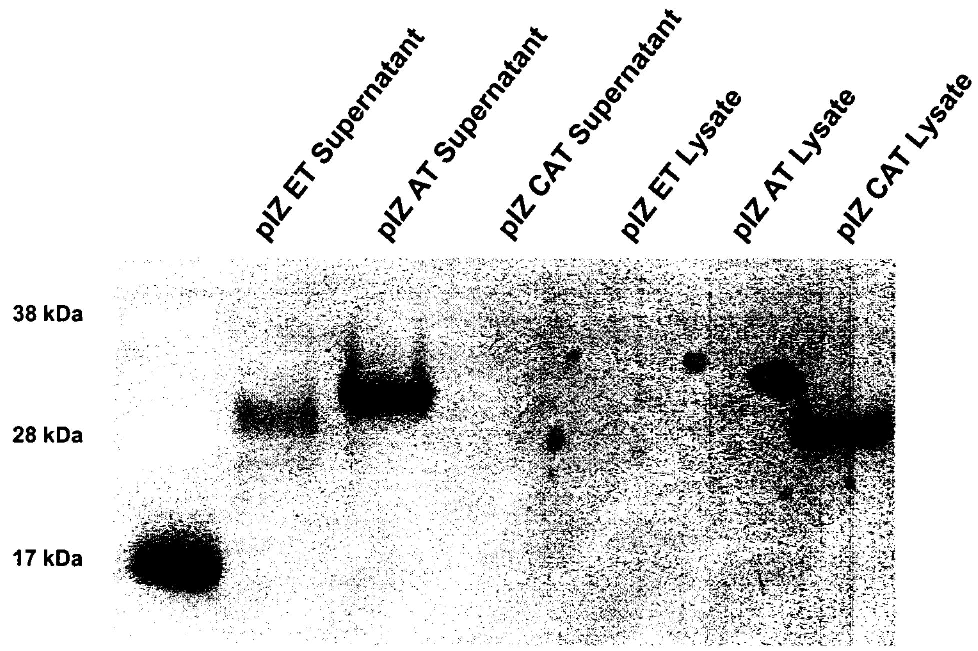

Figure 2.3: Western blotting analysis of recombinant proteins following transient expression with the pIZ expression vectors. Cellular supernatants and lysates were prepared from pIZ ET, pIZ AT and pIZ CAT transfected High Five cells. Equal volumes were loaded on a 10% Bis-Tris gel and probed with mouse α-V5 primary antibody. pIZ CAT acted as the positive control.

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Protein Assay Kit (Pierce Biotechnology, Rockford, IL), but their concentrations were low and fell below the linear range of the standard curve.

Immune sera collected from the mice vaccinated against either ET or AT was analyzed for the presence of trypsin specific antibodies by western blot. Analysis of pooled sera from the anti-AT immune group demonstrated that both vaccination regimens induced an AT specific humoral immune response as evidenced by the presence of antibodies (Figure 2.4). There was no statistical difference between antibody levels produced from the two vaccination regimens. An attempt to find anti-ET antibodies in the sera from either ET vaccination regimens was not successful.

The mosquitocidal potential of each of the target genes was determined using both vaccination regimens. It was demonstrated that neither anti-ET nor AT immune responses had a significant effect on mosquito survivorship (Figure 2.5). Individual mice in each group acted as internal replicates for the mosquitocidal studies. In the DNA vaccination regimen there were four mice per group, except for empty vector group which contained three. The DNA: alphavirus prime-boost regimen only includes data from three mice due to low feeding rates.

Serum collected from mice immunized against ET and AT was tested for its ability to inhibit DENV-2 infectivity of *A. aegypti*. I found that immune sera from mice vaccinated with ET and AT expression plasmids had no significant effect on MIR. There was a slight increase in infection rates of 5 % and 9%, compared to the empty vector group, for both ET and AT immune groups, respectively (Figure 2.6 A). The low number of replicates did not permit for statistical analysis. RII did not seem to differ between the experimental and control groups (Figure 2.6 C). Interestingly, when anti-AT immune

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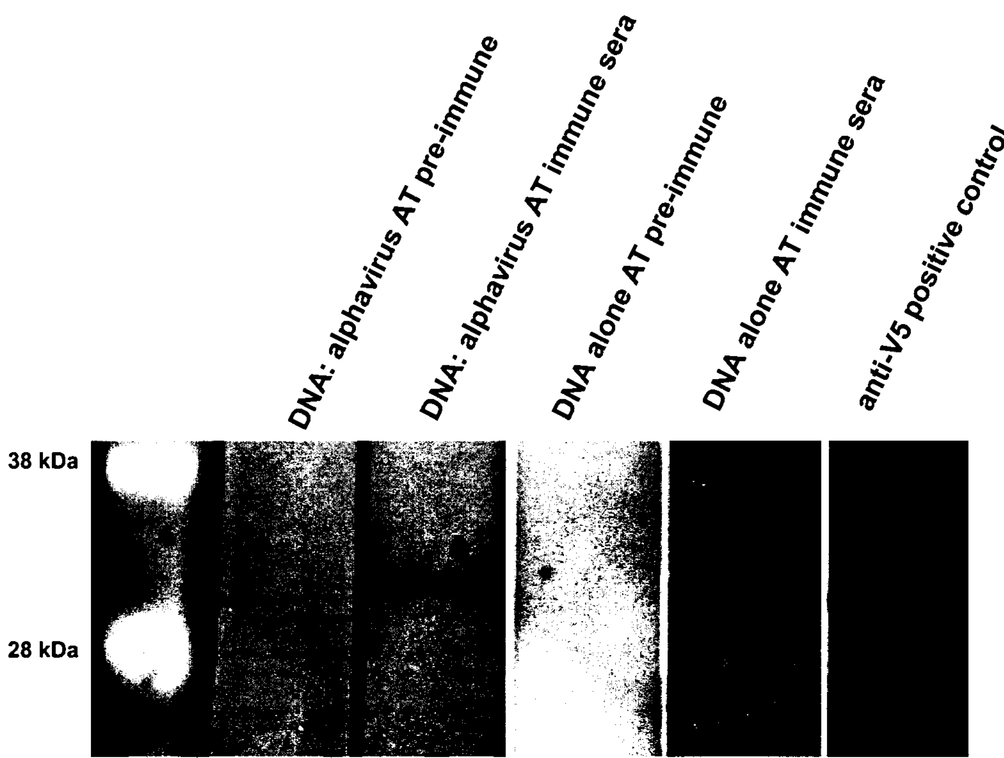

Figure 2.4: Reactivity of pre- and post-immune sera collected from mice following vaccination with AT. Pooled pre- and post-immune sera from mice vaccinated against AT with either the DNA or DNA: alphavirus prime-boost vaccination regimens were tested for reactivity against recombinant AT purified from the pIZ expression system. The positive control was probed with the α-V5 antibody.

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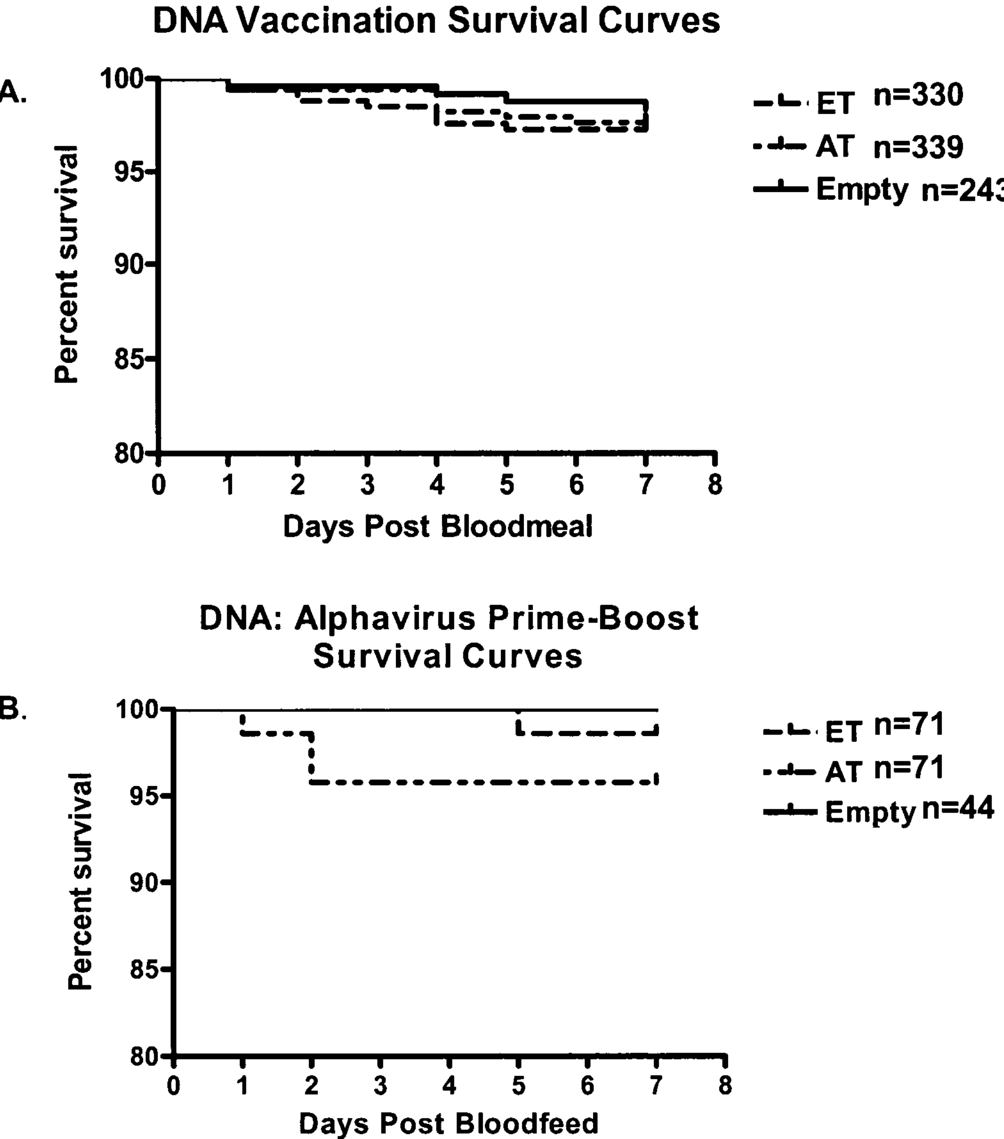

Figure 2.5: Kaplan Meier survival curves of mosquitoes after bloodfeeding upon mice vaccinated against ET or AT. Individual mice from each experimental and control group were fed upon by uninfected *A. aegypti* two weeks after the final vaccination. Survivorship of bloodfed mosquitoes collected from each vaccinated mouse was monitored for 7 days. Log rank analysis of the Kaplan Meier survival curves from each individual mouse revealed that there was no difference between the survival curves of mosquitoes fed upon mice from the same vaccinated group. Mosquito survival data within groups were pooled and a log rank test assessed the difference between experimental groups. (A) Mosquito survivorship after feeding upon mice vaccinated with DNA alone or (B) DNA: alphavirus prime-boost regimens. n= the total number of pooled bloodfed mosquitoes per vaccinated group.

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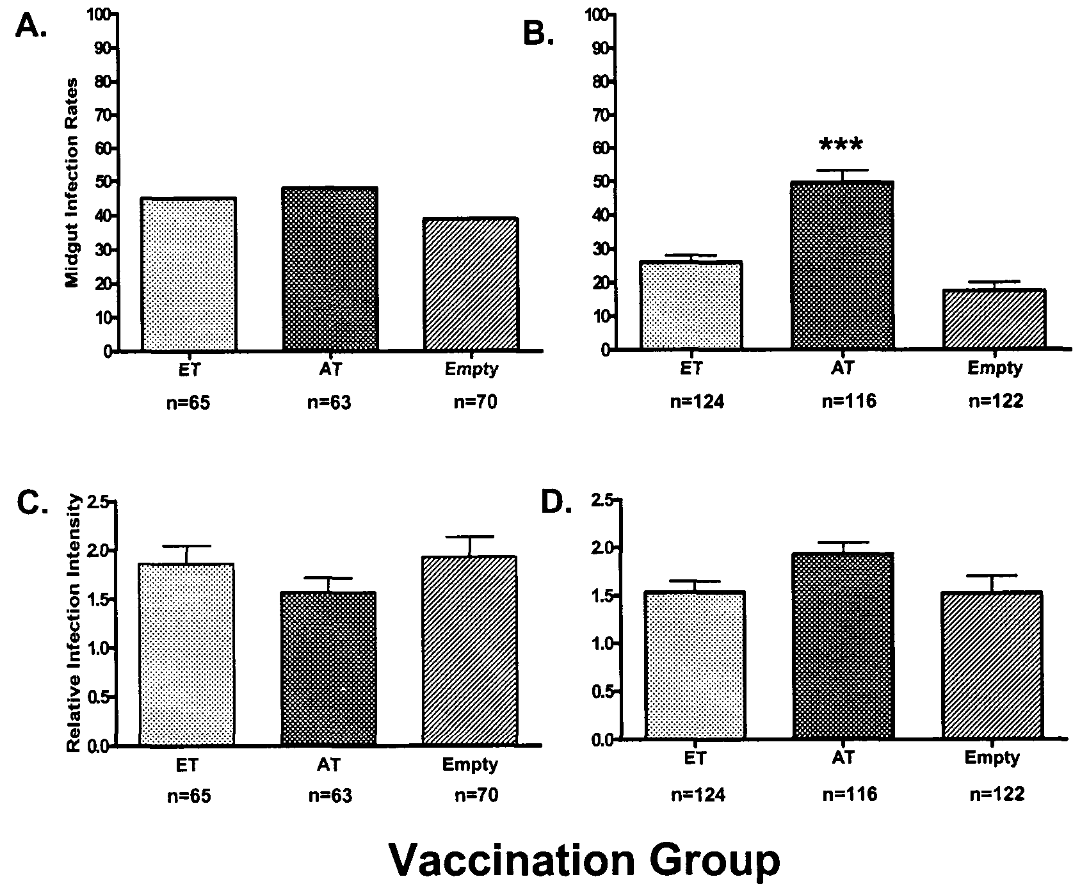

Figure 2.6: ***A. aegypti*** **midgut infection rates and relative infection intensity of DENV-2 following an infectious bloodmeal in the presence of immune sera collected from mice vaccinated with ET or AT.** DENV-2 titers for the DNA vaccination experiment were 8 x 10^5 pfu/ ml for the empty control group, 6.67 x 10^5 pfu/ ml for the anti-ET sera group and 7.63 x 10^5 pfu/ ml for the anti-AT sera group. (A) MIR and (C) RII for each group after DNA vaccination. An average of the DENV-2 titers from two replicates for the DNA: alphavirus prime-boost vaccination experiments were 4.4 x 10^5 pfu/ ml for the empty control group, 6.3 x 10^5 pfu/ mlfor the anti-ET sera group and 5.3 x 10^5 pfu/ ml for the anti-AT sera group. (B) MIR and (D) RII for each group after DNA: alphavirus prime-boost vaccination. (*) indicates a significant difference between experimental group and the control group.

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sera from mice vaccinated with the prime-boost regimen was tested, it significantly increased MIR compared to either the control or anti-ET groups ($p<0.0001$) (Figure 2.5 B). Furthermore, RII were consistently higher in the anti-AT immune group ($p<0.0665$), although these results were not significant (Figure 2.5 D). MIR and RII for the anti-ET immune group were not significantly different than the empty vector control group.

Discussion

The development of mosquitocidal and transmission blocking vaccines as novel control strategies for several medically relevant vectors has been evaluated on numerous occasions (Kay & Kemp, 1994). The use of TBV as means to control parasitic diseases such as plasmodium and leishmania is well documented, but their application for disruption of arboviral transmission cycles has been understudied (Carter, 2001, Tonui, 1999). The literature suggests that some arboviruses may utilize the proteolytic enzymes in the arthropod midgut as a means to enhance infectivity (Ludwig et al., 1989, Mertens et al., 1996, Molina-Cruz et al., 2005). The objective of this aim was to evaluate the transmission blocking potential of vaccines targeting these enzymes. Specifically, immune sera from mice vaccinated against either early or abundant trypsin were tested for their ability to inhibit DENV-2 infection of *Aedes aegypti.*

Initially, ET and AT were cloned into a common mammalian expression vector, pcDNA3.1$^+$, to be used for DNA vaccinations. Prior to vaccinations, expression of each of the proteins was assessed in HEK 293T cells. Using previously developed ET and AT specific antibodies it was demonstrated that each of the proteins was produced and secreted (Figure 2.1). These results suggested that ET and AT, in the context of this

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vector, would be expressed *in vivo* and that adequate levels of antigen would be present for immune recognition and induction.

The production of recombinant 5'dsMRE16 viruses containing each of the genes of interest (GOI) was successful. Attempts to verify production of MRE16 derived ET and AT were performed in C6/36 cells. Western blotting analysis revealed that AT was expressed from the 5'dsMRE16 construct, but levels of ET induction were not detectable (Figure 2.2). These results were surprising considering that numerous gene products have been shown to be expressed from the double subgenomic promoter. For this reason I believe that the problem exists at the point of detection. Perhaps the antibody, which was originally produced in 1996, retained very low levels of reactogenicity (Noriega et al., 1996a). The ability of the antibody to identify ET produced by the DNA expression vector in HEK 293T cells and not in the 5'dsMRE16 virus may be a reflection of the amount of recombinant protein produced in each system.

Utilizing the Insect Select System I was able to produce His tagged ET and AT each with a V5 epitope. Western blot analysis revealed that each of the proteins is secreted, as expected, and can be purified by affinity chromatography (Figure 2.3). It was determined on multiple occasions that AT was more efficiently expressed in High Five cells than ET. The reasons for this drastic difference in expression levels may be related to the regulatory factors associated with ET transcripts. In the context of mosquitoes, ET is transcribed within the first day post emergence and pools of mRNA accumulate in epithelial cells (Noriega et al., 1996a). Upon bloodmeal acquisition, transcripts are immediately translated (Noriega et al., 1996a). The regulatory mechanisms controlling the stability of these RNAs and the exact factors inducing their

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translation are not known (Noriega & Wells, 1999). On the other hand, AT is regulated at the transcriptional level (Barillas-Mury et al., 1995). Despite my ability to detect ET with the α-V5 antibody, I was unable to detect it with the α-ET specific antibody. Again this may be a result low reactogenicity and low expression levels.

Detection of AT specific antibodies induced by both vaccination regimens was detectable by western blot (Figure 2.4). This demonstrates that an AT specific immune response was induced and may be responsible for the increase in DENV-2 infectivity observed. The results presented in this chapter would have been further supported had the inhibitory effects of the immune sera been demonstrated by *in vivo* trypsin activity assays. Due to small quantities of immune sera these assays were not performed.

I was unable to demonstrate that either vaccination regimen induced anti-ET specific antibodies. This may have been a result of low quantities of recombinant protein antigen used in the western blots, low ET immunogenicity or poor *in vivo* expression of ET resulting in an undetectable immune response. In light of these results it is difficult to draw conclusion about the role of ET in DENV-2 infectivity of *A aegypti* or its applicability as a TBV target.

Vaccination of mice with either ET or AT had no significant impact on the survivorship of mosquitoes that fed upon these mice (Figure 2.5). There was a trend of increased mortality for each of the experimental groups, albeit slight. These results differ from those found by Odhiembo and Beier (personal communication Dr. Brian Foy). They found an increase in *Anopheles gambiae* mortality that fed upon mice vaccinated with *A. gambiae* midgut trypsins. Cumulatively, these data suggest that midgut trypsins

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are not an ideal target for mosquitocidal vaccines in *A. aegypti*, but may be useful targets against *A. gambiae*.

The ability of immune sera specific for each of the target proteins to inhibit DENV-2 infection of *A. aegypti* was assessed. Surprisingly, I found that sera from mice vaccinated with the DNA constructs actually increased the MIR. Anti-ET and anti-AT sera increased infection rates by 5% and 9% compared to controls, respectively. Serum from mice vaccinated with the prime-boost regimen also increased the MIR. In fact, anti-AT serum significantly increased MIR on the order of 2.5-fold compared to the control group (Figure 2.6). These data do not support the results from prior studies, in fact, they contradict them. Previously, Molina-Cruz *et. al.* reported that inhibition of early phase tryptic digestion by STI significantly decreased DENV-2 infectivity (Molina-Cruz et al., 2005). Recent evidence suggests that the STI used in those experiments may have been contaminated. Furthermore, this proposed contaminant may be responsible for drastic alterations in the physiological state of the midgut (Lu et al., 2006). It was demonstrated that feeding STI resulted in reduction in oviposition rates, ablation of peritrophic matrix 1 formation and expulsion of the bloodmeal. These physiological effects were not observed when egg white trypsin inhibitor or a synthetic trypsin inhibitor (AEBSF) was fed to *A. aegypti* (Lu et al., 2006). In light of this fact, it is difficult to determine what role, if any, midgut trypsins contribute to vector competence. Due to the increase in MIR observed when feeding with anti-AT immune sera, my results suggest that AT would not be suitable target for TBV development. Furthermore, the limited effect observed in this study suggests that antibody binding of AT allows for increased DENV-2 infectivity,

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although this cannot be definitively stated without proof of immune sera binding and inhibition of AT activity.

The ability of anti-AT immune sera to increase midgut infection rates is interesting considering that arboviruses are thought to infect the midgut within the first few hours after uptake and AT does not begin to be expressed until 8 hpbm. Perhaps the highly conserved three dimensional structures of serine proteases may explain the effects observed. Chymotrypsin-like serine proteases which include trypsins, chymotrypsins and elastases contain two parallel six-stranded beta-barrels that form the substrate recognition pocket (Blow, 1971). Vaccination with AT may have produced antibodies that recognize structural epitopes conserved among this subfamily of serine proteases. Given that ET begins to accumulate within 1 hpbm it could be possible that cross-reactive antibodies specific for AT may bind to and inhibit ET activity. This inhibitory effect would limit the virus's exposure to proteolytic enzymes during the initial stages of infection and increasing the likelihood of infection. This hypothesis would explain the effects observed following co-feeds of DENV-2 and anti-AT immune sera. Provided with sufficient quantities of anti-AT immune sera and access to recombinant ET, *in vivo* early phase trypsin activity assays and western blots could be performed to test this hypothesis.

I also observed that the anti-AT immune sera could increase midgut infection intensities, albeit not significantly. The RII is a semi-quantitative approach to assessing the ability of the virus to overcome the midgut infection barrier. Initially, it was believed that an increase in midgut RII would correlate to an increase in dissemination rates and a reduction in the extrinsic incubation period. Interestingly, neither of these assumptions was correct (Bosio et al., 1998, Miller & Mitchell, 1991, Myles et al., 2004).

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Nevertheless, RII is still a good indicator for determining the rate and degree of replication efficiency in the mosquito midgut.

The results presented in this chapter suggest that AT is not a promising target for a DENV-2 TBV. At the same time these results underscore the fact that DENV-2 interacts with midgut proteins, suggesting that development of a TBV may be possible once a suitable target is identified. This study highlights the deficiency in our understanding of virus/ vector interactions, specifically, the importance of midgut serine proteases in DENV-2 infectivity of *A. aegypti*. Perhaps analysis of the virus and vector proteases, individually, would be beneficial to understanding this system more fully. Nevertheless, further investigation into the role of these proteolytic enzymes in vector competence is warranted.

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Chapter 3

Analysis of the Role of E-glycoprotein Potential Trypsin Cleavage Sites on DENV-2 Infectivity of *Aedes aegypti*

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Hypothesis

The objective of this aim was to characterize the role of the DENV-2 E-glycoprotein potential trypsin recognition sites on infectivity of the vector *Aedes aegypti*. I hypothesized that disruption of one or more of the solvent exposed potential trypsin sites within domain III will reduce the ability of the virus to infect the primary vector, *A. aegypti*.

1. Introduction

DENV is a member of the family *Flaviviridae* and has an enveloped, positive-sense, single-stranded RNA genome of approximately 10.8 Kb. The genome encodes 10 genes, three of which are structural genes; capsid (C), pre-membrane (prM) and E-glycoprotein (E) (Chambers et al., 1990, Lindenbach & Rice, 2001). During viral maturation prM and E are closely associated in the endoplasmic reticulum (ER), and this association prevents an irreversible conformational change in E during egress through acidic cellular compartments (Heinz et al., 1994). The nucleocapsid buds through ER acquiring an envelope containing the prM:E and the immature virus particle is subsequently transported to the Golgi apparatus, where prM is cleaved by cellular furin. This results in a conformational change within E and the maturation of the virion (Elshuber et al., 2003, Stadler et al., 1997).

The E glycoprotein is the predominant structural protein on the exterior of mature virions and 90 homodimers are layered parallel to the lipid bilayer (Kuhn et al., 2002, Zhang et al., 2004). In the context of the virion, E is responsible for attachment, penetration and fusion with host cells (Anderson et al., 1992, Chen et al., 1996b, He et

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al., 1995, Modis et al., 2004). The E monomer is 495 amino acids in length and is composed of three domains (I-III) with domain III being the most distal projection. Domains I and II are involved in for membrane fusion, while competitive inhibition assays have revealed that domain III functions as the viral attachment peptide (VAP)(Chin et al., 2007, Modis et al., 2004). Attachment studies, in which cryo-electron microscopy analysis of soluble E (sE) coupled with the carbohydrate recognition domain of dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN), suggest that viral glycosylation motifs within domain II act as the initial sites of attachment. The authors speculated that this initial attachment allows the virus to be properly positioned so that domain III is accessible to the primary receptor (Pokidysheva et al., 2006). To date, neither the primary receptor nor the region on domain III that acts as the VAP have been identified. Even less is known about the virus/ receptor interactions within the mosquito vector *Aedes aegypti*.

Monoclonal antibody epitope mapping, in which DENV-2 was proteolytically digested with serine proteases *ex vivo*, revealed that there are multiple sites within E that are sensitive to serine proteases (Roehrig et al., 1998, Wang et al., 1999). These experiments provided a rough estimate of the location of these cleavage events, although without further analysis by mass spectrometry-based mapping the exact locations of these cleavage sites remain unknown. Domain III has 11 potential trypsin recognition sites, Lys (K)/ Arg (R)-X (as along as X is not a Pro), which may act as targets for proteolytic processing or degradation. The importance of these sites in receptor recognition and/ or binding has not been evaluated.

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The mosquito midgut is a highly proteolytic environment that is equipped to quickly digest nutrients in the bloodmeal. To date, four midgut serine proteases have been identified in *A. aegypti*. Furthermore, these midgut serine proteases have been implicated in conferring susceptibility to DENV-2. Quantitative trait loci (QTL) mapping experiments have identified two of these midgut proteases which maybe associated with DENV-2 susceptibility (Bennett et al., 2005b, Bosio et al., 2000). In addition, Molina-Cruz *et. al.* found that inhibition of trypsin activity significantly decreased DENV-2 infectivity of *A. aegypti* (Molina-Cruz et al., 2005). Similar results have been documented in other viral/ vector systems (Ludwig et al., 1989, Mertens et al., 1996). These results are not surprising considering that many gut associated viruses, i.e. rotaviruses, utilize host proteases for enhancement of infectivity (Arias et al., 1996).

This evidence suggests that proteolytic processing of DENV-2, probably within domain III due to its positioning and reported involvement in host cell binding, may be required for efficient infectivity of *A. aegypti*. Using an infectious cDNA clone of DENV-2 and site-directed mutagenesis (SDM) techniques, I tested the importance of the domain III potential trypsin cleavage sites in mosquito infectivity. Specifically, the four most solvent exposed sites, as determined from structural data, were selected as the most rational targets for these experiments.

2. Materials and Methods

Mosquito Rearing

The Rexville (RxD) strain of *A. aegypti*, originally colonized from Puerto Rico, was used for these experiments. The mosquitoes were maintained at a constant

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temperature of 28°C and a relative humidity of 80%. The photoperiod was set to a 14:10 light: dark cycle.

Cell Lines and Maintenance

Vertebrate and invertebrate cell lines were used in these experiments. C6/36 cells are an *A. albopictus* cell line derived from whole mosquito cellular suspensions. They are maintained in Liebowitz-15 medium (L-15) with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% penicillin/ streptomycin and 1% L-glutamine. Cells are grown in incubators 28°C without CO_2. Vero and LLC-MK2 cells are African green and rhesus monkey kidney cell lines, respectively. They were grown and maintained in DMEM with 10% FBS, 1% non-essential amino acids, 1% penicillin/ streptomycin and 1% L-glutamine at 37°C in the presence of CO_2. L-15 or DMEM medium containing 2% FBS was used during infections of all the cell lines.

Alignments

Protein alignments were initially generated using CLUSTALW (www.align.genome.jp/). Alignments were then manually adjusted using a previously generated E-glycoprotein amino acid sequence as a template (Rey et al., 1995). The viruses used for the alignment are listed as follows: virus, strain and NCBI accession number. DENV-2 16681 (AAB58782), DENV-1 Hawaii-01P (AAZ42313), DENV-3 Nicaragua24/94 (AAW31406), DENV-4 CN78-42 (ABO27189), JEV VN105/Vietnam/2002/mosquito (AAR26619), YFV V-528A/Colombia/1979/mosquito (AAA92706), and TBEV 4387/B7 (CAA54069). Multiple DENV-2 sequences were also

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analyzed for conservation of the 11 potential trypsin sites, although alignments were not generated.

Site-Directed Mutagenesis

Initially, because of its use in my other studies, attempts were made to mutagenize an infectious clone of a low-passage Jamaica 1409 strain, but it proved to be difficult to manipulate (Pierro et al., 2006). In its place, I used the infectious clone pD2/ic-30P encoding DENV-2 strain 16681 which was generously provided by Dr. Richard Kinney (Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Disease). Strain 16681 was the original DENV-2 strain used for infectious clone production and was recovered from a DHF patient in Thailand in 1964, but had been passaged numerous times prior to assembly of the infectious clone (Kinney et al., 1997).

Upon examination of the three-dimensional structure of sE, four potential trypsin cleavage sites, K305, K307, K310 and K361 were chosen as targets for SDM because of their solvent accessibility. Each of the four sites is located in a β-strand on the internal face of domain III. Mutagenesis primers were designed so that single nucleotide changes would be introduced in order to convert each of the Lys to a semi-conserved Gln. In the case of the production of the quadruple mutant (KquadQ) the first primer set used introduced nucleotide changes corresponding to sites K305, K307 and K310. Subsequently, a second round of mutagenesis was applied to introduce the final mutation at K361.

SDM was performed on pD2/ic-30P using the QuickChange II XL Site-Directed Mutagenesis Kit according to manufacturer's protocol (Stratagene, Cedar Creek, TX).

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Briefly, 40 ng of pD2/ic-30P was mixed with 10X reaction buffer, dNTP's, 125 ng of the forward and reverse primers, QuickSolution™, PfuUltra high fidelity DNA polymerase and brought to a final volume of 50 µl in ddH_2O. The PCR parameters were as follows; 95°C for 30 seconds followed by 20 cycles of 95°C for 30 seconds, 55°C for 1 minute and 68°C for 14 minutes. Finally, a 68°C extension step was applied for 7 minutes. Products were treated with *Dpn*I and then transformed into electrocompetent *E. coli* cells and plated on LB agar with ampicillin. Colonies were screened with E protein specific primers, grown-up, and isolated with Qiagen's Mini-Prep Kit (Qiagen, Valencia, CA). Subsequently, the entire E gene was sequenced in order to confirm the desired mutations, but no others had been introduced.

Mutant Virus Production

Once the mutations had been verified, viral RNA was produced via an *in vitro* RNA transcription reaction (Kinney et al., 1997). Briefly, 5 µg of infectious clone plasmid DNA was linearized with *Xba*I and treated with proteinase K. The reaction was phenol: chloroform extracted and ethanol precipitated. Subsequently, 1 µg of linear DNA was incubated in the presence of rNTPs, m^7-GpppA cap analog, 5X transcription buffer and T7 RNA polymerase at 39°C for 1 hour. Upon completion of the transcription reaction, 12 µl of positive sense ssRNA were electroporated into 4×10^6 C6/36 cells using the following settings: 250 V, 25 Ω and 550 µF. Cells were placed in flasks at 28°C for 8 days at which point they were screened for the presence of viral antigen by indirect immunofluorescence. If viral antigen was present a p(0) stock was collected.

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The p(0) stocks were amplified once on C6/36 cells to produce the p(0)p(1) working stocks and titrated by plaque assay on LLC-MK2 cells.

Viral Growth Curves

C6/36 and Vero cells were infected at a multiplicity of infection (MOI) of 0.01 with the pD2/ic-30P infectious clone derived wild type (wt) 16681 virus (16681icd) and each of the mutant viruses. 50 µl aliquots of infectious cell culture supernatant were collected every 2 days for the next 14 days. An aliquot was also collected pre-adsorption, time-point 0, in order to verify that input viral doses were equivalent. Growth curves were repeated in triplicate and titrated by plaque assay on LLC-MK2 cells.

Artificial Infectious Bloodmeals

The16681icd and each of the mutant viruses were inoculated at an MOI of 0.01 on C6/36 cells and allowed to incubate for 7 days, which point all but 1 ml of cell culture supernatant was removed and replaced with fresh 2% L-15 medium. Four days later cell culture supernatant and cells were mixed 1:1 with defibrinated sheep blood. The blood/ virus meal was placed in a water jacketed membrane feeder maintained at 37°C and mosquitoes were allowed to feed for 1 hour (Rutledge et al., 1964). The infectious bloodmeal titers are an average of the three replicates: 16681icd 3.54 x 10^6 pfu/ ml, K305Q 3.99 x 10^6 pfu/ ml, K307Q 4.44 x 10^6 pfu/ ml, K310Q 4.89 x 10^6 pfu/ ml and K361Q 3.33 x 10^6 pfu/ ml. Mosquitoes were maintained in the insectaries for 10 days, at which point 30 individual midguts were collected for determination of midgut infection rates (MIR) by indirect immunofluorescence assay (IFA). At day 17 post infection paired

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midguts and heads were obtained from 24 individuals for determination of dissemination rates (DR) by IFA.

Indirect Immunofluorescence Assay

IFAs were performed according to the protocol outlined in Bennett *et. al.* (2002). Briefly, dissected midguts and heads were fixed in either 4% paraformaldehyde or acetone, respectively. Midguts were washed and stained in PBS+0.1% Triton X100, while PBS was used for the heads. Initial staining was carried out with the E-glycoprotein specific murine monoclonal antibody, 4G2. The samples were washed and the secondary stain was applied, which included biotinylated sheep anti-mouse antibody (Amersham, Arlington Heights, IL) and 0.005% Evans blue counter stain. Finally, samples were developed with streptavidin-fluorescein (Amersham) and visualized for fluorescence. MIR was reported as a percentage determined by dividing the number of positive midguts by the total number tested. DR was determined by dividing the total number of positive heads by the total number of positive midguts. Relative infection intensities (RII) were determined for midguts 10 dpi. This was done by scoring positive midguts on a scale of 1-4 then totaling these values and dividing by the number of positive midguts.

Statistical Analysis

Statistical analyses were carried out using SAS 9.1 (SAS Institute Inc., Cary, NC). Each data set was analyzed using analysis of variance (ANOVA) in which the proc mixed procedure was applied. A randomized block design was fit where the three replicates

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composed the random block effect and the groups were the fixed effect.

Results

Prior to undertaking these experiments, several DENV-2 E-glycoprotein amino acid sequences, generated from more than 20 geographically and chronologically distinct isolates, were evaluated to determine whether the potential trypsin recognition sites were conserved. I found that all 11 sites within domain III were conserved (data not shown).

Analysis of the E-glycoprotein alignments revealed that K305 was conserved in DENV-2, DENV-4, JEV, YFV and TBEV, but this site had been replaced with a Ser and Thr in DENV-1 and DENV-3, respectively. DENV-2 and DENV-1 both contained a Lys at position 307, although none of the other viruses analyzed contained this potential trypsin recognition motif. Interestingly, all viruses analyzed contained a Lys at position 310, except for TBEV, which contained an Arg. Finally, DENV serotypes 1-3 contained a Lys at position 361, but DENV-4 and the other three viruses analyzed had Lys 361 replaced with small nucleophilic amino acids except for YFV, which had an Asn (Figure 3.1).

I successfully mutated pD2/ic-30P to contain each of the desired mutations, K305Q, K307Q, K310Q and K361Q including the quadruple mutant. Furthermore, the sequencing data of E verified that no other unintended mutations were introduced. Production of infectious viral particles was successful for each of the single mutations as well as the wt 16681icd, but I was unable to recover virus particles from the quadruple mutant. Analysis of the *in vitro* transcribed RNA on a 1% agarose gel revealed that

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DENV-2     MRCIGMSNRDFVEGVSGGSWVDIVLEHGSCVTTMAKNKPTLDFELIKTEAK          60
DENV-1     MRCVGIGNRDFVEGLSGATWVDVVLEHGSCVTTMAKDKPTLDIELLKTEVT          60
DENV-3     MRCVGVGNRDFVEGLSGAPWVVVVLEHGGCVTTMAKNKPTLDIELQKTEAT          60
DENV-4     MRCVGVGNRDFVEGVSGGAWVDLVLEHGGCVTTMAQGKPTLDFELTKTTAK          60
JEV        FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEAS          60
YFV        AHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAID          60
TBEV       SRCTHLENRDFVTGTQGTTRVTLVLELGGCVTITAEGKPSMDVWLDAIYQE          60

DENV-2                                                                 120
DENV-1                                                                 120
DENV-3                                                                 120
DENV-4                                                                 120
JEV                                                                    120
YFV                                                                    120
TBEV                                                                   120

DENV-2                 EYTIVITPHSGEEHAV-GNDT----GKHGKEIKITPQSSITEAEL  175
DENV-1                 KYSVIVTVHTGDQHQV-GNET----TEHGTIATITPQAPTSEIQL  175
DENV-3                 KYTVIITVHTGDQHQV-GNET----QGVTAEITPQASTTEA-I-L  173
DENV-4                 EYTVVVTVHNGDTNAV-GNDI----SNHGVTATITPRSPSVEVEL  175
JEV                    KYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVTPNAPSITLKL  180
YFV                    QYVIRAQLHVGAKQENWNT--------DIKTLKFDALSGSQEAEF  172
TBEV                   KIVYTVKVEPHTGDYVAANETHSG--RKTASFTVS--SEKTILTM  176

DENV-2     TGYGTVTMECSPRTGLD-                                          230
DENV-1     TDYGALTLDCSPRTGLD-                                          230
DENV-3     PEYGTLGLECSPRTGLD-                                          228
DENV-4     PDYGELTLDCEPRSGID-                                          230
JEV        GDYGEVTLDCEPRSGLNT                                          231
YFV        TGYGKATLECQVQTALD-                                          224
TBEV       GEYGDVSLLCRVASGVDL                                          231

DENV-2                                                           GHLKC  286
DENV-1                                                           GHLKC  286
DENV-3                                                           GHLKC  284
DENV-4                                                           GHLKC  286
JEV                                                              GHLKC  287
YFV                                                              GHVAC  284
TBEV                                                             GHVTC  284

DENV-2     RLRMDKLQLKGMSYSMCT-GKFKVVKEIAETQHGTIVIRVQYEGDGSPCKIPFEIMDLEK  345
DENV-1     RLKMDKLTLKGISYVMCT-GSFKLEKEVAETQHGTVLVQVKYEGTDAPCKIPFSSQDEKG  345
DENV-3     RLKMDKLELKGMSYAMCT-NTFVLKKEVSETQHGTILIKVEYKGEDVPCKIPFSTEDGQG  343
DENV-4     KVRMEKLRIKGMSYTMCS-GKFSIDKEMAETQHGTTVVKVKYEGAGAPCKVPIEIRDVNK  345
JEV        RLKMDKLALKGTTYGMCT-EKFSFAKNPADTGHGTVVIELTYSGSDGPCKIPIVSVASLN  346
YFV        RVKLSALTLKGTSYKMCT-DKMSFVKNPTDTGHGTAVMQVKVPKGA-PCRIPVMVADDLT  342
TBEV       EVGLEKLKMKGLTYTMCDKTKFTWKRAPTDSGHDTVVMEVTFSGTK-PCRIPVRAVAHGS  343
                               A                B                C
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                 *     *         *                      *   *
DENV-2           RHVL-GRLITVNPIVT--EKDSPVNIEAEPPFGDSYIIIGVEPGQLKLNWFKKGSSIGQM  402
DENV-1           VTQN-GRLVTANPIVT--DKEKPVNIEAEPPFGESYIVVGAGEKALKLSWFKKGSSIGKM  402
DENV-3           KAHN-GRLITANPVVT--KKEEPVNIEAEPPFGESNIVIGIGDNALKINWYKKGSSIGKM  400
DENV-4           EKVV-GRIISSTPFAE--NTNSVTNIELEPPFGDSYIVIGVGDSALTLHWFRKGSSIGKM  402
JEV              DMTPVGRLVTVNPFVATSSSNSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAGSTLGKA  406
YFV              AAVNKGILVTVNPIAS--TNDDEVLIEVNPPFGDSYIIVGTGDSRLTYQWHKEGSSIGKL  400
TBEV             PDVNVAMLITPNPTIE---TNGGGFIEMQLPPGDNIIYVG----ELSHQWFQKGSSIGRV  396
                       D               E        F             G

DENV-2           FETTMRGAKRMAILGDTAWDFGSLGGVFTSIGKALHQVFGAIYGAAFSGVSWTMKILIGV  462
DENV-1           FEATARGARRMAILGDTAWDFGSIGGVFTSVGKLVHQIFGTAYGVLFSGVSWTMKIGIGI  462
DENV-3           FEATARGARRMAILGDTAWDFGSVGGVLNSLGKMVHQIFGSAYTALFSGVSWVMKIGIGV  460
DENV-4           FESTYRGAKRMAILGETAWDFGSVGGLFTSLGKAVHQVFGSVYTTMFGGVSWMVRILIGF  462
JEV              FSTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMSWITQGLMGA  466
YFV              FTQTMKGAERLAVMGDAAWDFGSAGGFFTSVGKGIHTVFGSAFQGLFGGLSWITKVIMGV  460
TBEV             FQKTKKGIERLTVIGEHAWDFGSAGGFLSSIGKAVHTVLGGAFNSIFGGVGFLPKLLLGV  456

DENV-2           IITWIGMNSRSTSLSVTLVLVGIVTLYLGVMVQA  496
DENV-1           LLTWLGLNSRSTSLSMTCIAVGMVTLYLGVMVQA  496
DENV-3           LLTWIGLNSKNTSMSFSCIAIGIITLYLGAVVQA  494
DENV-4           LVLWIGTNSRNTSMAMTCIAVGGITLFLGFTVQA  496
JEV              LLLWMGVNARDRSIALAFLATGGVLVFLATNVHA  500
YFV              VLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA  494
TBEV             ALAWLGLNMRNPTMSMSFLLAGGLVLAMTLGVGA  490
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Figure 3.1: Amino acid alignment of the four dengue virus serotypes along with a representative virus from each of the other three flavivirus serocomplexes. Domains I-III are shaded red, yellow and blue, respectively. The C-terminal region in black represents the insoluble region followed by the transmembrane region shaded green. Above the alignment, paired solid black symbols (♦, ■, ◄, ▼, ► and ▲) mark conserved cysteines and their respective disulfide bond counterparts (Nowak & Wengler, 1987, Rey et al., 1995). (*) denotes the 11 potential trypsin cleavage sites for DENV-2. The underlined regions (A-G) of DIII correspond with β-strands as determined by the TBEV-sE subunit structure (Rey et al., 1995).

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introduction of all four mutations produced a ssRNA species that migrated much quicker than the wt or single mutants (Lane 11, Figure 3.2).

Growth curve analysis revealed that each of the mutant viruses grew similarly to the wt 1668licd in C6/36 and Vero cells. Viruses reached a peak titer of 6.5-7 $\log_{10}$ plaque forming units (pfu)/ ml by 8 dpi in C6/36 cells. Interestingly, I found that each of the viruses reached peak titers by 6 dpi infection when grown in mammalian cells, but only achieved concentrations of 5.5-6 $\log_{10}$ pfu/ ml (Figure 3.3). Initial experiments revealed that virus produced in mosquito cells infected mosquitoes much more efficiently than virus produced in mammalian cells (data not shown). Consequently, the viruses used in all subsequent infection assays were produced in mosquito cells. Surprisingly, I found that one mutation, K305Q, significantly increased MIR compared with the control ($p<0.0492$) (Figure3.4A). This increase in MIR was not accompanied by an increase in infection intensity as RII values did not differ significantly (Figure 3.4B). Introduction of mutations at the other three sites had no affect on MIR or RII compared to the wt 1668licd. I also determined that all of the mutant viruses produced for these experiments disseminated at the same rate as the control virus (Figure 3.4C).

Discussion

Gut-associated vertebrate viruses, such as rotaviruses, utilize host proteolytic enzymes for enhancement of infectivity (Arias et al., 1996). Similarly, some arboviruses, including DENV-2, have been implicated in utilizing gut proteases in order to enhance infectivity in their invertebrate vectors (Ludwig et al., 1989, Mertens et al., 1996, Molina-Cruz et al., 2005). It is believed that domain III of the E-glycoprotein, which mediates

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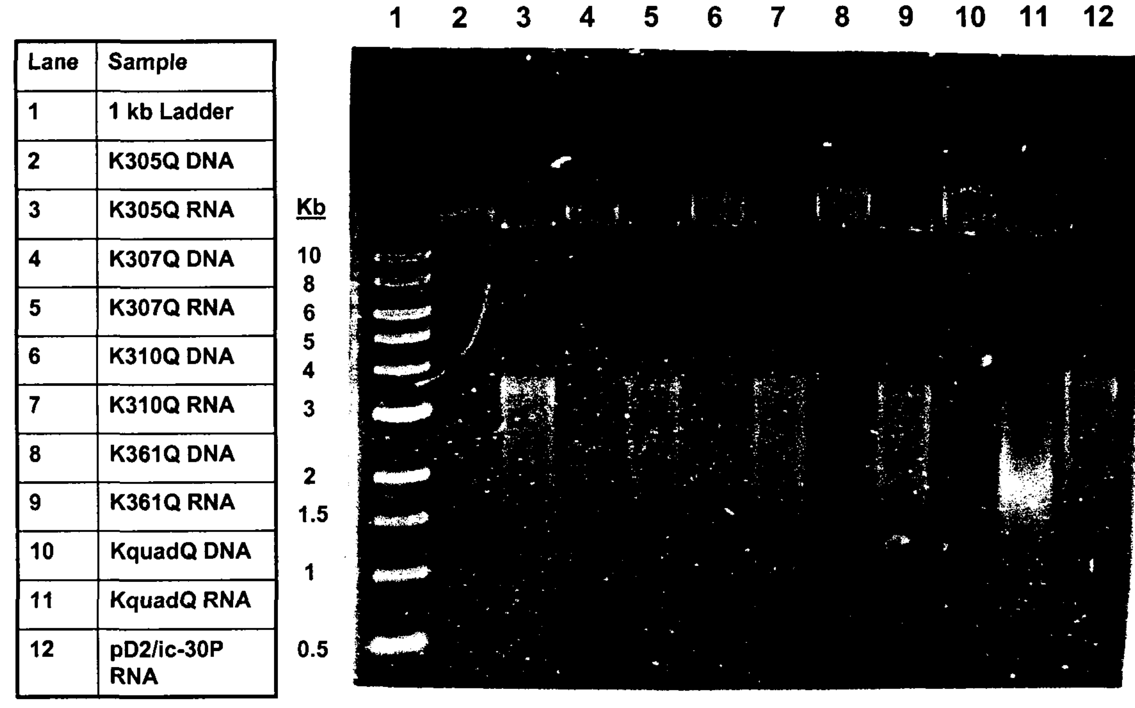

Lane	Sample
1	1 kb Ladder
2	K305Q DNA
3	K305Q RNA
4	K307Q DNA
5	K307Q RNA
6	K310Q DNA
7	K310Q RNA
8	K361Q DNA
9	K361Q RNA
10	KquadQ DNA
11	KquadQ RNA
12	pD2/ic-30P RNA

Figure 3.2: Linearized infectious clone DNA and *in vitro* transcribed RNA. Upon verification that each of the mutant viruses had the correct mutation; DNA was linearized and subsequently used for *in vitro* transcription reactions. Equal volumes of linearized infectious clone DNA and in vitro transcribed RNA for each mutant were loaded on a 1% agarose gel and stained with ethidium bromide in order to visualize RNA stability and migration rates.

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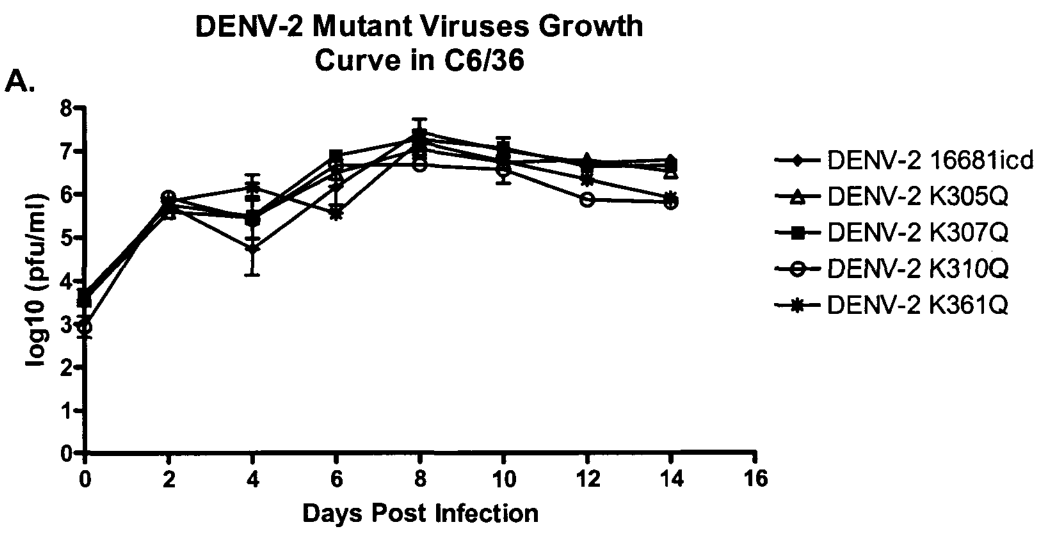

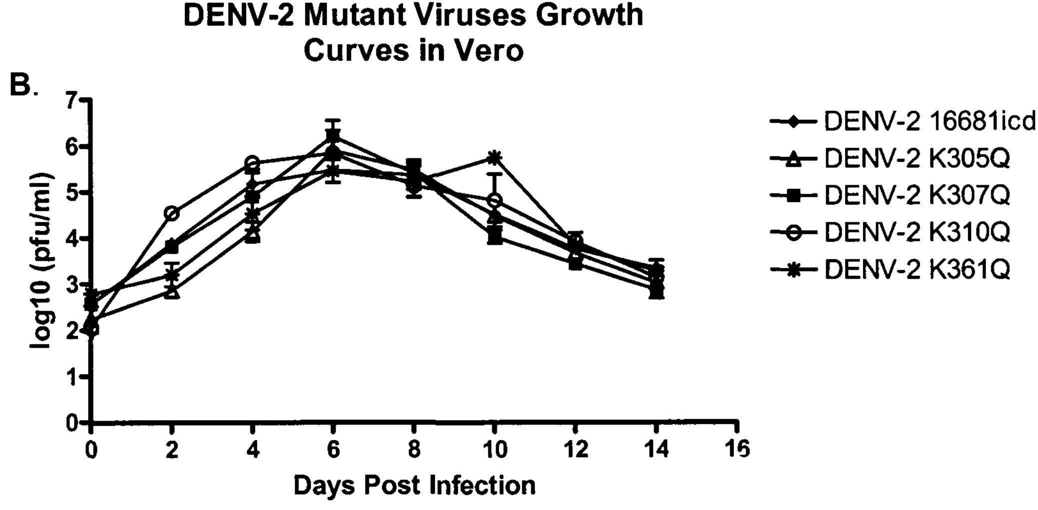

Figure 3.3: Mutant virus growth curves. Growth kinetics of each of the mutant viruses was assessed in cell culture prior to infection assays. Each virus was inoculated onto either C6/36 cells (A) or Vero cells (B) at an MOI of 0.01. An input sample was collected immediately after inoculation and represent time point 0; subsequently samples were collected every 48 hours for the next 14 days and samples were titrated by plaque assay on LLc-MK2 cells.

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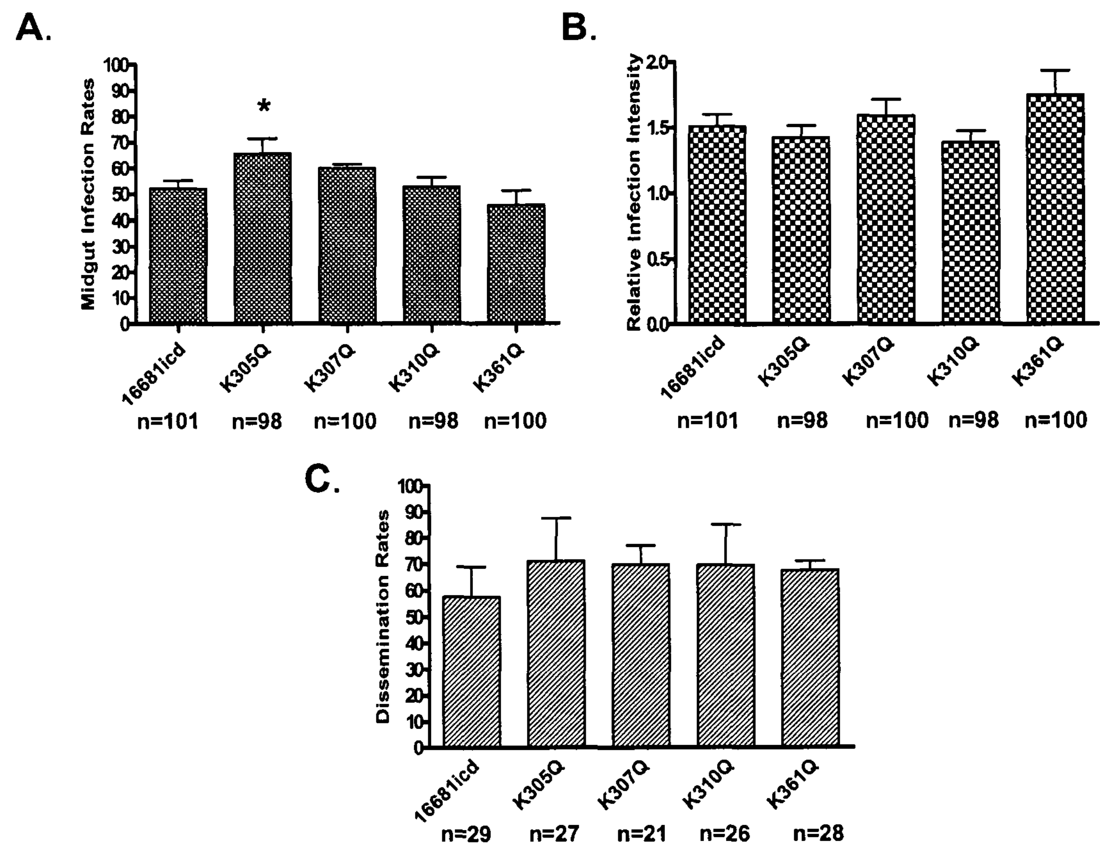

Figure 3.4: Mutant virus infection rates in *A. aegypti*. Mutant virus titers bloodfed to mosquitoes were 16681icd 3.54 x 10^6 pfu/ ml, K305Q 3.99 x 10^6 pfu/ ml, K307Q 4.44 x 10^6 pfu/ ml, K310Q 4.89 x 10^6 pfu/ ml and K361Q 3.33 x 10^6 pfu/ ml. The titers are an average over from the three replicates. (A) MIR and (B) RII were determined 10 dpi by IFA. (C) DR was determined 17 dpi by IFA. Sample size values under (C) correspond to the number of IFA positive midguts per each group analyzed. The DR was determined by dividing the number of IFA positive heads by the number of positive midguts. (*) indicates the mutant virus significantly increased infectivity compared to the control 16681icd virus.

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cellular attachment, may be the target of proteolysis. In fact, *in vitro* analysis has demonstrated that domain III is sensitive to trypsins, although the precise location of these cleavage events remains uncertain (Roehrig et al., 1998, Wang et al., 1999). The objective of this study was to selectively target potential trypsin recognition sites within domain III of DENV-2 for SDM and test the effect of these mutations on viral infectivity in *A. aegypti*.

Analysis of sequence alignments revealed that the 11 potential trypsin recognition sites found within domain III are highly conserved among all DENV-2 isolates. Yet, this was not the case when the other three serotypes were added to the analysis. Specifically, the four sites analyzed in this study were only partially conserved across serotypes, but one trypsin recognition motif, K/R 310, was maintained in all flaviviruses analyzed (Figure 3.1). Furthermore, three other trypsin recognition sites, K334, R351 and K394, were highly conserved, although due to their inaccessible location within sE these are probably not involved in the enhancement of viral infectivity (personal communication Dr. Yorgo Modis).

Considering that all four DENV serotypes are maintained in the same transmission cycle and utilize the same vector, the divergence found at the four most solvent exposed trypsin recognition sites, aside from K310, was surprising. There are three possible explanations for these results; 1) proteolytic processing of DENV occurs at site K310, 2) processing is not a prerequisite for efficient DENV infectivity of *A. aegypti* or 3) the mechanism by which each serotype infects the midgut differs. Each of the three explanations is viable and further investigation is warranted.

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The production of infectious viral particles from each of the infectious clone variants was successful with the exception of the quadruple mutant. Analysis of the *in vitro* transcribed positive sense ssRNA revealed that it migrated at a much faster rate than the other ssRNA species. Furthermore, electroporation of this mutant ssRNA molecule into C6/36 cells did not result in production of infectious virus particles. This result was observed on multiple occasions (Figure 3.2). These data suggest that the RNA species produced was either truncated or that the additive effect of all four mutations drastically altered the secondary structure of the ssRNA molecule. Considering that only the E-glycoprotein gene was sequenced following the site-directed mutagenesis, it is possible that a stop codon may have been unexpectedly introduced elsewhere in the genome. This would result in a truncated genome and account for the faster migration of the genomic RNA on the gel and my inability to recover virus particles (Figure 3.2). This is unlikely considering that the infectious clone template is only ~14 kb in length and the Pfu Ultra polymerase used for the mutagenesis reactions has an error rate of 3.9×10^{-6} mutations per base pair per duplication.

It is more likely that introduction of the four nucleotide changes may have altered the secondary structure of the input RNA genome. These alterations may have led to a fatal fold in the E-glycoprotein gene making it more vulnerable to intracellular nucleases. Another explanation may be that introduction of the four mutations may have altered the orientation or accessibility of important translation or replication sequences such as the 5' and 3' un-translated regions (Alvarez et al., 2005, Chiu et al., 2005, Holden et al., 2006).

The growth curve data show that each of the viruses, including the wt 16681icd, grew to higher titers in mosquito cells as compared to mammalian cells (Figure 3.3).

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These results are not surprising, considering that prior to the initial infectious clone construction the parental virus had been serially passaged in C6/36 cells. Several studies have demonstrated that continuous passaging of a virus in a specific cell type confers adaptability and fitness gains (Chen et al., 2003, Ciota et al., 2007, Cooper & Scott, 2001, Greene et al., 2005). This may also account for the higher MIR observed when feeding virus derived from the mosquito cells (data not shown). Interestingly, this virus derived cell type preference is opposite of what has been reported for alphaviruses (Pierro et al., 2007). This discrepancy may be the result of differences in virus maturation and glycosylation which could affect host cell binding preferences.

One of the parameters used in this study was relative infection intensity. The RII is a semi-quantitative approach to assessing the ability of the virus to overcome the midgut infection barrier. Initially, it was believed that an increase in midgut RII would correlate with an increase in dissemination rates and a reduction in the extrinsic incubation period. Interestingly, neither of these assumptions was correct (Bosio et al., 1998, Miller & Mitchell, 1991, Myles et al., 2004). Nevertheless, RII is still a good indicator for determining the rate and degree of replication efficiency in the mosquito midgut.

The results obtained from my mutagenesis studies revealed that one site, K305, is important in determining MIR, although this mutation did not seem to affect RII or DR. The other three sites analyzed in this study had no effect on MIR, RII or DR (Figure 3.4). This suggests that K305 may represent the trypsin sensitive cleavage site previously described around amino acid 300 of the E-glycoprotein (Roehrig et al., 1998). Analysis of proteolytically treated DENV-2 fragments using mass spectrometry could reveal the

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exact location of this cleavage site. These results suggest that abolishment of the trypsin recognition motif at position K305 protects the virus from degradation within the highly proteolytic midgut environment. The sequence alignments demonstrated that this site is only conserved among DENV-2 and -4 (Figure 3.1), suggesting that the mechanisms limiting DENV infectivity in *A. aegypti* are not identical in all four serotypes. Evolutionary data implies that each of the four serotypes jumped from their sylvatic cycles in monkeys to humans independently (Holmes, 2003). These independent events could also occur in the vector, given that sylvatic cycles are maintained by arboreal *Aedes spp.* (*Stegomyia*) and urban cycles are maintained by *A. aegypti* (Diallo et al., 2003, Rudnick, 1965). It is possible that independent adaptation of the four serotypes to *A. aegypti* resulted in slightly differing mechanisms of midgut infectivity. Alternatively, mutation of K305 might not be relevant to disabling a critical tryptic site during viral infectivity and the observed results may be the result of this mutation affecting other aspects of the virus during 'lifecycle' such as viral attachment, fusion or E-glycoprotein maturation.

These results suggest that conservation of these trypsin recognition sites, specifically K305, in DENV-2 may be, in part, responsible for influencing viral infectivity of *A. aeygpti*. These results in conjunction with those from chapter I suggest that midgut proteases do not enhance midgut infections, but instead may be detrimental to viral infectivity. To date, the viral and/ or host genetic factors influencing vector competence remain unknown and further investigation is essential. Attempts to elucidate the midgut serine protease(s) responsible for this mode of action are necessary to fully understanding their importance in vector competence.

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Chapter 4

Dengue Virus Type 2 Interactions with *Aedes aegypti* Midgut Serine Proteases

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Hypothesis

The objective of this research aim was to investigate the importance of midgut serine proteases during the course of a DENV-2 infection in *Aedes aegypti*. I hypothesized that suppression of one or more of these midgut serine proteases would increase DENV-2 infectivity in *A. aegypti*.

1. Introduction

Dengue viruses (DENV) have re-emerged in the last 30 years as a serious public health threat in many tropical and sub-tropical regions of the world. Currently, there are 50-100 million cases of dengue fever worldwide with an estimated 3 billion people at risk (Gubler, 1996, Monath, 1994). The primary vector for DENV is *Aedes aegypti* is highly anthropophilic and endophagic making it an ideal vector for maintenance of the transmission cycle (Nelson, 1986, Tinker, 1964).

The intrinsic ability of *A. aegypti* to become infected, permit replication and eventually transmit DENV to the next human is referred to as vector competence (Hardy, 1988). The phenotypic and genotypic traits which regulate complex interactions between virus and vector are not well understood.

During the initial stages of infection of an arthropod, arboviruses are gut-associated. It has been well documented that gut-associated viruses, i.e. rotaviruses, utilize the proteolytic enzymes of the gut for viral enhancement of infection (Arias et al., 1996, Espejo et al., 1981, Estes et al., 1981). Similarly, previous studies have found evidence suggesting that La Crosse virus (LACV) and blue tongue virus (BTV) utilize

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vector midgut proteases for proteolytic processing in order to increase viral infectivity (Ludwig et al., 1989, Ludwig et al., 1991, Mertens et al., 1996).

Likewise, in the case of DENV-2 and *A. aegypti*, there have been studies suggesting that midgut proteases condition vector competence (Black et al., 2002). Genetic mapping experiments have identified multiple quantitative trait loci (QTL) that control vector competence at the midgut infection barrier (MIB), two of which are *early trypsin* and *abundant trypsin* (Bennett et al., 2005b, Bosio et al., 2000). Furthermore, *in vivo* studies, in which Soybean Trypsin Inhibitor (STI) was fed in the presence of an infectious DENV-2 bloodmeal, demonstrated that suppression of abundant trypsin accumulation significantly reduced DENV-2 midgut titers and delayed viral dissemination (Molina-Cruz et al., 2005).

Serine proteases, which include trypsins and chymotrypsins, are a diverse class of proteolytic enzymes designated by their Asp-His-Ser catalytic triad (Matthews et al., 1967). To date, there have been four midgut serine proteases identified in *A. aegypti* which are integral during bloodmeal digestion. Trypsins account for the primary mechanism of bloodmeal digestion and are expressed in a biphasic manner (Felix et al., 1991). Early trypsin (ET), the principal enzyme during the early phase, is immediately translated from a pool of mRNAs and reaches maximal concentrations and activity by 3 hours post bloodmeal (hpbm) and then precipitously declines (Noriega et al., 1996a, Pennington et al., 1995). Additionally, an experiment utilizing STI suggested that early phase digestion acts as a signal to induce abundant trypsin (AT) transcription and translation (Barillas-Mury et al., 1995). AT appears 8 hpbm, reaches peak concentrations by 24 hpbm and has been presumed to account for the majority of endoproteolytic

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activity during digestion (Barillas-Mury et al., 1991, Graf et al., 1988, Graf et al., 1986). Recently, the regulatory mechanisms controlling AT have been questioned in light of the fact that the STI used in many of the previous experiments may have been contaminated (Lu et al., 2006). Furthermore, despite the robust expression levels of AT, evidence has arisen suggesting that AT is not a trypsin, although its specific function has yet to be determined (see Chapter V). Another serine protease of *A. aegypti*, late trypsin (LT) a.k.a. 5G1, has been cloned and sequenced. It displays similar mRNA expression patterns as AT, although its significance in digestion has yet to be characterized (Kalhok et al., 1993). Finally, chymotrypsin (CHYMO) was the most recent serine protease to be associated with bloodmeal digestion in *A. aegypti*. CHYMO possesses similar characteristics to ET in that it is transcribed prior to feeding and immediately translated upon bloodmeal ingestion. However, unlike ET, its mRNA, protein and protease activity levels remain high through the duration of digestion (Jiang et al., 1997).

In light of these findings, I initially characterized the involvement of ET and AT during the course of a DENV-2 infection. Subsequently, this analysis was expanded to include LT and CHYMO. Utilizing double stranded RNA (dsRNA) suppression techniques and a panel of trypsin inhibitors, I evaluated the effect of each gene product on the midgut infection (MIR) and dissemination rates (DR) of DENV-2 in *A. aegypti*.

2. Materials and Methods

Mosquito Rearing

The Rexville (RxD) strain of *Aedes aegypti*, originally colonized from Puerto Rico, was used for these experiments. The mosquitoes were maintained at a constant

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temperature of 28°C and a relative humidity of 80%. The photoperiod was set to a 14:10 light: dark cycle.

DENV-2 Production

The DENV-2 strain utilized for these experiments was isolated from a dengue fever patient in Jamaica in 1983 (Pierro et al., 2006). The virus has subsequently been passaged many times in C6/36 cells and is referred to as JAM1409hp. Briefly, C6/36 cells, which are an *Aedes albopictus* cell line, were infected at a multiplicity of infection (MOI) of 0.01. Subsequently, the medium was replaced 7 days post infection (dpi), harvested four days later and used for infectious bloodmeals.

RNA Extractions and cDNA Synthesis

Whole body RNA extractions were performed on pools of five unfed and five 24 hpbm fed female mosquitoes using TRIzol according to manufacturer's protocol (Invitrogen, Carlsbad, CA). The RNA was subsequently used for production of a cDNA pool with an oligo-d(t) primer and SuperScript II reverse transcriptase (RT)(Invitrogen).

Double Stranded RNA Synthesis

The genes of interest (GOI) were PCR amplified using gene specific primers designed to amplify ~500 b.p. fragments from the 3' region of each of the mRNA's. Each primer set, Table 4.1, included a T7 promoter sequence in both the forward and reverse primers. Products were amplified using the following settings; 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds

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followed by a final 10 minute extension step at 72°C. PCR products were amplified with a 2XMasterMix containing Taq polymerase (Invitrogen). The PCR fragments were purified via Qiagen's PCR purification kit, quantified with a spectrophotometer and subsequently used for dsRNA production (Qiagen, Valencia, CA).

dsRNA molecules were synthesized using the T7 Megascript kit (Ambion, Austin, TX) according to manufacturer's protocol. Samples were resuspended in 50 μl of phosphate-buffered saline (PBS), quantified and brought to a final concentration of 1 μg/μl in PBS. A fragment of the *Escherichia coli* β-galatosidase (B-gal) gene was used as the template to produce the control dsRNA (Keene et al., 2004), and the group denoted Mix was a mixture of 500 ng each of ET and AT dsRNA.

dsRNA Injections and Artificial Bloodmeals

Adult female mosquitoes 4-5 days post emergence were cold anesthetized and injected intrathoracically with ~500 ng of dsRNA. Following a three day recovery period mosquitoes were given an artificial bloodmeal containing a 1:1 ratio of JAM1409hp cell culture medium and defibrinated sheep blood. The infectious bloodmeal was placed in a water jacketed membrane feeder maintained at 37°C and mosquitoes were allowed to feed for 1 hour (Rutledge et al., 1964). The infectious bloodmeal titers ranged from 8 x 10^4 to 5.33 x 10^6 pfu/ ml for the dsRNA injection experiments and 5.33 x 10^4 to 1.4 x 10^6 pfu/ ml for trypsin inhibitor experiments. Following feeding mosquitoes were cold anesthetized and five unfed mosquitoes were collected and placed at -80°C for analysis of ET and CHYMO transcript levels. At 24 hpbm five more mosquitoes were collected and placed at -80°C for analysis of AT, LT and CHYMO transcript levels. At 7 dpi 30

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Primer Name	5'- Sequence - 3'	Position
T7-Bgal F	TAA TAC GAC TCA CTA TAG GGG TCG CCA GCG GCA CCG CGC GCC TTT C	————
T7-Bgal R	TAA TAC GAC TCA CTA TAG GGC CGG TAG CCA GCG CGG ATC ATC GG	————
T7-ET F	TAA TAC GAC TCA CTA TAG GGG CAT CAT AGT GAA AGT CAA ATC GG	Nucleotides 271-295
T7-ET R	TAA TAC GAC TCA CTA TAG GGC TCG GAA ACC TCT CGG ATC CAT TG	Nucleotides 736-759
T7-AT F	TAA TAC GAC TCA CTA TAG GGC ACC GCC ACG AGA AGT ACA ACC CAC	Nucleotides 307-331
T7-AT R	TAA TAC GAC TCA CTA TAG GGC CCT GCT CAC AGT CCA GTC TTC TGC	Nucleotides 756-774
T7-LT F	TAA TAC GAC TCA CTA TAG GGC GAA ATA CGA TGA AGT TAC CAC CGA AC	Nucleotides 345-369
T7-LT R	TAA TAC GAC TCA CTA TAG GGA ATC ATT TCA TTT AAG ACA TGC AGT TC	Nucleotides 817-843
T7-CHYMO F	TAA TAC GAC TCA CTA TAG GGA CAT CGT CCA CGA GGA CTA TCA AGG AGG	Nucleotides 324-350
T7-CHYMO R	TAA TAC GAC TCA CTA TAG GGA CGA CGT AAT GGG AAA CTC CGG CAA ACA C	Nucleotides 751-778
Actin QPCR F	CGC TCG TTG TCG ACA ATG G	————
Actin QPCR R	CAT ACC GAC CAT CAC ACC C	————
ET QPCR F	CCC AAA GCC AAC CAA CCT	Nucleotides -61 5' NCR
ET QPCR R	CGA CCC TCC GCA GAA ATG	Nucleotides 184-201
AT QPCR F	GTT CAC TTC AAC GGT GGT TTT C	Nucleotides 3-24
AT QPCR R	AGA ACT TGG AAT GGG AAC TGA C	Nucleotides 104-125
LT QPCR F	CTT CCC ACT TCT GTG GAG GAT C	Nucleotides 185-206
LT QPCR R	GAT GGC GGT TGA CCT TCT TAA C	Nucleotides 322-343
CHYMO QPCR F	GGT AGC TTT CCT GCT CGT TG	Nucleotides 18-37
CHYMO QPCR R	GTA TTG GAG TAT GCG GTC TTG	Nucleotides 222-242

Table 4.1: Primers used for dsRNA synthesis and Q-RT-PCR. The four genes used in these experiments have the following accession numbers: ET (NCBI accession #X64362), AT (NCBI accession #M77814), LT (NCBI accession #X64363) and CHYMO (NCBI accession #U56423). Underlined sequences correspond with the T7 promoter sequence. T7 primer sets were used for PCR amplification of genes used for dsRNA synthesis. The Q-RT-PCR primer sets were designed to amplify ~150bp fragment from each of the genes so as to not overlap with the dsRNA the regions used for dsRNA silencing of the genes.

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midguts from each dsRNA treatment group were collected and triturated with pellet pestles and a tissue grinder. The midguts homogenates were filtered through an Acrodisc 0.2 µm pore size Supor membrane syringe filter (Pall Life Sciences, Ann Arbor, MI). Samples were subsequently titrated on LLC-MK2 cells by plaque assay. Finally, at 14dpi paired midguts and heads were collected from 24 mosquitoes per group for indirect immunofluorescence assay (IFA) to determine dissemination rates (DR).

Q-RT-PCR

Total RNA was extracted in 500 µl of TRIzol from the five unfed and five 24 hpbm mosquitoes (Invitrogen). RNA was resuspended in 50 µl of nuclease free H_2O and quantified using a spectrophotometer. Individual sample RNA concentrations were standardized within experimental replicates and stored at -80°C until Q-RT-PCR analysis was performed.

Q-RT-PCR reactions were completed using an Opticon 2 Real-Time Thermocycler (Bio-Rad, Hercules, CA). Primers, in Table 1, were designed to amplify ~150 bp fragments from the 5' of the mRNA. Amplification products were chosen so as not to overlap with the dsRNA region used for silencing. Actin was chosen as the control gene. I used the Qiagen SYBR Green Quantitect Q-RT-PCR kit for analysis of each of the genes (Qiagen). Each sample was analyzed in duplicate with control and GOI primer sets. Reactions were run according to manufacturer's protocols, except reaction volumes were reduced to 20 µl. The reaction parameters were; 30 minute 50°C RT reaction followed by 15 minutes at 95°C and a 40 cycle PCR reaction with the following settings: 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 30 seconds and the plate was

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read at 76°C for 1 second. A final 10 minute extension period at 72°C was applied followed by melting curve analysis being read every 0.2°C/ second between 70°C-95°C.

Data from the Q-RT-PCR reaction were analyzed using the Opticon 3 software (Bio-Rad). Melting curves were assessed in order to confirm amplification of our target gene and threshold values were set at 0.02. The data were copied to a Microsoft Excel spreadsheet for analysis (Microsoft, Seattle, WA). Briefly, the $\Delta C(t)$ value was determined for each sample by averaging the experimental and control replicates. The average control value was subtracted from the average experimental value, and these values were then used to determine the statistical significance between the B-gal control group and experimental groups. The $\Delta\Delta C(t)$ values were then determined by subtracting the average $\Delta C(t)$ value of the B-gal group from the average $\Delta C(t)$ value of the experimental group. From this a normalized target value was determined and converted to percent decrease.

Activity Assays

Trypsin and chymotrypsin activity assays were performed using the colorimetric substrates N_α-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-BAPNA) and N-succinyl-Ala-Ala-Pro-Phe-P-nitroanilide (Suc-AAPF-pNA), respectively (Sigma-Aldrich St. Louis, MO). These reagents were resuspended at a concentration of 200mM in DMSO.

Three days after injection, mosquitoes were offered an artificial bloodmeal containing either 50% FBS, 40% PBS, 1mM ATP and a pinch of phenol red or defibrinated sheep blood. FBS meals were provided to experimental groups to be

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analyzed for early phase activity because hemoglobin in the blood routinely interfered with the absorbance readings. This did not seem to be a problem at the 24 hour time-point so whole blood was provided to those groups to be analyzed for late phase protease activity.

For analysis of early phase protease activity, one midgut equivalent was mixed with activity buffer (50 mM Tris-HCl and 10 mM $CaCl_2$, p.H. 7.0) and the respective colorimetric substrate and incubated at 37°C for 30 minutes. Analysis of late phase proteolytic activity was performed on $1/10^{th}$ of a midgut and reactions were incubated for 5 minutes at 37°C. Absorbance values were read at 405 nm. Each sample was tested in triplicate and each experiment was performed three times. Activity is reported as the percentage of activity relative to the B-gal dsRNA injected control groups.

Trypsin Inhibitors

The trypsin inhibitors used for these experiments were soy bean trypsin inhibitor (STI) (Fluka Biochemika), egg white trypsin inhibitor (EWTI) and a synthetic serine protease inhibitor 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (Sigma-Aldrich). Each sample was brought to a concentration of 1mM in PBS and diluted to 100μM in the bloodmeals. Trypsin activity, midgut infection rates (MIR) and DR were evaluated.

Indirect Immunofluorescence Assay

IFAs were performed according to the protocol outlined in Bennett *et. al.* (Bennett et al., 2002). Briefly, dissected midguts and heads were fixed in either 4%

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paraformaldehyde or acetone, respectively. Midguts were washed and stained in PBS+0.1% Triton X100, while PBS was used for the heads. Initial staining was carried out with the flavivirus E-glycoprotein specific murine monoclonal antibody, 3H5. The samples were washed and the secondary stain applied, which included biotinylated sheep anti-mouse antibody (Amersham, Arlington Heights, IL) and 0.005% Evans blue counter stain. Finally, samples were developed with streptavidin-fluorescein (Amersham) and visualized for fluorescence.

Statistical Analysis

Statistical analyses were carried out using SAS 9.1 (SAS Institute Inc., Cary, NC). Each data set was analyzed using analysis of variance (ANOVA) in which the proc mixed procedure was applied. A randomized block design was fit where the three replicates composed the random block effect and the groups were the fixed effect.

3. Results

dsRNA silencing of the four target midgut serine proteases was assayed through reduction in mRNA transcript levels as determined by Q-RT-PCR as well as protease specific activity assays. ET transcript levels were decreased by 98.7% ($p<0.0001$), which reduced early phase tryptic activity to 18% of controls ($p<0.0021$) (Figure 4.1 A & B). Furthermore, reduction of ET transcript levels had no effect on AT transcript levels as previously reported (Figure 4.2 A) (Lu et al., 2006). Similar levels of ET transcript reduction were observed for the Mix group despite containing half the amount of injected ET dsRNA (97.5%, $p<0.001$) (Figure 4.1A). Suppression of ET had no effect on late

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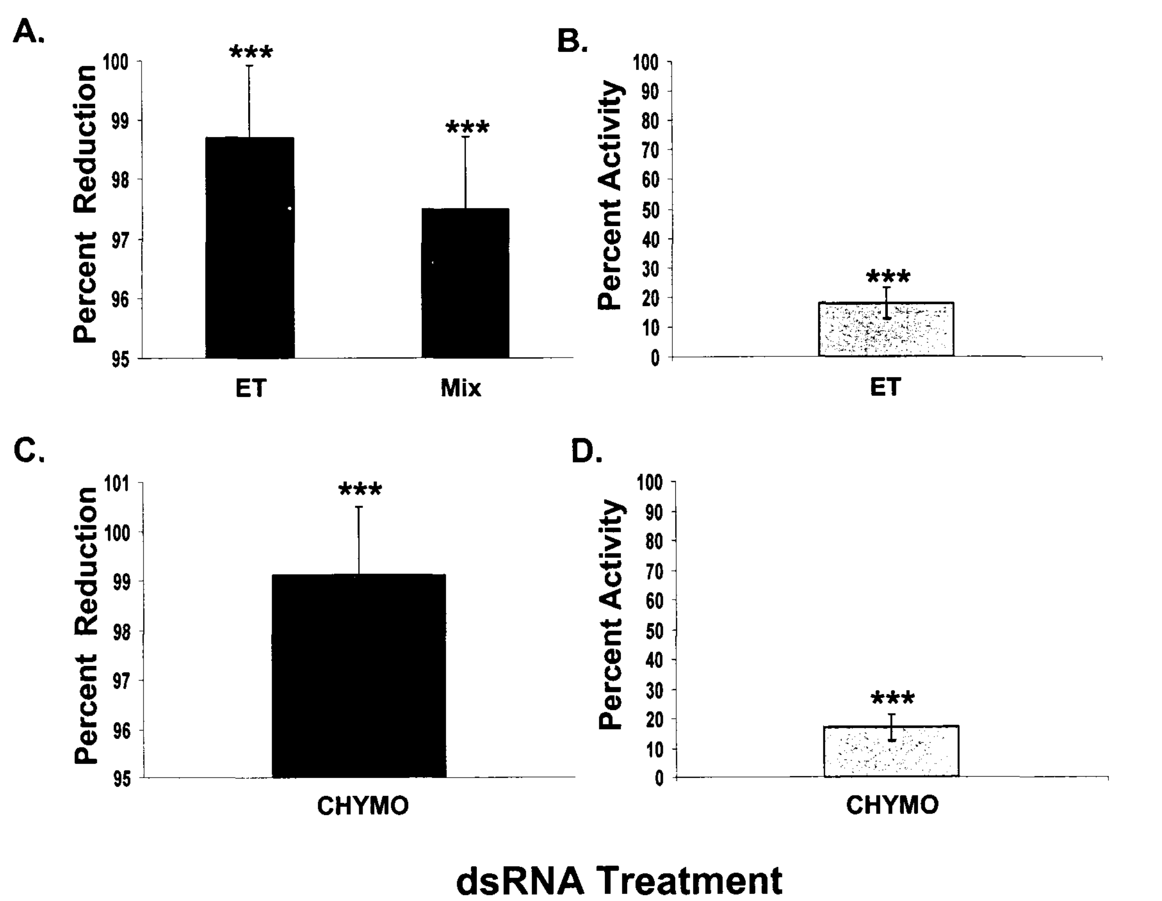

Figure 4.1: The effects of dsRNA silencing on ET and CHYMO transcript levels and enzymatic activity. The values are plotted relative to the B-gal control group. (*) indicate groups significantly different from the B-gal injected controls. Percent reduction in respective transcript levels as determined by Q-RT-PCR on unfed mosquitoes and percent enzymatic activity in mosquitoes 2 hpbm. (A) ET transcript levels and (B) early phase tryptic activity after injection with ET or Mix dsRNA. (C) CHYMO transcript levels and (D) early phase chymotryptic activity after injection with CHYMO dsRNA.

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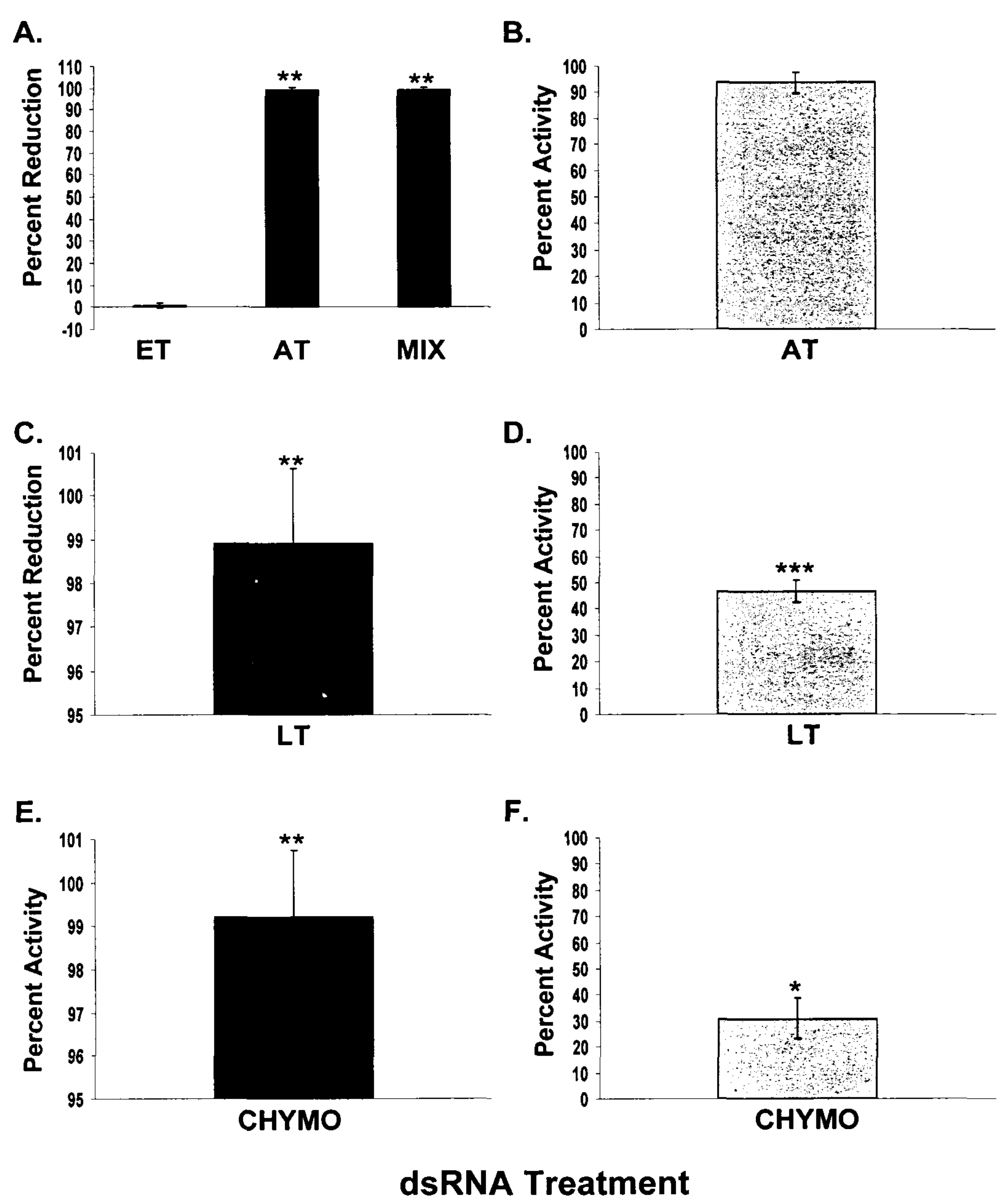

Figure 4.2: The effects of dsRNA silencing on AT, LT and CHYMO transcript levels and enzymatic activity 24 hpbm. The values are plotted relative to the B-gal control group. (*) indicate statistically significant differences compared to the B-gal control group. Percent reduction in respective transcript levels as determined by Q-RT-PCR and percent enzymatic activity in mosquitoes 24 hpbm. (A) AT transcript levels and (B) late phase tryptic activity after injection with either ET, AT or Mix dsRNA. (C) LT transcript levels and (D) late phase tryptic activity after injection with LT. (E) CHYMO transcript levels and (F) late phase chymotryptic activity after injection with CHYMO dsRNA.

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phase tryptic activity, as expected (data not shown).

A significant reduction in AT transcript levels was recorded in both the AT (99.4%, $p<0.001$) and Mix (99.3%, $p<0.001$) groups, as expected (Figure 4.2A). Despite near complete ablation of AT transcript levels in mosquitoes 24 hpbm, these results had no effect on late phase tryptic activity (Figure 4.2 B).

The effect of dsRNA suppression of CHYMO transcript levels was measured in unfed and 24 hpbm mosquitoes. I determined that a reduction in CHYMO transcript levels of 99.1% (p,0.0007) in unfed mosquitoes corresponded to a 83% decrease in chymotrypsin activity during early phase digestion ($p<0.0013$) (Figure 4.1 C & D). During late phase digestion depletion of CHYMO transcripts were on the order of 99.2% ($p<0.0015$) and resulted in late phase chymotryptic activity levels 31% of the control levels ($p<0.0117$) (Figures 4.2 E & F).

Finally, the depletion of LT mRNA correlated with a 98.9% reduction ($p<0.0038$) in transcript levels in mosquitoes 24 hpbm (Figure 4.2 C). Surprisingly, nearly 40% ($p<0.0001$) of late phase tryptic activity remained despite the significant levels of reduction observed at the transcript level (Figure 4.2 D).

Early and late phase tryptic activity was evaluated for each of the three trypsin inhibitors. I was able to significantly reduce trypsin activity in the presence of STI ($p<0.0004$) and AEBSF ($p<0.0064$) during the first two hours of digestion, but by 24 hpbm, the inhibitory effects had become negligible (data not shown). Interestingly, the presence of EWTI ($p<0.7862$) had no discernable effect on early or late phase tryptic activity (Figure 4.3).

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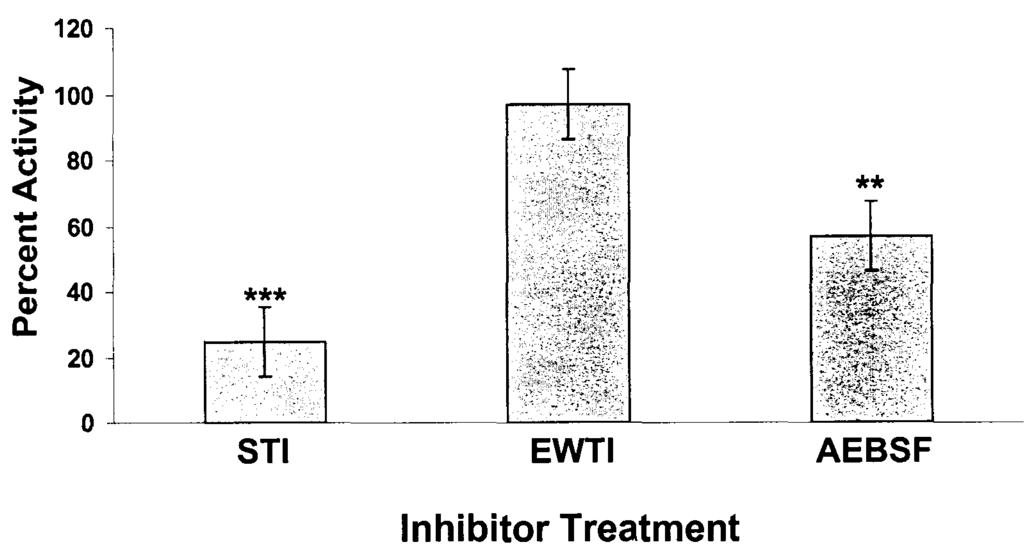

Figure 4.3: The effects of trypsin inhibitors on early phase tryptic digestion in *A. aegypti*. Mosquitoes were fed either STI, EWTI, AEBSF or an equal volume of PBS, which acted as the control. Mosquitoes were evaluated for early phase tryptic activity 2 hpbm. Values are reported as percent enzymatic activity compared to the PBS fed control group. (*) indicate a significant reduction in tryptic activity.

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Upon confirmation of transcript suppression, MIR and DR were assessed for each of the experimental groups. It was determined that suppression of ET or AT had no effect on MIR or the intensity of infection as determined by plaque titration (Figure 4.4 A and 4.4 C, respectively). These experiments were then expanded to include LT and CHYMO and again suppression of these genes had no significant effect on DENV-2 MIR and titers (Figure 4.4 B and 4.4 D, respectively). It was observed that suppression of LT resulted in higher MIR, although due to large variability in MIR from experiment to experiment, this result was only significant when a one-tailed T-test was applied ($p<0.03905$). Similarly, suppression of each of the GOI had no effect on DR (Figure 4.4 E & F).

The effects of the exogenously-supplied trypsin inhibitors on DENV-2 infection rates were studied in order to complement my dsRNA suppression results. Furthermore, it allowed me to repeat previous experiments in which STI was used and expand upon the repertoire of inhibitors tested. Co-feeding DENV-2 with STI ($p<0.0023$) and EWTI ($p<0.0114$) significantly increased MIR, while AEBSF ($p<0.1897$) had no effect, as determined by IFA (Figure 4.5 A). Despite the increased percentage of midguts infected, I found no effect on DR (Figure 4.5 B).

4. Discussion

Arthropods acquire an arbovirus infection through acquisition of a bloodmeal from an infected vertebrate host. The bloodmeal and virus are then transported to the highly proteolytic environment of the midgut for degradation. To this end, arboviruses may have had to adapt to and possibly take advantage of this proteolytic environment. In

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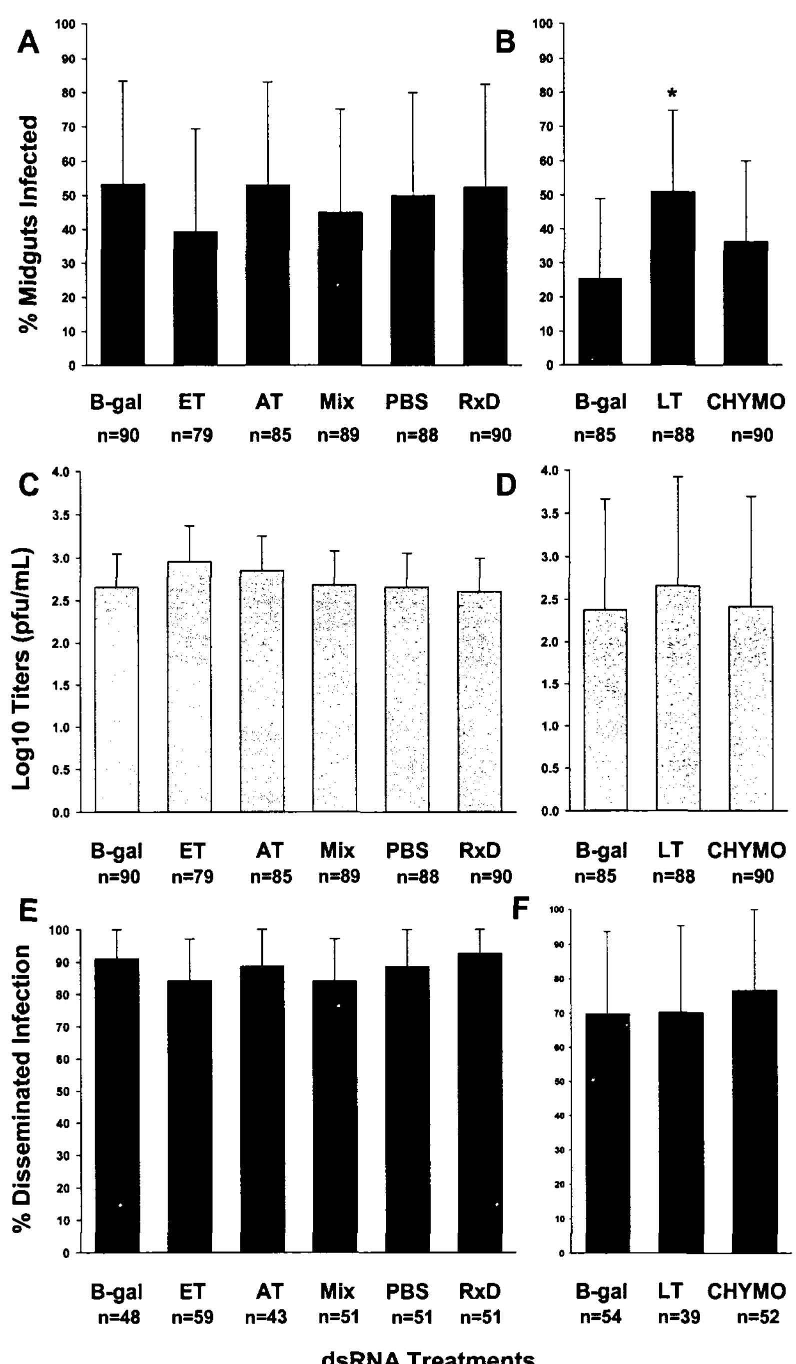

Figure 4.4: The effects of dsRNA suppression of midgut serine proteases on DENV-2 infectivity. (A & B) Midgut infection rates 7 dpi as determined by plaque titrations, (C & D) titers of DENV-2 in midguts 7 dpi and (E & F) DENV-2 dissemination rates 14 dpi. B-gal values can be found in duplicate because experiments on the right were performed at a later time-point compared to the corresponding graph to the left. Sample size values under (E & F) correspond to the number of IFA positive midguts per each group analyzed. (*) Value is not significant by the standard two-tailed T-test, but is significant with a one-tailed T-test.

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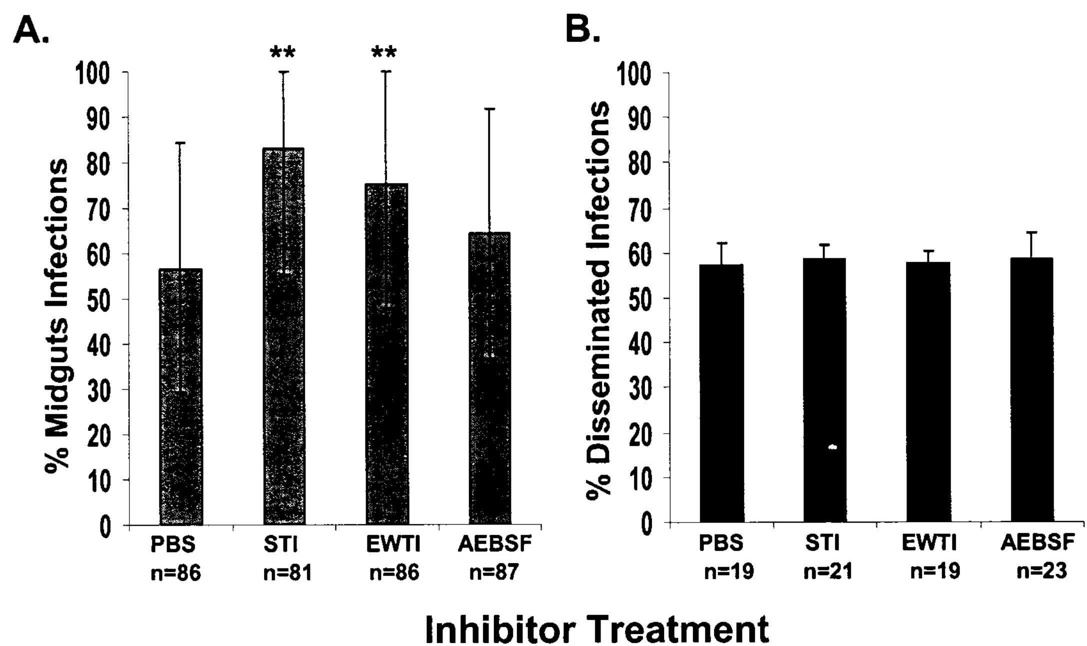

Figure 4.5: The effects of trypsin inhibitors on DENV-2 infectivity. (A) Midgut infection rates of DENV-2 7 dpi and (B) dissemination rates 14 dpi as determined by IFA. Sample size values under (B) correspond to the number of IFA positive midguts per each group analyzed. (*) indicate that the experimental groups were significantly different than the PBS control group.

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fact, the involvement of midgut serine proteases in arbovirus infectivity of vectors has been implicated on numerous occasions (Bennett et al., 2005a, Bosio et al., 2000, Ludwig et al., 1989, Mertens et al., 1996, Molina-Cruz et al., 2005). This study was performed in order to determine if specific midgut serine proteases are required for DENV-2 to effectively infect *Aedes aegypti.*

The use of dsRNA suppression allowed for highly specific analysis of each of the four identified midgut serine proteases. Suppression levels for each of the genes were determined at the timepoints corresponding to their peak levels of expression as previously reported (Barillas-Mury et al., 1991, Jiang et al., 1997, Kalhok et al., 1993, Noriega et al., 1996a, Pennington et al., 1995). The very potent and specific suppression of ET had no effect on AT transcript levels or late phase tryptic activity, validating data by Lu *et. al.* that challenged previous data suggesting a link between ET activity and AT transcription (Figure 4.2) (Lu et al., 2006). Although early phase tryptic activity was not ablated, but only reduced by 82% in these experiments (Figure 4.1), it could be argued that the remaining 18% of ET activity was enough to activate AT transcription. However, addition of di-isopropyl-fluorophosphate (a potent serine protease inhibitor) during trypsin activity assays of dsRNA ET treated mosquitoes did not further suppress tryptic activity (Lu et al., 2006). These results suggested that dsRNA suppression of ET was sufficient to reduce almost all early phase serine protease hydrolysis of DL-BAPNA.

The levels of AT suppression and late phase tryptic activity were analyzed by Q-RT-PCR and DL-BAPNA assays, respectively. Surprisingly, despite the highly significant reduction in AT transcript levels as recorded by Q-RT-PCR there was no reduction in late phase tryptic activity (Figure 4.2). These results imply that either 1)

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redundancy exists within late phase tryptic enzymes thereby masking the effects of AT suppression, or 2) AT is not a trypsin. One may exclude the first explanation, because suppression of LT, another late phase tryptic enzyme, resulted in a 60% reduction in late phase tryptic activity (Figure 4.2). Furthermore, alignment data showed that AT lacks the critical Asp 189 residue commonly associated with trypsins (Kalhok et al., 1993). The original authors concluded that AT may be a unique variant of the classical trypsin archetype, but our data suggest otherwise (Barillas-Mury et al., 1991). Further studies should address the identity and function of this protein. It is highly up-regulated 24 hpbm and is closely related to trypsins in amino acid sequence, yet it lacks tryptic activity. As previously stated, suppression of LT coincided with a significant decrease in transcript levels as well as late phase tryptic activity. However, there was still 40% activity compared to controls, suggesting that there may be other active trypsins during late phase digestion which remain to be identified.

I observed that the presence of 100 μM STI and AEBSF significantly reduced early phase tryptic activity (Figure 4.3). Previously, it was demonstrated that each of these compounds could maintain a level of 50% suppression for up to 18 hpbm (Lu et al., 2006). However, my data showed that by 24 hpbm the inhibitory effects of STI and AEBSF had become negligible (data not shown). Interestingly, the presence of EWTI had no inhibitory effects during early or late phase digestion, which is consistent with a previous report (Shahabuddin et al., 1995), but others have found that EWTI can inhibit trypsin activity as effectively as STI or AEBSF (Lu et al., 2006). This discrepancy might be explained by differences in the source and/ or purity of this compound.

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I found that suppression of each of my target genes resulted in no discernible effects on the ability of DENV-2 to infect the midgut or disseminate to secondary tissues. In fact, suppression of LT increased the MIR in each of the three replicates, but these results were not significant considering the large degree of variability of MIR between experiments (Figure 4.4).

QTL mapping data suggests that several regions on chromosome II that include the linkage map marker genes *early trypsin* and *abundant trypsin,* are associated with midgut infection rates with DENV-2 (Bennett et al., 2005b, Bosio et al., 2000). These regions can be many centimorgans in length and encode hundreds of genes. In contrast to previous data suggesting otherwise, my data shows that the *early* and *abundant trypsjn* genes in these QTLs do not by themselves control the DENV-2 MIB phenotype. Thus, it is likely that other genes chromosomally positioned near *early* and/ or *abundant trypsin* are the true genes involved in this phenotype.

Parallel to the dsRNA suppression experiments, I evaluated whether multiple trypsin inhibitors would affect DENV-2 infectivity of *A. aegypti.* In these experiments STI, EWTI and AEBSF were tested independently for their effects on MIR and DR. I observed that AEBSF had no effect on DENV-2 MIR or DR, but that EWTI and STI significantly increased MIR. STI and EWTI did not differ from the control group in DR (Figure 4.5).

The differences observed in MIR between the gene silencing and trypsin inhibitor experiments may be the result of redundancy within mosquito digestion. dsRNA suppression specifically targets individual genes, but the trypsin inhibitors target all trypsins in the midgut. Considering that numerous trypsins have been identified,

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suppression of one may not have a significant effect on DENV-2 infections, but inhibition of all of the trypsins may significantly alter the outcome of the infection.

These results for the effects of STI on DENV-2 infectivity greatly differ from those previously reported. Molina-Cruz *et. al.* reported a significant decrease in vRNA and E-glycoprotein accumulation in midguts 7 days post feeding in the presence of STI. Furthermore, they noticed delayed dissemination kinetics compared to controls (Molina-Cruz et al., 2005). The discrepancy observed between these and the current experiments might be explained by the purity of the STI used. Recently, researchers have demonstrated that the STI used in previous experiments designed to investigate the signal transduction cascade of AT may have been contaminated (Lu et al., 2006). Furthermore, this contaminant may have had global physiological effects, resulting in reduced AT transcription, peritrophic matrix 1 (PM1) formation and oviposition rates, as well as expulsion of the bloodmeal. Considering that Molina-Cruz *et. al.* used these very same parameters to demonstrate trypsin activity reductions, I speculate that the STI used in their experiments may also have been contaminated. It is difficult to conclude whether the results they presented were a consequence of global physiological changes as a result of a contaminant or a reduction in early phase tryptic activity.

The DL-BAPNA colorimetric assay was used as a means to measure trypsin activity, and the STI used in these experiments could effectively inhibit trypsin activity. Furthermore, the STI had lost its inhibitory effect by 24 hpbm and there was no noticeable reduction in oviposition rates (data not shown). This evidence suggests that the STI used in my experiments did not contain a contaminant with global physiological

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effects. Therefore, these results appear to more accurately depict the true nature of trypsin inhibition on DENV-2 infectivity.

My results suggest that serine proteases do not proteolytically activate the virus, but rather that proteolytic activity within the midgut actually limits virus infectivity. During an infectious bloodmeal viable viral particles are introduced into the midgut and will either infect the mosquito, be degraded or expelled in the feces. Some speculate that the fate of the virus is determined within the first hour of infection prior to PM1 formation, which begins to form 5 hpbm (Perrone & Spielman, 1988), or accumulation of digestive enzymes. My data suggest that this may not be the case. I found that LT, which doesn't begin to be expressed until ~8 hpbm, had the greatest effect on DENV-2 MIR. One can envision that during the early periods of LT expression, virus particles, remaining in the bloodmeal retain the ability to infect the vector. Upon secretion of LT, these residual particles are degraded, thereby reducing the possibility of the midgut eventually being infected.

The literature lends support to both hypotheses. Researchers have found that western equine encephalitis virus (WEEV), a member of the family *Togaviridae*, infects the midgut of *Culex tarsalis* within 1-2 hpbm prior to PM1 formation or accumulation of digestive enzymes (Houk et al., 1985). However, another study found that St. Louis encephalitis virus (SLEV), which is a flavivirus like DENV, can persist in the midgut of *Culex pipiens* for up to 8 hours before infecting the midgut epithelium (Whitfield et al., 1973). These two contrasting studies highlight the fact that each virus/ mosquito system is unique and the rate at which viruses infect the midgut is dependent upon the virus and mosquito species (Hardy et al., 1983).

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The results presented in this chapter more completely describe the role of serine proteases during a DENV-2 infection of *Aedes aegypti*, but much remains unknown about *A. aegypti* vector competence. The results also emphasize our poor understanding of the basic physiology of mosquito digestion, specifically midgut proteases and signify the importance of future research on this topic.

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Chapter 5

Comparative Genomics of *Aedes aegypti* Midgut Serine Proteases

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Hypothesis

The objective of this aim was to better characterize the role of each of the four identified *Aedes aegypti* midgut serine proteases, particularly abundant trypsin (AT). I hypothesized that, in light of some recent evidence, AT is not a trypsin and may be providing some other function in bloodmeal digestion.

1. Introduction

Serine proteases are a diverse group of proteolytic enzymes and have been identified in all kingdoms, as well as viruses. This diversity is illustrated by their involvement in many physiological processes (Hedstrom, 2002). Serine proteases are characterized by their Asn-His-Ser catalytic triad and are appropriately named due to the presence of a hallmark nucleophilic Ser residue at the active site (Blow, 1997). Despite the highly conserved catalytic triad residues, phylogenetic analysis revealed that there are four distinct families within this clan of proteases (Dodson & Wlodawer, 1998). Of particular interest is the chymotrypsin subfamily of serine proteases, which includes trypsins, chymotrypsins, elastases and a few recently identified serine collagenases. These enzymes have the same basic three dimensional structures and are arranged as two six-stranded beta barrels and contain catalytic, substrate recognition and zymogen activation domains (Blow, 1971).

Alignment data have revealed that each of these enzymes has specific amino acids that are highly conserved and are critical in determining substrate specificity. Trypsins preferentially cleave basic residues Lys/Arg and are classified by the presence of an Asp residue at position 189. Conversely, chymotrypsins have a Ser at position 189 that allows

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the binding pocket to open up and accept large aromatic substrates, such as Phe/Tyr/Trp. On the other hand elastases are characterized by the presence of a Val and Thr at positions 216 and 226, respectively (Hedstrom, 2002). Due to the small number of identified serine collagenases, little is known about their binding pockets or substrate specificities.

The acquisition of a bloodmeal by adult female *Aedes aegypti* mosquitoes is critical for many processes. The nutrients provided in the blood are used for energy production, egg maturation and as a source of replenishment of maternal reserves (Zhou et al., 2004). Digestion of the proteins found in the blood involves a plethora of exo- and endo-proteolytic enzymes (Pennington & Wells, 2005). The principal luminal enzymes responsible for much of this degradation are endoproteolytic serine proteases. Of these, trypsins account for the majority of proteolytic activity during bloodmeal digestion. To date, four serine proteases have been identified in the midgut of *A. aegypti.*

Midgut trypsins have a unique biphasic secretion pattern (Felix et al., 1991). Early phase tryptic expression, which occurs within the first few hours following a bloodmeal, is characterized by the secretion of one enzyme, early trypsin (ET). ET is transcribed within the first 24 hours post emergence and remains untranslated in intracellular pools, until a bloodmeal is imbibed (Noriega et al., 1996a). ET transcription is regulated by juvenile hormone, but the regulatory mechanisms influencing ET mRNA stability and its eventual translation have not been elucidated (Noriega et al., 1997, Noriega & Wells, 1999). Upon induction of translation ET is released into the lumen of the midgut where it reaches peak concentrations of 300 ng/ midgut by 3 hours post bloodmeal (hpbm) (Pennington et al., 1995). Levels precipitously decline and by 8 hpbm

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can no longer be detected. Interestingly, little protein degradation occurs during this initial phase of digestion and the exact function of ET is not well understood. Initially, it was believed that ET was involved in the transcriptional activation of abundant trypsin (AT), but recent evidence suggests that this is not the case (Barillas-Mury et al., 1995, Lu et al., 2006).

Characterization of late phase tryptic digestion has reveled that AT is the predominant proteolytic enzyme from 8-36 hpbm (Graf et al., 1986). It is secreted by the posterior midgut and reaches a maximal concentration of 5-6 μg/ midgut 24 hpbm (Graf et al., 1988). Recently, its classification as a trypsin has been questioned. Alignment analysis of AT revealed that it has a Ser at position 189, which is typical of chymotrypsins (Kalhok et al., 1993). Furthermore, initial purification of AT, which eventually led to cloning and sequencing of the gene, was performed with an anion exchange column that was not specific for trypsins (Barillas-Mury et al., 1991, Graf & Briegel, 1985).

The midgut also expresses a chymotrypsin (CHYMO) during bloodmeal digestion. Similar to ET, it is transcribed post-emergence and is immediately translated following bloodmeal uptake. Unlike ET, its transcript and protein levels remain high through the duration of bloodmeal digestion and chymotryptic activity can be detected from 2-36 hpbm. Peak levels of protein expression occur 24 hpbm and reach a maximal concentration of 300 ng/ midgut (Jiang et al., 1997).

Finally, another late phase trypsin gene has been cloned and sequenced. Late trypsin (LT), formerly designated 5G1, is significantly up-regulated following a bloodmeal and maximal transcript levels can be found 24 hpbm. Furthermore,

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sequencing data revealed that, unlike AT, it contains an Asp at amino acid 189, which is characteristic of trypsins (Kalhok et al., 1993). Despite the sequencing and expression data, its significance in digestion has yet to be characterized.

Phylogenetic analysis, sequence alignments, and dsRNA suppression were used to better characterize the function of each of the four identified *A. aegypti* serine proteases in bloodmeal digestion. Based on the findings a new uniform and descriptive classification scheme is proposed.

Materials and Methods

Amino Acid Sequence Alignments and Phylogenetic Analysis

The sequences used for the alignments were taken from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein&itool=toolbar). Some recently cloned *A. aegypti* serine protease sequences used in the analysis were generously provided by Dr. Jun Isoe (University of Arizona). The species and protein names along with their NCBI accession numbers are reported in the alignment figure caption. Multiple sequence alignments were performed using the ClustalW program (http://align.genome.jp/). Initially, all serine protease sequences were analyzed using a distance matrix and neighbor-joining algorithm in which the Jones-Taylor-Thornton model was tested (Jones et al., 1992). The sequences were not manually aligned before this analysis and leader sequences were included in the test. Subsequently, the mature peptide sequences for each of the branches were manually aligned, with bovine chymotrypsin as the template, and a maximum likelihood analysis was performed using the Jones-Taylor-Thornton model (Jones et al., 1992). Phylogenetic analysis using these

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sequences was performed with PHYLIP version 3.67 (Felsenstein, 1996). Bootstrap support was evaluated based on 1,000 replicates. These phylogenetic analyses were performed with the considerable assistance of Dr. William Black IV.

Mosquito Rearing

Adult female *Aedes aegypti*, strain Rexville D (RxD), were used for all of the experiments. They were maintained at a constant temperature of 28°C with a relative humidity of 80% and a 14:10 light: dark photoperiod.

Double Stranded RNA Synthesis

Each of the genes tested in this chapter was amplified from one of two cDNA pools. The first pool was made from whole body RNA extracted from adult female mosquitoes 4 days post emergence using TRIzol according to manufacturer's instructions (Invitogen, Carlsbad, CA). The second pool was constructed from whole body RNA extracted from adult females 24 hpbm. For each of the genes tested in these experiments I designed primer sets that would amplify ~500 bp fragments from the 3'region of each of the mRNAs. All primers included a T7 promoter sequence for double stranded RNA (dsRNA) production. Primer sets are listed in Table 5.1. Products were amplified by PCR using the following settings; 95°C for 5 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds followed by a final 10 minute extension step at 72°C. PCR products were amplified with a 2XMasterMix containing Taq polymerase (Invitrogen). The PCR fragments were purified using Qiagen's PCR

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Primer Name	5'- Sequence - 3'	Position
T7-Bgal F	TAA TAC GAC TCA CTA TAG GGG TCG CCA GCG GCA CCG CGC GCC TTT C	————
T7-Bgal R	TAA TAC GAC TCA CTA TAG GGC CGG TAG CCA GCG CGG ATC ATC GG	————
T7-ET F	TAA TAC GAC TCA CTA TAG GGG CAT CAT AGT GAA AGT CAA ATC GG	Nucleotides 271-295
T7-ET R	TAA TAC GAC TCA CTA TAG GGC TCG GAA ACC TCT CGG ATC CAT TG	Nucleotides 736-759
T7-AT F	TAA TAC GAC TCA CTA TAG GGC ACC GCC ACG AGA AGT ACA ACC CAC	Nucleotides 307-331
T7-AT R	TAA TAC GAC TCA CTA TAG GGC CCT GCT CAC AGT CCA GTC TTC TGC	Nucleotides 756-774
T7-LT F	TAA TAC GAC TCA CTA TAG GGC GAA ATA CGA TGA AGT TAC CAC CGA AC	Nucleotides 345-369
T7-LT R	TAA TAC GAC TCA CTA TAG GGA ATC ATT TCA TTT AAG ACA TGC AGT TC	Nucleotides 817-843
T7-CHYMO F	TAA TAC GAC TCA CTA TAG GGA CAT CGT CCA CGA GGA CTA TCA AGG AGG	Nucleotides 324-350
T7-CHYMO R	TAA TAC GAC TCA CTA TAG GGA CGA CGT AAT GGG AAA CTC CGG CAA ACA C	Nucleotides 751-778
T7-CxTry Hom F	TAA TAC GAC TCA CTA TAG GGG TGC GTG TCG GTT CCA GCC AGC ATG	Nucleotides 268-292
T7-CxTry Hom R	TAA TAC GAC TCA CTA TAG GGC TCC ACT GAC TTC GGC CAC CCA ATC	Nucleotides 765-790

Table 5.1: Primers used for dsRNA synthesis. This table is slightly modified from Table 4.1 and includes the names, sequence and locations of the primers used. Underlined sequences correspond with the T7 promoter sequence. The CxTry Homolog is from an uncharacterized midgut serine protease sequence of interest provided by Dr. Jun Isoe.

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purification kit, quantified and subsequently used for dsRNA production (Qiagen, Valencia, CA).

dsRNA molecules were synthesized using the T7 Megascript kit (Ambion, Austin, TX) according to manufacturer's protocol. Samples were resuspended in 50 μl of phosphate-buffered saline (PBS), quantified and brought to a final concentration of 1 μg/μl in PBS. A fragment of the *Escherichia coli* β-galatosidase (B-gal) gene was used as the template to produce the control dsRNA in all of the experiments (Keene et al., 2004).

dsRNA Injections and Artificial Bloodmeals

Adult female Rx D mosquitoes 4-5 days post emergence were cold anesthetized and injected with ~500 ng of dsRNA. Following a three day recovery period, mosquitoes were offered an artificial bloodmeal containing either 50% FBS, 40% PBS, 1mM ATP and a pinch of phenol red or defibrinated sheep blood. FBS meals were offered to the experimental groups that were to be analyzed for early phase activity because hemoglobin in the blood routinely interfered with the absorbance readings. This did not seem to be a problem at the 24 hour time-point so whole blood was provided to those groups to be analyzed for late phase activity. Upon completion of feeding, nine mosquitoes per group were individually placed in separate cartons with oviposition cups and five days later eggs were counted.

Activity Assays

Trypsin, chymotrypsin and elastase activity assays were performed using the colorimetric substrates N_{α}-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (DL-

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BAPNA), N-succinyl-Ala-Ala-Pro-Phe-P-nitroanilide (Suc-AAPF-pNA) and N-succinyl-Ala-Ala-Ala-P-nitroanilide, respectively (Sigma-Aldrich St. Louis, MO). These reagents were resuspended at a concentration of 200mM in DMSO. For analysis of early phase activity, one midgut equivalent was mixed with activity buffer (50 mM Tris-HCl and 10 mM $CaCl_2$, p.H. 7.0) and the respective colorimetric substrate and allowed to incubate at 37°C for 30 minutes. Analysis of late phase proteolytic activity was performed on $1/10^{th}$ of a midgut and reactions were incubated for 5 minutes at 37°C. Absorbance values were determined using a plate reader at 405 nm. Each sample was tested in triplicate and each experiment was performed three times. Activity was reported as percentage of activity relative to the B-gal injected control groups.

Abundant Trypsin Western Blot

Western blotting analysis of dsRNA suppression of AT was performed with the XCell Sure Lock Minigel system and NuPAGE reagents according to manufacturer's instructions (Invitrogen). Samples were collected from homogenized midguts 24 hpbm, denatured and equal volumes loaded onto a 10% Bis-Tris gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% non-fat powdered milk in phosphate buffered saline with 0.1% Tween-20 (PBST) for 2 hours at room temperature (RT). The membrane was washed twice with PBST for 10 minutes and incubated with the primary antibody, diluted 1:500 in blocking buffer, overnight at 4°C. Rabbit α-AT originally produced by Dr. Carolina Barillas-Mury in 1995 was generously provided by Dr. Irma Sanchez-Vargas (Barillas-Mury et al., 1995). Membranes were subsequently washed twice with PBST and probed with a horseradish peroxidase (HRP) conjugated goat α-

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rabbit secondary antibody (ZyMax, San Francisco, CA), diluted 1:5000 in blocking buffer, for 2 hours at RT, washed, developed with the ECL Plus Western Blotting Detection Kit (Amersham, Arlington Heights, IL) and visualized with a StormImager (GMI Inc., Ramsey, MN).

Statistical Analysis

Statistical analysis of proteolytic activity and oogenesis was performed using SAS 9.1 (SAS Institute Inc., Cary, NC). Each data set was analyzed using a Type-III analysis of variance (ANOVA), which allowed me to determine which variables were fixed and which were random.

Results

A total of 32 insect serine proteases, along with bovine chymotrypsin and crab collagenase, were used for the phylogenetic analysis. The analysis included 31 Dipteran and 1 Lepidopteran sequences from the following insect families; 1 Sphingidae, 1 Drosophilidae, 2 Brachycera, 1 Simuliidae, 1 Hypodermatidae and 25 Culicidae. Some of the samples labeled *A.a.*Tryp I-XV were not complete sequences. The relationship of these serine proteases was analyzed with a distance matrix and statistical confidence evaluated by bootstrap analysis. This initial analysis revealed that the insect serine proteases consistently segregated into three branches (Figure 5.1).

Branch A consisted of many well characterized trypsins from multiple species and has a bootstrap support of 73% (Figure 5.1). As the branch length extended along the cluster the trypsins diverge in accordance with the established phylogenetic relationships

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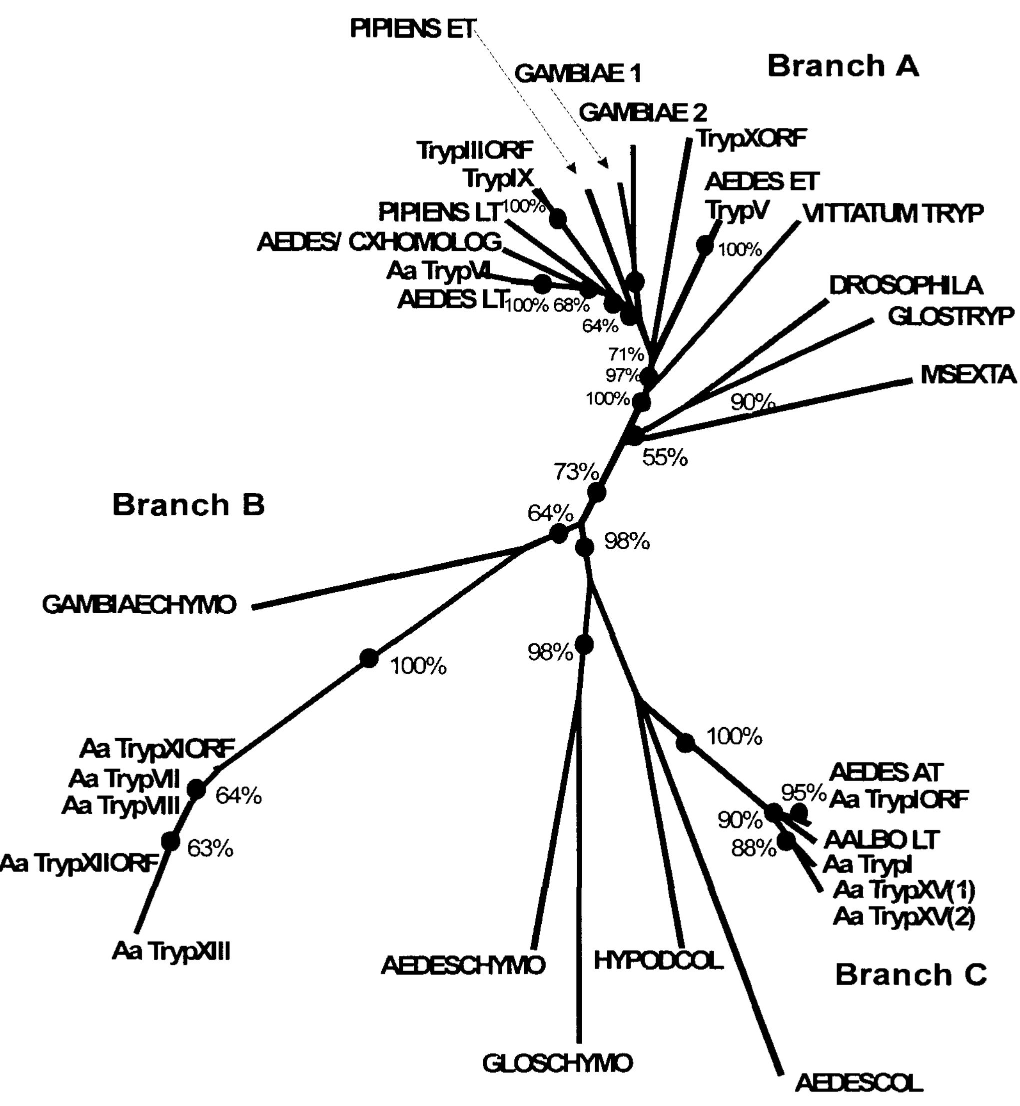

Figure 5.1: Distance/ Neighbor Joining analysis of insect serine proteases. This analysis was conducted with mature peptide and leader sequences. *A. aegypti* ET (X64362); *A.a.* LT (X64363); recently cloned *Aedes/ Cx* Homolog and *A.a.* sequences Tryp III ORF, TrypV, Tryp VI, Tryp IX, Tryp X ORF, Tryp VII, TrypVIII, Tryp XI ORF, Tryp XII ORF, Tryp XIII, Tryp I, TrypI ORF, Tryp XV 1 and Tryp XV 2; *C. pipiens* ET (AY029276); *C.p.* LT (U65412); *A. gambiae* Tryp 1 and Tryp 2 (Z22930); *S. vittatum* Tryp (L08428); *G. moristans* Tryp (AF252869); *D. melanogaster* alpha-Tryp (M96372); *M. sexta* alkaline Tryp (L16805); *A. gambiae* chymotrypsin (Z18887); *A. aegypti* AT (M77814); *A.a.* CHYMO (U56423); proposed *A.a.* serine collagenase; *A. albopictus* LT (AF268665); *G. moristans* chymotrypsin (AF252868); *H. linateum* collagenase (P08897)

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based on insect morphology (Wheeler et al., 2001). Furthermore, there seems to have been a recent expansion in midgut trypsins within the family Culicidae. This branch contained ET, LT and an *A. aegypti* CxTry homolog, as expected. The mature peptides from the Branch A sequences were further analyzed using a maximum likelihood estimation (Figure 5.2). The results from the maximum likelihood estimation corroborate the results from the neighbor-joining analysis tree. The *Manduca sexta* alkaline trypsin (MSEXTA), *Drosophila melanogaster* alpha-trypsin (DROSPHILA) and *Glossinia moristans* trypsin (GLOSTRYP) group together (42.5% bootstrap support). Subsequently, the two Dipteran sequences segregate with 68.2% confidence. The larger upper branch contains the remaining Dipteran trypsins, including *Simulium vittatum* trypsin (VITTATUMTRYP) (bootstrap support 67.7%). Interestingly, one *Aedes* sequence, AaTrypX, segregates independently from the other Culicidae sequences (bootstrap support 41%) (Figure 5.2).

Branch B (bootstrap support of 64%) is composed of *Anopheles gambiae* chymotrypsin and numerous *A. aegypti* serine protease sequences. The *A. gambiae* chymotrypsin quickly diverges from the *A. aegypti* sequences, which are tightly clustered (100% bootstrap support) and segregates independently of the other insect chymotrypsins found in Branch C (Figure 5.1). The maximum likelihood analysis confirms these results. The *Aedes* sequences are highly conserved (95% bootstrap support) whereas the *A. gambiae* CHYMO sequence is quite divergent and segregates independently using Maximum Likelihood analysis (Figure 5.3). In addition, the *A. gambiae* CHYMO demonstrates little, if any, similarity to the prototypical bovine chymotrypsin.

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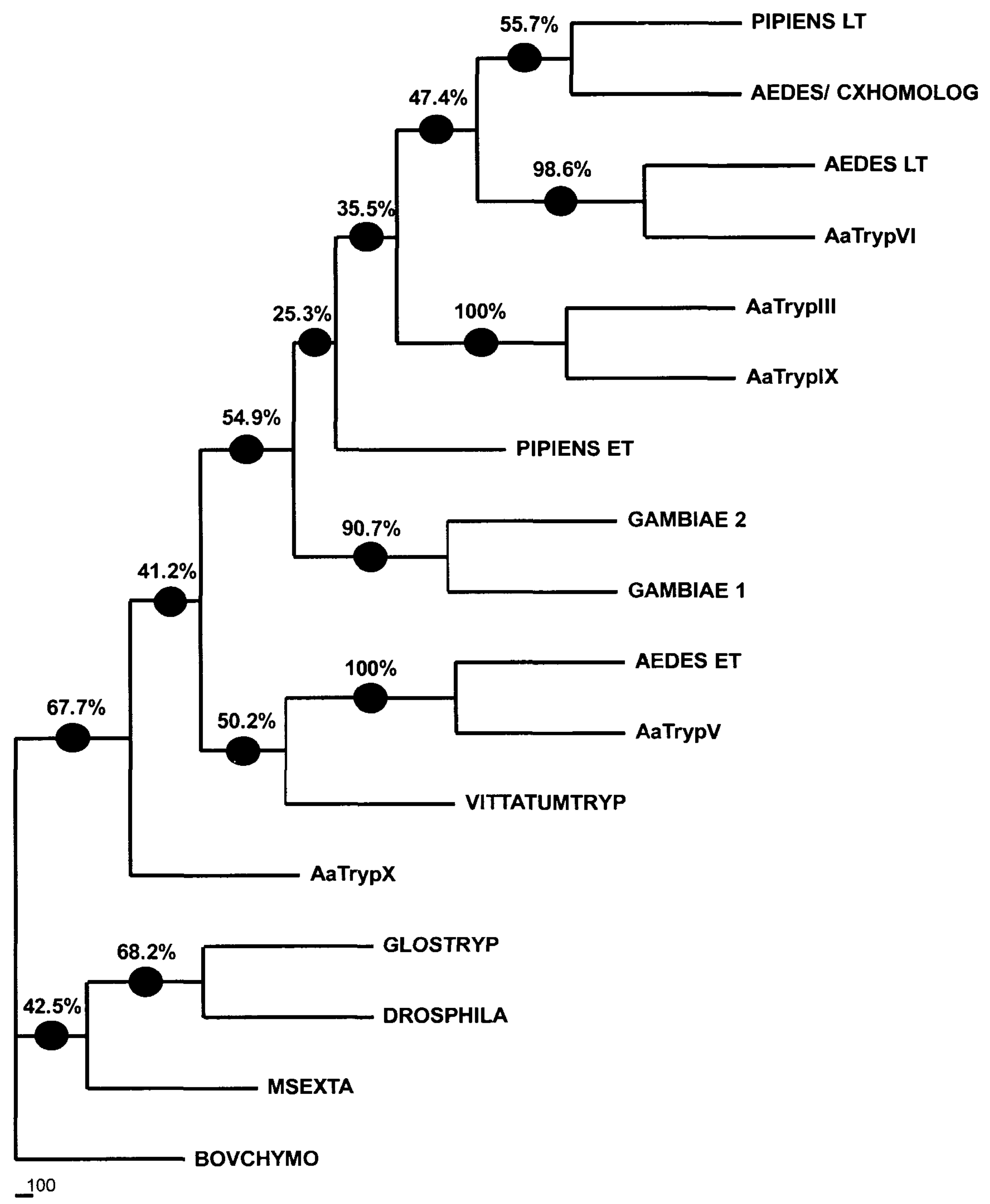

Figure 5.2: Maximum Likelihood analysis of Branch A. This analysis was performed with mature peptide sequences. Bovine chymotrypsin was added to assist with manual alignments and to act as an outlier. *B. taurus* chymotrypsin A (1EX3A); *A. aegypti* ET (X64362); *A.a.* LT (X64363); recently cloned *Aedes/ Cx* Homolog and *A.a.* sequences Tryp III ORF, TrypV, Tryp VI, Tryp IX and Tryp X ORF; *C. pipiens* ET (AY029276); *C.p.* LT (U65412); *A. gambiae* Tryp 1 and Tryp 2 (Z22930); *S. vittatum* Tryp (L08428); *G. moristans* Tryp (AF252869); *D. melanogaster* alpha-Tryp (M96372); *M. sexta* alkaline Tryp (L16805).

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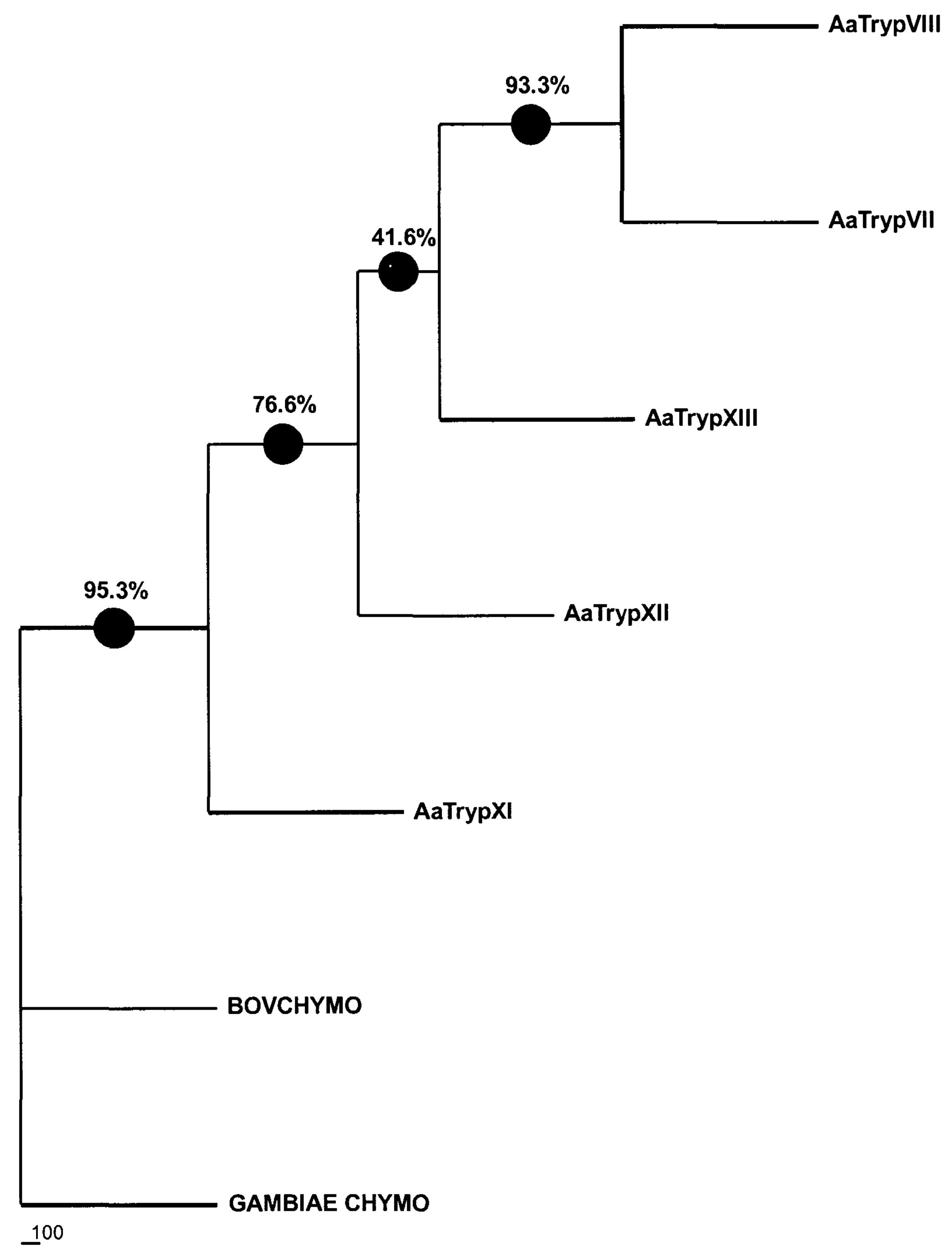

Figure 5.3: Maximum Likelihood analysis of Branch B: Mature peptide sequences were used for this analysis. Bovine chymotrypsin was added to aid with manual alignments and to act as an outlier. . *B. taurus* chymotrypsin A (1EX3A); *A. gambiae* chymotrypsin (Z18887); recently cloned *A. aegypti* sequences Tryp VII, TrypVIII, Tryp XI ORF, Tryp XII ORF and Tryp XIII.

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Finally, Branch C contains the *A. aegypti* and *G. moristans* chymotrypsin sequences, which diverge early along this branch (bootstrap support of 98%) and a cluster of proteins containing numerous *Aedes* sequences and *Hypoderma lineatum*, botfly, serine collagenase (HYPOCOL) (Figure 5.1). Within the 'serine collagenase' cluster there is a smaller cluster containing several of the *Aedes* sequences, including AT. One protein, a proposed *A. aegypti* serine collagenase (AEDESCOL), diverges early and segregates with the botfly serine collagenase (Figure 5.1). Further analysis by maximum likelihood reveals that the two chymotrypsin sequences group together (bootstrap support 96.8%) independent from the other sequences (Figure 5.4). The remaining sequences, including *Uca pugilator* serine collagenase (CRABCOL), cluster together with 94% confidence. The proposed AEDESCOL segregates with the crab collagenase, 76.3%, while the remaining *Aedes* sequences group with the botfly serine collagenase (bootstrap support 69.9%) (Figure 5.4).

The sequence alignments were performed on each of the three branches identified from the distance/ NJ tree analysis independently using only the mature peptides. All three of the alignments show that all but one of the sequences analyzed contained the conserved catalytic triad and six cysteines corresponding with the three disulfide bonds. The proposed *A. aegypti* serine collagenase does not have the traditional His57-Asp102-Ser195 catalytic triad, but rather Val57-Asn102-Gly195 at these positions.

The Branch A alignment reveals that all of these sequences contain the hallmark Asp189 characteristic of trypsins (Figure 5.5). In the Branch B alignment all of the *Aedes* sequences contain an Asp189, yet interestingly the *A. gambiae* chymotrypsin, which was expected to have a Ser at this position, has a Gly in its place. Furthermore, it

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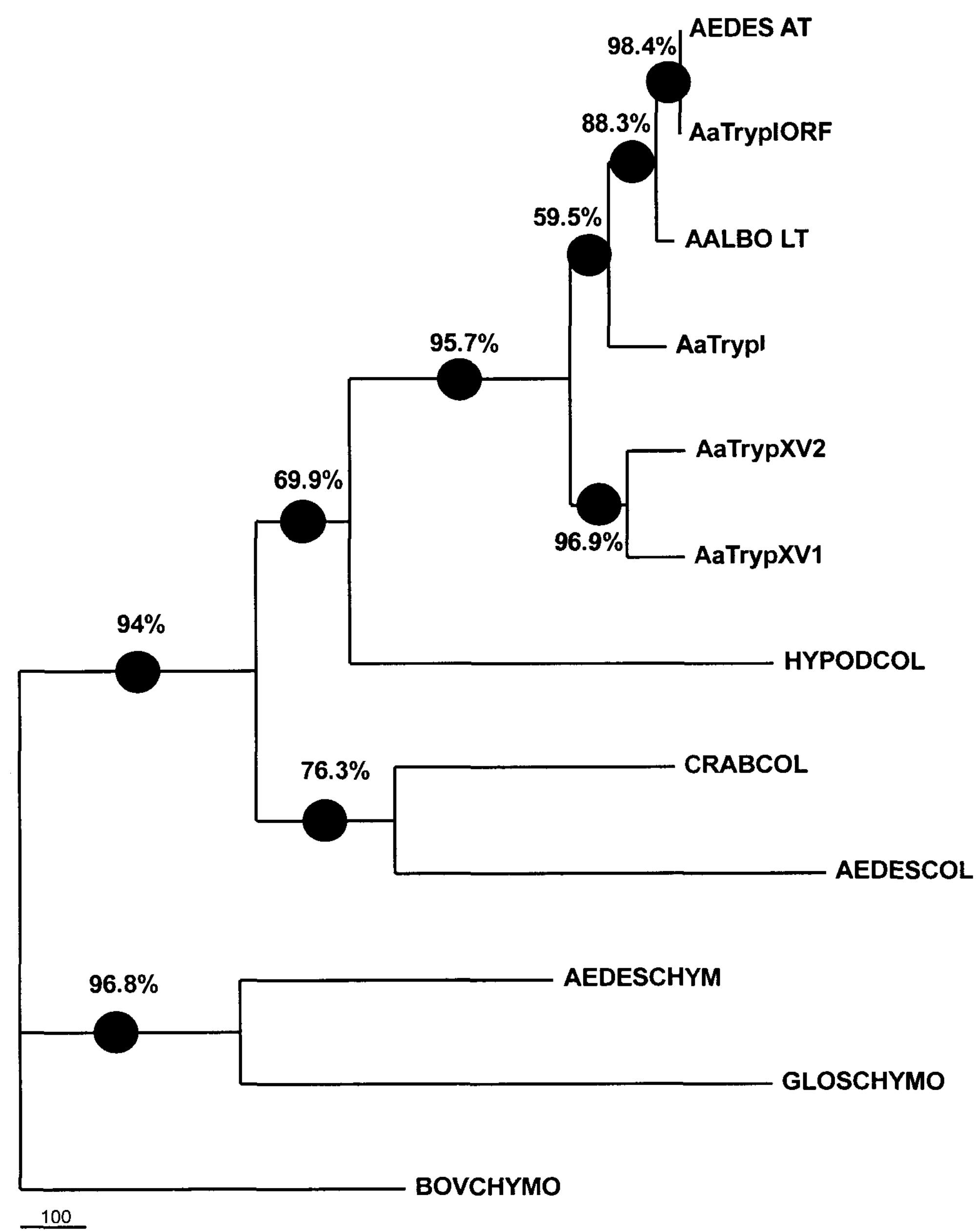

Figure 5.4: Maximum Likelihood analysis of Branch C. Only the mature peptide sequences were used for this analysis. Bovine chymotrypsin was added as an outlier and to assist with the manual alignments. Crab collagenase was added to this analysis because it is the most extensively characterized serine collagenase. *B. taurus* chymotrypsin A (1EX3A); *A. aegypti* AT (M77814); recently cloned *A. aegypti* sequences Tryp I, TrypI ORF, Tryp XV 1 and Tryp XV 2; *A.a.* CHYMO (U56423); proposed *A.a.* serine collagenase; *A. albopictus* LT (AF268665); *G. moristans* chymotrypsin (AF252868); *H. linateum* collagenase (P08897); *U. pugilator* crab collagenase (U49931).

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```
                  ♦                            ▲             *▲
BOVCHYMO          IVNGEEAVPGSWPWQVSLQDKTG----FHFCGGSLINENWVVTAAHCGV-   60
AaTrypIIIORF      IVGGYEVDIADVPFQISLQHDFR-----HFCGGSIISPKWVLTAAHCAA-   87
AaTrypIX          IVGGYEVDIADVPFQISLQHGFG-----HFCGGSIISPKWVLTAGHCAG-   87
PIPIENS ET        IVGGFEVDIKDVPYQVSLRSFGS-----HICGGSIISKRWILTAAHCAS-   81
AEDES LT          IVGGFEVPVEEVPFQVSLSGVGSS----HFCGGSLLSERWVMTAGHCAA-   55
AaTrypVI          IVGGFEVPVEEVPFQVSLSRVGSS----HFCGGSLLSERWVMTAGHCAS-   84
AEDES/CXHOMOLOG   VVGGYEVDVKDIPFQVSLQNTYG-----HFCGGSIISERWVLTAGHCAS-   83
PIPIENS LT        IVGGFEIDILEVPYQISLQ--SYG----HFCGGSIIGENWVLTAGHCAD-   84
GAMBIAE 1         IVGGFEIDVSDAPYQVSLQYNKR-----HNCGGSVLSSKWVLTAAHCTA-   91
GAMBIAE 2         VVGGFQIDVSDAPYQVSLQYFNS-----HRCGGSVLDNKWVLTAAHCTQ-   94
AaTrypXORF        IVGGIAVNISDYPYQVSLQ--RNQ----HFCGGSVLNDRWILTAAHCTKG   78
AEDES ET          IVGGFQIDIAEVPHQVSLQRSGR-----HFCGGSIISPRWVLTRAHCTT-   71
AaTrypV           IVGGFQIDIAEVPHQVSLQRSGR-----HFCGGSIISPRWVLTRAHCTT-   71
VITTATUM TRYP     IVGGEMTDISLIPYQVSVQTAISSYGFIHHCGGSIISPRWVVTAAHCAQ-   79
MSEXTA            IVGGSTTTIQQYPTIVALLFSRNGNTFFQACGGIILNNRNVLTAAHCPH-   73
DROSOPHILA        IVGGSATTISSFPWQISLQ--RSGS---HSCGGSIYSANIIVTAAHCLQ-   74
GLOSTRYP          IVNGVETTIEKRPYQVSLQSAVSGS---HFCGGSIISEDIIVTAAHCVS-   73

                                                              *
BOVCHYMO          TTSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSL-TI-NNDITLLKL  108
AaTrypIIIORF      TSEDPQMNIRVGSTKHTQGGQLVAVKRAVQHPEFDFA-TI-DYDFALLEL  135
AaTrypIX          SSEDPQMNIRVGSTLHTQGGQLVAVKRVVQHPEYDST-TI-DYDFALLEL  135
PIPIENS ET        SADRPKETIRVGSSEKGSGGQILKLKRIVQHPQYDGS-II-DYDFSLLEL  129
AEDES LT          SGQTN-LQVRIGSSQHASGGQLIKVKKVNRHPKYDEV-TT-DYDFALLEL  103
AaTrypVI          SGQTN-LQVRIGSSQHASGGQLFKVKKVNRHPKYDEV-TT-DYDFALLEL  132
AEDES/CXHOMOLOG   PRDHG-LKVRVGSSQHASGGQLYKVAKIHQHPQYNPS-TI-DYDFCLLEL  131
PIPIENS LT        DNDVG-LNVRVGSSLHGSGGQLVPVKPVIQHPQYNPS-TI-DFDFALLEL  132
GAMBIAE 1         GASTSSLTVRLGTSRHASGGTVVRVARVVQHPKYDSS-SI-DFDYSLLEL  139
GAMBIAE 2         GLDPSSLAVRLGSSEHATGGTLVGVLRTVEHPQYDGN-TI-DFDFSLMEL  140
AaTrypXORF        ITNASVLKIRAGSTEVRSGGILAQVRDIYFHPKQN-SWS--NYDFSLLEL  125
AEDES ET          NTDPAAYTIRAGSTDRTNGGIIVKVKSVIPHPQYNGD-TY-NYDFSLLEL  119
AaTrypV           NTDPAAYTIRAGSTDRTNGGIIVKVKSVIPHPQYNGD-TY-NYDFSLLEL  119
VITTATUM TRYP     -KTNSAYQVYTGSSNKVEGGQAYRVKTIINHPLYDEE-TT-DYDVALLEL  127
MSEXTA            GDAVNRWRVRSGSTYANSGGAVHNLNRVRIHPNFNRR-TL-DNDIAIMRT  121
DROSOPHILA        SVSASVLQVRAGSTYWSSGGVVAKVSSFKNHEGYNAN-TM-VNDIAVIRL  122
GLOSTRYP          GSNPSQLKVRLGSTYNNEGGIVVGVKALKYHEKFNND-VLW-HDIAVLKL  121

BOVCHYMO          STAASFS-QT--VSAVCLPSASDDFAAGT-TC-VTTGWGLTRYTNANTPD  153
AaTrypIIIORF      DEELQLD-DGFY--AVELPEQDESVEDGSCL-QV-SGWGNTQSAT-ESGV  179
AaTrypIX          DEKLQLN-DEFY--AVELPEQDEPVEDGSCL-QV-SGWGNTQSAT-ESGV  179
PIPIENS ET        AEELELD-DS--HTTIALPEQDEPVTDGA-ICRV-SGWGNTQSSA-QSNK  173
AEDES LT          EETVTFS-DS--CAPVKLPQKDTPVNEGTCL-QV-SGWGNTQNPS-ESSE  147
AaTrypVI          EETVTFS-DS--CAPVKLPQKDAPVNEGTCL-QV-SGWGNTQNPA-ESSE  176
AEDES/CXHOMOLOG   EKAITFS-EV--SQSVDLPKLNDPVQDGT-L-LQVSGWGYTQNPS-ESRD  175
PIPIENS LT        EQPVQLS-EEFF--PVELPEQDQEVEDGQ-L-LQVSGWGYTQNPS-ESNE  176
GAMBIAE 1         EDELTFS-DA--VQPVGLPKQDETVKDGT-M-TTVSGWGNTQSAA-ESNA  183
GAMBIAE 2         ETELTFS-DL--VQPVELPEHEEPVEPGT-M-ATVSGWGNTQSAV-ESSD  184
AaTrypXORF        KEPLKLS-KGI--QPISLPTHGDSFEDGT-LCEV-SGWGNTRNAN-ESSL  169
AEDES ET          DESIGFS-RSI--EAIALPDASETVADGA-MCTV-SGWGDTKNVF-EMNT  163
AaTrypV           DESIGFS-RSI--EAIALPDASETVADGA-MCTV-SGWGDTKNVF-EMNT  163
VITTATUM TRYP     AEPIVMNY-K--TAAIELAEVGEEVETDA-M-AIVSGWGDTKNFG-EEPN  171
MSEXTA            TSNIAFN-NA--AQPARIAGANYNLGDNQ-V-VWAAGWGAIRSGG-PSSE  165
DROSOPHILA        SSSLSFS-SSI--KAISLATYNP--ANGASA-AV-SGWGTQSSGSSSIPS  165
GLOSTRYP          EKPVKQS-STI--RYIEMAKKVP--KTGTPA-VV-SGWGTKCFLTCPLSP  164
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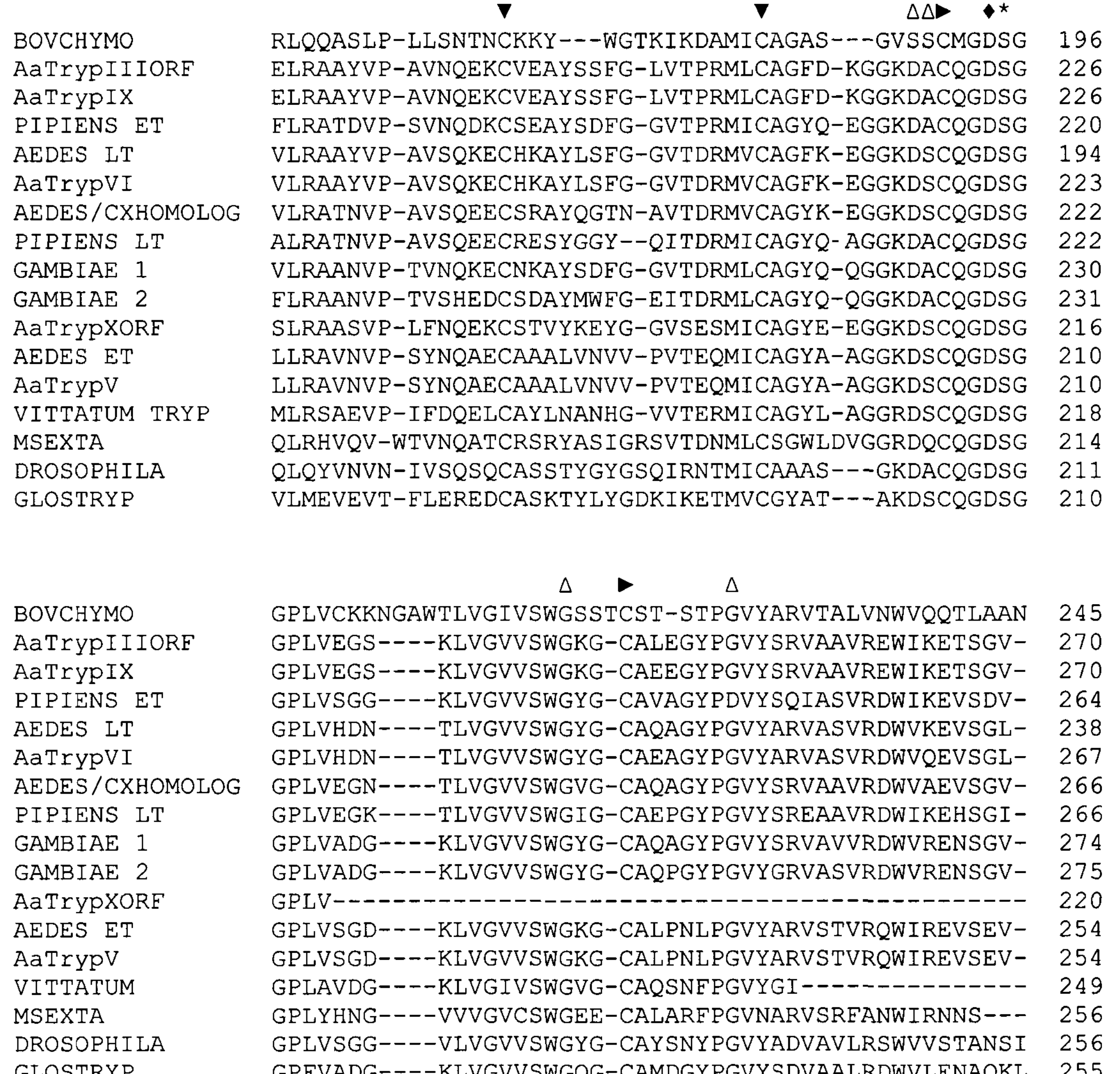

Figure 5.5: Alignment of Branch A (Trypsin) sequences. Numbering is based on Bovine alpha-chymotrypsinogen. (♦) Beginning of mature pepide that starts with Ile/ Val 16 which is buried in Asp 194, (▲) disulfide bridge between 42-58, (*) catalytic triad residues, (▼) disulfide bridge between 168-182, (Δ) accessory catalytic residues and (►) disulfide bridge between 191-220. *B. taurus* chymotrypsin A (1EX3A); *A. aegypti* ET (X64362); *A.a.* LT (X64363); recently cloned *Aedes/ Cx* Homolog and *A.a.* sequences Tryp III ORF, TrypV, Tryp VI, Tryp IX and Tryp X ORF; *C. pipiens* ET (AY029276); *C.p.* LT (U65412); *A. gambiae* Tryp 1 and Tryp 2 (Z22930); *S. vittatum* Tryp (L08428); *G. moristans* Tryp (AF252869); *D. melanogaster* alpha-Tryp (M96372); *M. sexta* alkaline Tryp (L16805).

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has an Asp at 226, which is usually a Gly (Figure 5.6). Alignment analysis of Branch C demonstrated that many of these sequences contained either a Gly or Ser at position 189. As expected CHYMO has a Ser at this position, which is typical of chymotrypsins. Although similar to the *A. gambiae* chymotrypsin, the *G. moristans* chymotrypsin has a Gly at this position. Interestingly, all of the *Aedes* sequences, except CHYMO, and the *H. linateum* collagenase have a Pro at position 190. In most characterized serine proteases this site is typically filled by a small nucleophilic amino acid (Figure 5.7).

Early and late phase tryptic and chymotryptic activity were determined following dsRNA suppression of my target genes. It was found that suppression of ET resulted in an 81% reduction in early phase tryptic activity ($p<0.0005$) (Figure 5.8 A). At the same time suppression of CHYMO resulted in an 87% reduction in early phase chymotryptic activity ($p<0.0004$) and 75% ($p<0.0001$) in late phase chymotryptic activity (Figure 5.8 A and B, respectively). I found that suppression of LT or CxTryp Homolog resulted in a 65% ($p<0.0007$) and 36% ($p<0.0233$) reduction in trypsin activity 24 hpbm, respectively (Figure 5.8 B). Surprisingly, suppression of AT had no effect on tryptic or chymotryptic activity at either timepoint tested. Furthermore, there does not seem to be elastase activity in the midgut of *A. aegypti* (data not shown).

To verify that AT levels were indeed suppressed, western blot analysis was performed on midguts from dsRNA injected mosquitoes 24 hpbm. The analysis revealed that dsRNA suppression of AT was very potent and almost completely eliminated AT protein accumulation (Figure 5.9).

Finally, the results of the oogenesis study from dsRNA injected mosquitoes demonstrated that suppression of ET ($p<0.0374$) and AT ($p<0.005$) significantly reduced

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```
                   ♦                                ▲                       *
BOVCHYMO           IVNGEEAVPGSWPWQVSLQDKTGF-------HFCGGSLINENW-VVTAAH  57
AaTrypVII          IIGGFPAQQSSTLHQVSIRQKSVDLALFGSGHFCGGSLINDR-TVLTAAH  76
AaTrypVIII         IIGGFPAQQSSTLHQVSIRQKSVDLALFGSGHFCGGSLINDR-TVLTAAH  76
AaTrypXIII         IIGGFPAQQSSTLHQVSIRQKSVDLALFGSGHICGGSLINDR-TVLTAAH  74
AaTrypXIIORF       IIGGFPAQQNSTRHQVSIRQKSVDLALFGSGHICGGSLINDR-TVLTAAH  76
AaTrypXIORF        --DVFPAQQSSTRHQVSIRRKSVDLALFGSGHFCGGSLINNR-TVLTAAH  72
GAMBIAE_CHYMO      VVGGEVAKNGSAPYQVSLQVPGWG-------HNCGGSLLNDRW-VLTAAH  74

                   ▲
BOVCHYMO           CGVT--------TSDVVVAGEFDQGSSSEKIQKLKIAKVFKNSKYNSLTI  99
AaTrypVII          CLVNEEASYFRVVGGELNRLLQTQNTVIANVSKVIIHESYNPNTFANDIG  126
AaTrypVIII         CLVNEEASYFRVVGGELNRLLQTQNTVIANVSKVIIHESYNPNTFANDIG  126
AaTrypXIII         CLVNEEASYFRVVGGGLNRLLQTQNTVIANVSKVIIHESYNPNTFANDIG  124
AaTrypXIIORF       CLVNEEASYFRVVGGELNRLLQTQNTVIANVSKVIIHESFDLKTKANDIG  126
AaTrypXIORF        CLVNEEGKRRVASYFRVVGGGLNRLLQTQNTVIANVSKVIIHESYNPNTF  122
GAMBIAE_CHYMO      CLVGHAPGDLMVLVGTNSLKEGG--ELLKVDKLLYHSRYNLPRFHN----  118

                      *
BOVCHYMO           NN--DITLLKLSTAASFS-QTVSAVC-LPS-ASDDFAAGTTCVTTGWGLT  144
AaTrypVII          LLILD-------KPVESTHQTLRTI-ELATC--RPIAG-SICQTTGWGIT  165
AaTrypVIII         LLILD-------KPVESTHQTLRTI-ELATC--RPIAG-SICQTTGWGIT  165
AaTrypXIII         LLILD-------KPVESSHQTLRTI-ELVTC--RPIAG-SICQTTGWGTT  163
AaTrypXIIORF       LLILD-------KPVESSHQTLRTI-ELATC--RPIAG-SICQTTGWGTT  165
AaTrypXIORF        AN--DIGLLILDKPVESSHQTLRTI-ELATC--RPIAG-SICQTTGWGTT  164
GAMBIAE_CHYMO      ----DIGLVRLEQPVQFS-ELVQSV-EYSE-KAVPAN--ATVRLTGWGRT  159

                                             ▼                 ▼
BOVCHYMO           RYTNA-NTPDRLQQ--ASLPLLSNTNCKKYWG---TKIKDAM--ICAG--  184
AaTrypVII          NYSHPMETVELMAVNVTIQPI---ESCNGTGSY-NGDILDGM--LCAGEI  209
AaTrypVIII         NYSHPMETVELMAVNVTIQPI---ESCNGTGSY-NGDILDGM--LCAGEI  209
AaTrypXIII         KYGLPMVTVELMAVNVTIQPI---ESCNGTGSY-NGTILDDM--LCAGEI  207
AaTrypXIIORF       EYDLPMVTVELMAVNVTIQPI---ESCNGTESY-NGTILDGM--LCAGEI  209
AaTrypXIORF        KYDLPMATVELMAVNVTIQPI---ESCNGTGSY-NGTILDGM--LCAGEI  208
GAMBIAE_CHYMO      SANGPSPTLLQSLNVV-TLSN---EDCNKKGG-DPGYT-DV-GHLCTLTK  203

                       ΔΔ►  ♦*                       Δ    ►       Δ
BOVCHYMO           ASGVSSCMGDSGGPLVCKKNGAWTLVGIVSWGSSTCSTST-PGVYARVTA  233
AaTrypVII          TGGKDSCQGDSGGPLVCGG----FLAGIVSHGY-GCGLASYPGIYSDVVH  254
AaTrypVIII         TGGKDSCQGDSGGPLVCGG----FLAGIVSHGY-GCGLASYPGIYSDVVH  254
AaTrypXIII         --------------------------------------------------  207
AaTrypXIIORF       TGGKDSCQGDSGGPLVCGG----FLAGIVSHG-EGCGWASYPGLYSDVVH  254
AaTrypXIORF        TGEKDSCEGDSGGPLVCGG----FLAGIVSHG-KRCGSSSYPEIYSDVVH  253
GAMBIAE_CHYMO      TG-EGACNGDSGGPLVYEG----KLVGVVNFG-VPCALG-YPDGFARVSY  244

BOVCHYMO           LVNWVQQT-LAAN---------------------------  245
AaTrypVII          FREWIDK-HMHTGGGENVKFTVVGLVAVCMVHLVRAVWVV  293
AaTrypVIII         FREWIDK-HMHTGGGENVKFTVVGLVAVCMVHLVRAVWVV  293
AaTrypXIII         --------------------------------------  207
AaTrypXIIORF       FREWIDK-HMHTSGGENVKFTVVGLVAVCMVHLVKAVWVV  293
AaTrypXIORF        FREWIDK-HMYTSGGENVKFTVVGLVSVCMVHLVRAVWFV  292
GAMBIAE_CHYMO      YHDWVRTT-MANNSK-------------------------  268
```

Figure 5.6: Alignment of Branch B sequences. Designation of symbols is the same as Figure 5.5. *B. taurus* chymotrypsin A (1EX3A); *A. gambiae* chymotrypsin (Z18887); recently cloned *A. aegypti* sequences Tryp VII, TrypVIII, Tryp XI ORF, Tryp XII ORF and Tryp XIII.

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               ♦                               ▲              *▲
BOVCHYMO       IVNGEEAVPGSWPWQVSLQDKTG------FHFCGGSLINENWVVTAAHCGVTTS  63
AEDESCHYMO     IVGGDEAEAHEFPYQISLQWNFNDGQTETMHFCGASVLNENFVLTAAHCKTAYS  79
GLOSCHYMO      IINGHEAEKGEAPFIVSLKTNS--------HFCGGSIIAENWVLTAGHCLIFD-  76
AaTrypIORF     VVNGQTATLGQFPFQVLLKVELS----QGRALCGGSLLSDQWVLTAGHCTDGAK  75
AEDES AT       VVNGQTATLGQFPFQVLLKVELS----QGRALCGGSLLSDQWVLTAGHCTDGAK  75
AALBO LT       VVNGQTATLGQFPFQVLVKVETP----QGRALCGGSLLSDQWVLTAGHCVEGAK  53
AaTrypI        VVNGQTATLGQFPYQVLLRIQFA----EGKALCGGSLLSNQWVLTAGHCTDGAK  75
AaTrypXV 1     ------------------------------------------------------
AaTrypXV 2     IVNGQTAAVGQFPYQALLKIQLP----QGRALCGGSLINAQWVLTAGHCTQGAT  75
HYPODCOL       IINGYEAYTGLFPYQAGLDITLQD---QRRVWCGGSLIDNKWILTAAHCVHDAV  81
AEDESCOL       IVGGQIASPGQIPYQAAILADIE----DGSGLCGGVLISANYVLTAAVCVNGAS  111
CRABCOL        IVGGVEAVPNSWPHQAALFIDDM----Y---FCGGSLISPEWILTAAHCMDGAG  91

                                                           *
BOVCHYMO       ----DVVVAGEFDQG--SSSEKIQKLKIAKVFKNSKYNSLTINNDITLLKLSTA  112
AEDESCHYMO     NTGFIEVVAAEHDVAVAEGSEQRRLV--AEFIVHEDYQGGVSPDDIAVIRVDKP  131
GLOSCHYMO      ---EFEIVAGLHSRNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDIGLIYVDKP  127
AaTrypIORF     -S-FEVTLGAVDFEDTTNDGRVVLTAT--EYHRHEKYNPLFATNDVAVVKLPTP  125
AEDES AT       -S-FEVTLGAVDFEDTTNDGRVVLTAT--EYHRHEKYNPLFATNDVAVVKLPTP  125
AALBO LT       -S-FEVTLGAVDFEDLTNDGRVVLTAT--EYYRHEKYNPLFATNDVAVVKLPKP  103
AaTrypI        -S-FEVTLGAVDFNSETDDGRVVLTAT--EYYRHEKYNPLFATNDVAVVKLPQP  125
AaTrypXV 1     ------------------------------------------------------
AaTrypXV 2     -S-FEITLGAVDMEQQSEDGRVVLVAS--EFYRHEKYNPLFASNDVAVVKLPNP  125
HYPODCOL       -S-VVVYLG----SAVQYEGEAVVNSE--RIISHSMFNPDTYLNDVALIKIP-H  126
AEDESCOL       -E-GTVILGAQNLQNENEDGQVRMDFTSSDVHVHEEYVEFIFRHNIAAIRLPQP  163
CRABCOL        F--VDVVLGAHNIR---EDEATQVTIQSTDFTVHENYNSFVISNDIAVIRLPSP  140

BOVCHYMO       ASFS-------QTVSAVCLPSA--SDDFAAGTTCVTTGWG-LTRYTNANTPDRL  156
AEDESCHYMO     FELN-------DKVKAVKLPKQ--LEQFDG--DVTLSGWGSVSTTVFPDYPDKL  174
GLOSCHYMO      FNLNALTRDGTAAVAKVNLPTG--KYESTG--EGKLYGWGRDNSGFLPN---IL  174
AaTrypIORF     VEFND-------RVQPVKLPTG---SDTFTDREVVVSGWGLQKN--GGNVADKL  167
AEDES AT       VEFND-------RVQPVKLPTG---SDTFTDREVVVSGWGLQKN--GGNVADKL  167
AALBO LT       VEFND-------RVQPVKLPSG---SDTFTDRKVVVSGWGLQKN--GGNVADKL  145
AaTrypI        VEFND-------RVHPVELPSG---PDSYANQEVVVSGWGLQKN--GGNVADKL  167
AaTrypXV 1     ---------------QPVSLPSG---SDSYADRKVIVSGWGLQQN--GGTVAQKL  35
AaTrypXV 2     VQFN-------ERIQPIQ-PTG---SDSYADRTVVVSGWGLQKS--GGNVAPKL  167
HYPODCOL       VEYTD-------NIQPIRLPSGEELNNKFENIWATVSGWGQS-----NTDTVIL  168
AEDESCOL       VAVTE-------RIRPAVLPAA-TDSRTFAGMQATISGFGRTSDA-STSFSDVL  208
CRABCOL        VTLTA-------AIATVGLPST-DVG---VGTVVTPTGWGLPSDS-ALGISDVL  182

                           ▼                ▼          ΔΔ►    ♦*
BOVCHYMO       QQASLPLLSNTNCKKYWG--TKIKDA-MICA-GASG--VSSCM--GDSGGPLVC  202
AEDESCHYMO     RKVVLPLVDYEQCDTLWGNDSALAKSNV-CA-GPIDGSKSACS--ADSGGPLVK  224
GLOSCHYMO      NTLDVNIIGYEECKKALPSDAPLDPVNI-CSYTADAT-DGAC--NGESGGPMVR  224
AaTrypIORF     QYAPLTVISNNECSKAYSPLVIKKST-L-CAKGEHK--ESPC--QGDSGGPLVL  215
AEDES AT       QYAPLTVISNNECSKAYSPLVIKKST-L-CAKGEHK--ESPC--QGDSGGPLVL  215
AALBO LT       QYAPLTVISNNECSKTYSPLVIKKTT-L-CAKGEHK--ESPC--NGDSGGPLVL  193
AaTrypI        QYAPLTVITNDECSQTYSPLVIKKTT-L-CAKGGNK--ESPC--NGDSGGPLVL  215
AaTrypXV 1     QFAPLKVITNLKCMKTYSPLVIKKTT-V-CAQGGEK--QSPC--NGDSGGPLVL  83
AaTrypXV 2     QFAPLKVITNSKCMKTYNPLVIKKTT-I-CAQGGEK--QSPC--NGDSGGPLVL  215
HYPODCOL       QYTYNLVIDNDRCAQEYPPGIIVEST-I-CGDT--CDGKSPC-F-GDSGGPFVL  216
AEDESCOL       RYVSNPIMTNADCGAGYYGDLIDGQK-M-CLAYFNT--RGPCI-GDDGGPLTVQ  257
CRABCOL        RQVDVPIMSNADCDAVYG--IVTDGN-I-CIDSTGG--KGTC--NGDSGGPLNY  228
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                           Δ     ►      Δ
BOVCHYMO       KKNGAWTLVGIVSWGSST-CS-TSTPGVYARVTALVNWVQQTLAAN-------   245
AEDESCHYMO     QSGEEVIQVGVVSWGAVP-CGSPRRPTVYAGVSHYVDWIEQQLRA--------   268
GLOSCHYMO      VTPDGTELVGIVSWGYVP-CASTTTPSIYTWTAAFEKWIEESIENYVVPAHLL   266
AaTrypIORF     E--GENVQVGVVSFGHAVGC-EQGYPGAFARLTSFVDWIKQKTGL--------   257
AEDES AT       E--GENVQVGVVSFGHAVGC-EQGYPGAFARLTSFVDWIKQKTGL--------   257
AALBO LT       E--GENVQVGVVSFGHAVGC-EQGYPGAFARLTSFVDWVKQKTGL--------   235
AaTrypI        E--GSKVQVGVVSFGHAAGC-ELGYPGAFARVTSFVDWVKKKTGL--------   257
AaTrypXV 1     E--GGFVQVGVVSFGHGTGC-ERGLPGAFARVTSFVDWIKQKTGL--------   125
AaTrypXV 2     E--GSNVQVGVVSFGHASGC-DRGLPGAFARLTSFSDWIKQKTGM--------   257
HYPODCOL       S--DKNLLIGVVSFVSGAGC-ESGKPVGFSRVTSYMDWIQQNTGIIF------   260
AEDESCOL       D-AGQSLLVGIFSFGSVVGC-ESQWPTVFVRITFYLDWIASHTDVVIGSH---   305
CRABCOL        N----GLTYGITSFGAAAGC-EAGYPDAFTRVTYFLDWIQTQTGITP------   270
```

Figure 5.7: Alignment of Branch C sequences. Designation of symbols is the same as Figure 5.5. *B. taurus* chymotrypsin A (1EX3A); *A. aegypti* AT (M77814); recently cloned *A. aegypti* sequences Tryp I, TrypI ORF, Tryp XV 1 and Tryp XV 2; *A.a.* CHYMO (U56423); proposed *A.a.* serine collagenase; *A. albopictus* LT (AF268665); *G. moristans* chymotrypsin (AF252868); *H. linateum* collagenase (P08897); *U. pugilator* crab collagenase (U49931).

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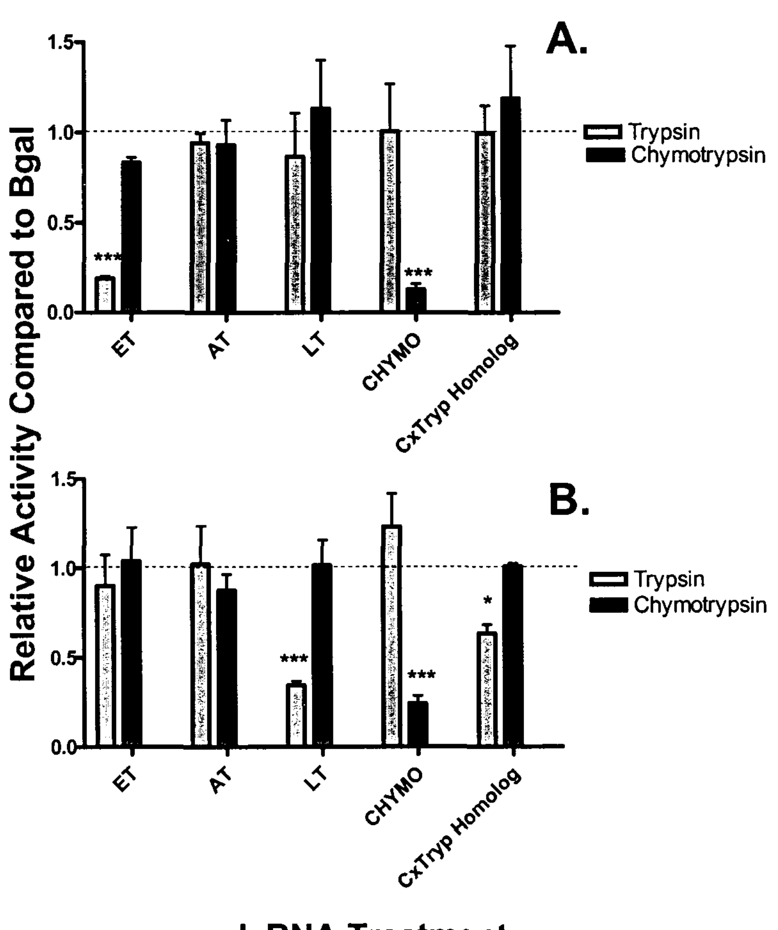

Figure 5.8: The effects of dsRNA suppression on early and late phase serine protease activity. ET, AT, LT, CHYMO and A. aegypti CxTryp Homolog were suppressed with dsRNA. (A) Early phase tryptic (light gray bars) and chymotryptic activity (dark gray bars) were tested 2 hpbm. (B) Late phase tryptic and chymotryptic activity were tested 24 hpbm. Relative activity is reported as a percentage of activity compared to the B-gal dsRNA injected groups. (*) indicate values are significantly different than the control group.

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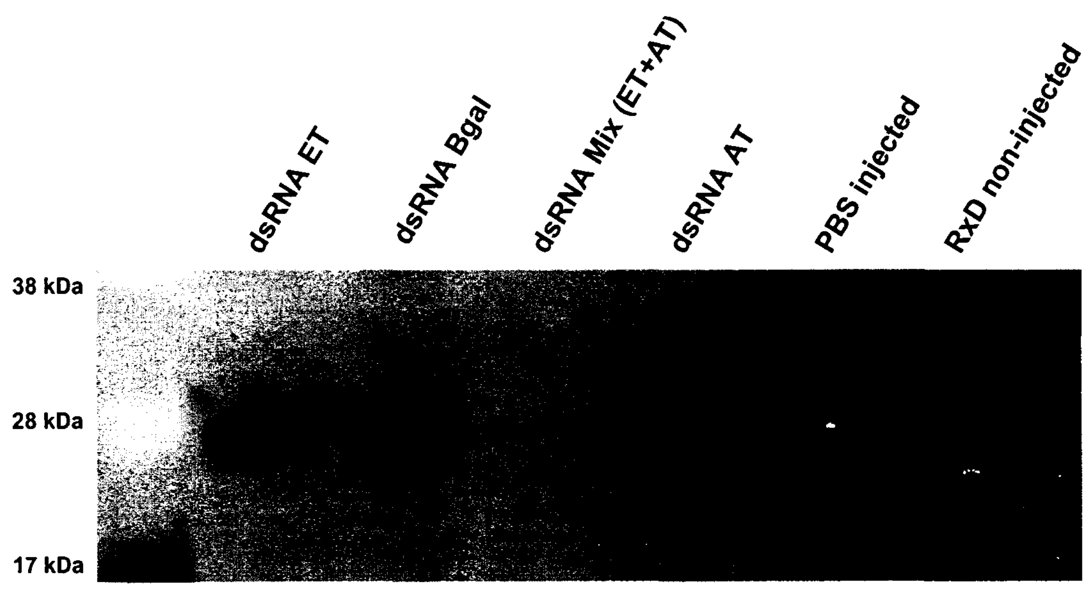

Figure 5.9: Effects of dsRNA suppression on AT protein accumulation. Samples were collected from dsRNA injected mosquitoes 24 hpbm and equal volumes loaded on a protein gel. The Mix group was injected with ~250 ng each of dsRNA ET and AT. The Bgal, PBS and non-injected groups acted as the controls.

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egg production (Figure 5.10). Egg production in the LT injected group was reduced as well, but this value was not quite significant ($p<0.0681$). Suppression of CHYMO did not have any effects on the ability of the mosquito to produce eggs.

Discussion

The *Aedes aegypti* midgut is equipped with numerous proteolytic enzymes that facilitate digestion of the bloodmeal and extraction of nutrients (Zhou et al., 2004). Midgut trypsins are the primary enzymes regulating digestion, which occurs in a biphasic manner (Felix et al., 1991). Initially, ET and AT were believed to be the two primary tryptic enzymes, but the identification of LT and the cloning of numerous other midgut associated serine proteases has changed this paradigm. Furthermore, the role of ET in the induction of AT transcription has been questioned (Lu et al., 2006). The completion of the *A. aegypti* genome and these recent findings has changed our understanding of mosquito digestion. This study was performed in order to expand our understanding of serine proteases on a genomic and functional level.

Phylogenetic analysis of the input protease sequences resulted in three consistent clusters, with strong bootstrap support (Figure 5.1). All of the mosquito sequences from Branch A were tightly clustered, with strong bootstrap support of 97%. In addition, all of these sequences contained the highly conserved Asp189, common to trypsins (Figure 5.5). The presence of ET, LT and *Aedes* Cx Homolog in this cluster is supported by the dsRNA suppression assays. In each case, suppression of the target gene resulted in a reduction in trypsin activity (Figure 5.8). The large number of proteins found in this tight cluster

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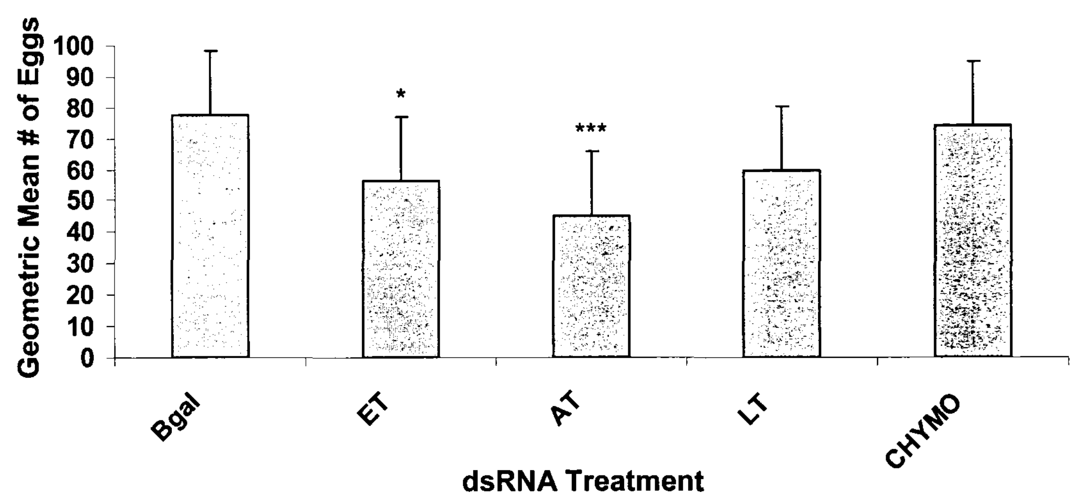

Figure 5.10: The effects of dsRNA suppression on egg production. In three independent replicates, previously injected mosquitoes, nine per group, were maintained and eggs collected. Eggs were counted and the geometric mean for each replicate was determined. The geometric means from the three replicates were analyzed by ANOVA. The egg laying rates for 27 mosquitoes per group. (*) indicates a significant reduction in the number of eggs laid compared to the B-gal dsRNA injected control.

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suggests that there has been a recent expansion of these genes. A similar pattern of *A. aegypti* gene expansion was found in Branch B and C as well (Figure 5.1). The recent completion and continued annotation of the *A. aegypti* genome has been very insightful into this matter. Recently, two serine protease gene clusters have been identified at supercontig positions 1.901 and 1.460. In fact, many of these sequences seem to be closely related to LT, which is probably the result of numerous gene duplications (personal communication Dr. Jun Isoe). These findings suggest that there may be numerous, as yet unidentified, serine proteases involved in bloodmeal digestion.

Analysis of the other two branches was quite surprising. I found that the chymotrypsins did not group with one another despite evidence confirming chymotrypsin substrate specificity for each of the three proteins (Jiang et al., 1997, Vizioli et al., 2001, Yan et al., 2001). The *A. gambiae* chymotrypsin grouped with a cluster of uncharacterized *A. aegypti* sequences, but diverged early along the branch (Figure 5.1). These results suggest that the *A. gambiae* chymotrypsin may be a unique variant of the prototypical chymotrypsin. In fact, activity assays showed that it could cleave the typical chymotrypsin substrates Phe and Tyr, as well as Leu, which is characteristic of elastase activity (Vizioli et al., 2001). These results are supported by the alignment data (Figure 5.6). Typically, chymotrypsins contain a Ser at position 189 and a Gly at 226, which forms an exposed substrate binding pocket allowing for access of large substrate side chains (Perona & Craik, 1995). Despite the rearrangement of the *A. gambiae* chymotrypsin binding pocket consisting of Gly189 and Asp226, it is still accessible, to some degree, to Phe and Tyr. At the same time the presence of the Asp226 may close the

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pocket just enough allowing for interactions with Leu as a substrate. Further investigation into its true activity is warranted.

The *A. aegypti* and *G. moristans* chymotrypsins grouped into Branch C and quickly diverged from the remaining members in this cluster (Figure 5.1). Both of these proteins contain the prototypical chymotrypsin architecture (Figure 5.7). Furthermore, dsRNA suppression of CHYMO demonstrated that it is specific for chymotrypsin substrates and does not affect trypsin activity and has no elastase activity (Figure 5.8).

The other members of Branch C grouped strongly with one another and segregated with the botfly and crab serine collagenases. AT grouped with this cluster (Figure 5.1). These results are not that surprising considering that dsRNA suppression of AT had no effect on trypsin and chymotrypsin activity (Figure 5.8). AT suppression was confirmed by western blot (Figure 5.9), so inadequate suppression cannot account for my results. This suggests one of two things; a) the resultant effect of AT suppression on tryptic or chymotryptic activity is masked by the presence of other serine proteases or b) AT is not a chymotrypsin or trypsin. The fact that LT suppression reduced late phase tryptic activity by 65% and CHYMO suppression reduced late phase chymotryptic activity by 75% implies that redundancy within the system cannot account for the inability of AT suppression to reduce tryptic or chymotryptic activity (figure 5.8). Combined with the phylogenetic and alignment data these results suggest that AT is not a trypsin or chymotrypsin and may represent a novel mosquito serine collagenase.

The significance of a serine collagenase in a hematophagous arthropods digestion of host blood is not known. Collagen is not a normal component of blood, but C1q, the initiator of the classical complement cascade, has a collagen-like sequence of 81 residues

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(Kishore & Reid, 2000). Thus, AT might be an inducible proteolytic defense mechanism required for degradation of C1q. In theory, this would block downstream effects of the complement pathway and protect the midgut epithelial cells from complement induced pathology. Alernatively, it may be an atypical digestive enzyme with as of yet unidentified substrate specificity.

The substrate specificity of serine collagenase is not well defined, but the botfly and crab collagenase do specifically cleave collagen. To date, the ability of AT to cleave collagen has not been demonstrated, but is being pursued by our collaborators at the University of Arizona. Furthermore, it was determined that crab collagenase had varying levels of trypsin, chymotrypsin and elastase activity, although this was not the case for botfly collagenase (Grant & Eisen, 1980, Lecroisey & Keil, 1985). It has been hypothesized that the broad substrate specificity of crab collagenase might, in part, be a result of repositioning of Asp189 and Gly226 to Gly189 and Asp226 (Grant et al., 1980). This architecture is similar to that of *A. gambiae* chymoypsin, which also possesses chymotrypsin and elastase activity. In addition, it was suggested that Gly216 in crab collagenase may act independently of a.a. 189 interactions during positioning of the substrate scissile bond (Tsu et al., 1997). In trypsins, Gly216 usually functions in substrate positioning via assistance from the substrate Lys/ Arg ionic interactions with Asp189 (Perona et al., 1995). Furthermore, analysis of the crystal structure of crab collagenase revealed that it contains two additional amino acids immediately following Gly216 which conditions this a.a. 189 independence and is the basis for its broad specificity (Tsu et al., 1997). The presence of these two additional amino acids was not abundantly obvious from the alignments in this study. Because their conclusions were

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based on comparisons between an arthropod collagenase and mammalian trypsin, it is difficult to conclude whether or not these two additional amino acids are basis for the broad specificity or a result of evolutionary divergence.

Nevertheless, the alignment data presented here reveals that both AT and botfly collagenase contain a Ser at 189 and a Gly at 216 and 226 (Figure 5.7). Due to these similarities, one might expect broad substrate specificity, like the crab collagenase. However, further analysis showed that crab collagenase along with the trypsins and chymotrypsins all contain small nucleophilic amino acids at position 190, which is thought to stabilize binding to polar amino groups (Perona & Craik, 1995). Both AT and botfly collagenase contain a hydrophobic Pro at this position. This alteration near the S1 binding site may account for their inability to hydrolyze trypsin, chymotrypsin or elastase substrates.

Another protein from Branch C, the proposed *A. aegypti* serine collagenase, contained a unique catalytic triad, Val57-Asn102-Gly195. To my knowledge, the architecture of this catalytic triad has not been described for other serine proteases. Annotation of the gene revealed that it contains characteristics similar to multiple catalytic types and is not exclusively a serine protease. This sequence was obtained from the *A. aegypti* Genome Project and does not represent a cDNA clone. Therefore, its significance in bloodmeal digestion, if any, remains undetermined.

Suppression of each of the genes was measured by reduction in serine protease activity. The activity assays determined that ET and, for the first time, LT and *Aedes* CxTryp Homolog, are true trypsins with a substrate preference for Lys/ Arg (Figure 5.8). Furthermore, suppression of these genes had no effect on chymotrypsin activity.

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Silencing of CHYMO significantly reduced early and late phase chymotryptic activity, but had no effect on trypsin activity. These results are not surprising considering the phylogenetic analysis and alignment data. As previously stated, AT suppression had no effect on chymotryptic or tryptic activity. Finally, this is the first study to look at elastase activity in the midgut. I found that there is no detectable level of elastase activity during early or late phase digestion (data not shown).

Analysis of the effect of silencing of each of these genes on oogenesis revealed that ET and AT significantly reduced egg production (Figure 5.10). These results are surprising considering that little protein degradation occurs during the early stages of digestion and the proteolytic nature of AT is unidentified. These data suggest that ET and AT are both critical for bloodmeal digestion and sequestration of nutrients for egg production.

Due to the poor understanding of midgut serine proteases and the lack of a uniform identification system, I propose that the nomenclature for the *A. aegypti* midgut serine proteases be standardized to be more descriptive. In Table 5.2 I have outlined the currently used and proposed name changes for the genes used in this study. I suggest that any and all genes be named A.a. SP (serine protease) followed by sequentially corresponding numerical value. This designation should remain as such until expression pattern data, functional activity assays and phylogenetic analyses are determined. Using ET as an example the following progression of naming events would be applied. First, the gene is identified as being a putative midgut serine protease and would be designated A.a. SP1. Expression data then determines that the putative protease is expressed during the early phase (within the first 8 hpbm) of digestion, it would then be referred to as A.a.

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Current Name	Proposed Name
Early Trypsin	A.a. EPT1
Abundant Trypsin	A.a. LPSP1
Late Trypsin	A.a. LPT1
Chymotrypsin	A.a. E/LPC1
Aedes CxTryp Hom.	A.a. LPT2
Serine Collagenase	A.a. SP1
A.a. Tryp I ORF	A.a. SP2
A.a. Tryp I	A.a. SP3
A.a. Tryp III ORF	A.a. SP4
A.a. Tryp V	A.a. SP5
A.a. Tryp VI	A.a. SP6
A.a. Tryp VII	A.a. SP7
A.a. Tryp VIII	A.a. SP8
A.a. Tryp IX	A.a. SP9
A.a. Tryp X ORF	A.a. SP10
A.a. Tryp XI ORF	A.a. SP11
A.a. Tryp XII ORF	A.a. SP12
A.a. Tryp XIII	A.a. SP13
A.a. Tryp XV 1	A.a. SP14
A.a. Tryp XV 2	A.a. SP15

Table 5.2: Proposed nomenclature changes for *Aedes aegypti* midgut serine proteases. The following abbreviations correspond to the new names; (EP) early phase, (LP) late phase, (E/LP) early and late phase, (SP) serine protease, (T) trypsin and (C) chymotrypsin. If it becomes apparent that there is a characterized collagenase the following abbreviation should be used (Col). The sequences used in this analysis that are not found in the databases can be found in Appendix A under the proposed nomenclature.

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E P(early phase)SP1. Upon confirmation of trypsin activity and phylogenetic relationship to other serine proteases the serine protease would then be renamed A.a. EPT (trypsin) 1. This new nomenclature is very descriptive and will unify the understanding of *A. aegypti* midgut serine proteases as more genes are identified.

The data presented in this chapter is seminal to our understanding of mosquito midgut physiology and highlights the complexity of mosquito digestion. Insight into the basic physiology of vectors is critical to comprehending virus/ vector interactions and can lead to novel control strategies. For example, characterization of the carboxypeptidase A gene expression in the midgut has allowed for the development of transgenic mosquitoes refractory to DENV-2 (Franz et al., 2006). Despite the knowledge gained from this research, much remains unknown and continued research in this field is needed. The incorporation of the proposed nomenclature scheme will ease the confusion as novel midgut serine proteases are discovered through annotation of the genome.

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Chapter 6

Summary and Conclusions

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Dengue virus is a significant public health threat in many tropical regions of the world. Currently, there are no vaccines or antivirals available and mosquito control programs are inadequate (Gubler, 1996, Gubler, 1998). DENV is transmitted between humans by the vector *Aedes aegypti.* Adult female mosquitoes acquire the virus through acquisition of a bloodmeal, which is pumped into the midgut for digestion. The midgut is a highly proteolytic environment and the ability of the virus to persist in this setting is paramount to eventual infection of the vector.

Gut-associated viruses, i.e. rotaviuses, have adapted to exploit the proteolytic nature of the mammalian alimentary canal. Rotavirus infectivity is significantly enhanced when its outer surface proteins are cleaved by trypsin (Espejo et al., 1981, Estes et al., 1981). Likewise, numerous arboviruses have been proposed to utilize invertebrate vector midgut proteases for viral enhancement (Ludwig et al., 1989, Mertens et al., 1996, Molina-Cruz et al., 2005). Specifically, studies have demonstrated that trypsin inhibition of *A. aegypti* midgut trypsins significantly reduced DENV infectivity (Molina-Cruz et al., 2005). These results were confirmed with ET suppression studies in which mosquitoes, previously infected with a dsSIN virus construct containing ET in the antisense orientation, had reduced DENV dissemination rates (personal communication Dr. Irma Sanchez-Vargas). It was concluded that trypsins, specifically ET, at least partly influenced vector competence.

The eventual control of DENV may depend upon the development of novel control strategies. One approach, TBV, has been proven to be effective in blocking malaria infection of *A. gambiae* (Carter, 2001). Its applicability in arbovirus systems is untested. The objective of this dissertation was to test the efficacy of a TBV targeting

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midgut serine protease and to further characterize the role of these proteases in DENV-2 infectivity of *A. aegypti.*

The transmission blocking potential of two midgut serine protease, ET and AT, was assessed in this dissertation. Utilizing a DNA vaccination and a DNA: alphavirus prime-boost vaccination regimen the ability to induce ET and AT specific antibodies was determined. Analysis of immune sera for ET and AT specific antibodies revealed that, in both vaccination regimens, an anti-AT immune response was induced; unfortunately I was unable to detect anti-ET antibodies. Antibodies are believed to be the primary effector molecules in TBVs, therefore the inability to detect an anti-ET immune response makes it impossible to validate its candidacy as a suitable TBV target. Analysis of the transmission blocking potential of anti-ET or AT immune sera revealed that anti-AT sera significantly increased DENV-2 MIR in *A. aegypti*. These results are not consistent with previous reports and suggest that trypsins may limit virus infectivity as opposed to enhancing it. Perhaps the more important outcome of this study was that it confirmed that proteins within the midgut directly interact with the virus and that transmission blocking vaccines are a realistic control option once a suitable target is identified.

To further evaluate the effects of midgut serine proteases on DENV infectivity, mutant viruses lacking potential trypsin recognition sites were produced. E-glycoprotein, specifically domain III, is responsible for attachment to and penetration of host cells (Anderson et al., 1992, Chen et al., 1996b, He et al., 1995). Domain III has 11 potential trypsin recognition sites, four of which are solvent exposed. In fact, *in vitro* digestion of DENV revealed that a fragment corresponding to domain III is produced after exposure to trypsin and chymotrypsin (Roehrig et al., 1998). Analysis of mutant viruses

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demonstrated that disruption of one of these sites, K305, increased the ability of the virus to infect the midgut. These results confirm those from the TBV experiments and lend further support to the hypothesis that exposure of DENV-2 to midgut proteases is detrimental to viral infectivity of the mosquito. The proteolytic enzymes responsible for these observed effects were evaluated in the next set of experiments.

To date four serine proteases have been partially characterized. ET and CHYMO are transcribed post emergence and immediately translated following bloodmeal uptake. ET levels reach maximal levels 3 hpbm, whereas CHYMO slowly accumulates and reaches peak concentrations 24 hpbm (Jiang et al., 1997, Noriega et al., 1996a). Previous studies have suggested that ET, in part, regulates transcriptional induction of AT, although this has recently been questioned (Barillas-Mury et al., 1995, Lu et al., 2006). AT and LT transcription are induced by a bloodmeal and reach maximal levels 24 hpbm (Barillas-Mury et al., 1991, Kalhok et al., 1993). Despite the expression pattern data, much remains unknown about the regulatory mechanisms controlling transcription and translation or their role in vector competence. Using dsRNA suppression to silence each of the genes, I found that a reduction in transcript levels corresponded to a decrease in activity for each of the genes except AT. This could have been the result of redundancy in late phase tryptic activity, although in light of the fact that suppression of LT resulted reduction in late phase tryptic activity, I concluded that AT was probably not a trypsin and served a different purpose in bloodmeal digestion.

The effect of each of these genes on DENV-2 infectivity was also determined. I found that suppression of the genes did not significantly affect midgut infection rates. The differences observed in MIR between the gene silencing and trypsin inhibitor

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experiments may be the result of redundancy within mosquito digestion. dsRNA suppression specifically targets individual genes, but the trypsin inhibitors target all trypsins in the midgut. Considering that numerous trypsins have been identified, suppression of one may not have a significant effect on DENV-2 infections, but inhibition of all of the trypsins with an inhibitor may alter the outcome of the infection.

Similarly, I would have expected to observe an increase in viral infectivity upon suppression of AT considering the TBV results. This discrepancy may be explained by the specificity of the antibodies developed against AT. Serine proteases, in general, have a highly conserved three-dimensional structure (Blow, 1971). It is feasible that antibodies recognizing structural epitopes may have had a broad serine protease inhibitory effect. This would explain why suppression of each of the genes individually had no effect on MIR. This conjecture is supported by the increase in infectivity observed when trypsin inhibitors were added to the bloodmeal. Again, these results contradict the published literature, but in three different experiments I have consistently observed increases in MIR upon manipulation of proteolytic activity of this virus/ vector system.

Given that suppression of AT did not affect late phase tryptic activity I aimed to elucidate its function in digestion. For this analysis I used gene annotation, comparative genomics and a panel of serine protease colorimetric substrates. The activity assays revealed that AT is neither a chymotrypsin nor an elastase. In fact, it was determined that *A. aegypti* do not contain elastolytic activity. Provided that AT was not a trypsin, chymotrypsin or elastase, I performed phylogenetic analyses and sequence alignments. I determined that AT clusters with botfly and crab serine collagenases with strong

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bootstrap support. Previous studies have demonstrated that each of these proteins can cleave collagen (Grant et al., 1980, Lecroisey & Keil, 1985). Collaborators at the University of Arizona are testing if AT can cleave collagen. Furthermore, the crab serine collagenase has broad substrate specificity whereas AT and the botfly serine collagenase do not (Grant & Eisen, 1980). This discrepancy may be the result of the hydrophobic Pro positioned next to the S1-substrate binding pocket in AT and botfly serine collagenase compared to the small nucleophilic amino acid in crab collagenase. These results suggest that AT may be a serine collagenase, the first to be identified in mosquitoes.

Overall, this study has demonstrated that trypsins do not proteolytically activate the virus, but that trypsin activity within the midgut actually limits virus infectivity. The significance of these results on vector competence has yet to be determined, but variable expression or activity of midgut serine proteases may contribute to differences in observed vector competence among populations of *A. aegypti*. Furthermore, the comparative genomics study highlighted the complexity of mosquito digestion. Understanding the basic physiology of vectors is critical to understanding arbovirus/ vector interactions and can lead to novel control strategies.

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Appendix A

Serine Protease Amino Acid Sequences

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A.a. LPT2

MARIIILLAVTLFAAVSGLSTGFHPLRPWWNSRTSGGRVVGGYEVDVKDIPFQVS
LQNTYGHFCGGSIISERWVLTAGHCASPRDHGLKVRVGSSQHASGGQLYKVAKI
HQHPQYNPSTIDYDFCLLELEKAITFSEVSQSVDLPKLNDPVQDGTLLQVSGWGY
TQNPSESRDVLRATNVPAVSQEECSRAYQGTNAVTDRMVCAGYKEGGKDSCQG
DSGGPLVEGNTLVGVVSWGVGCAQAGYPGVYSRVAAVRDWVAEVSGV

A.a. SP1

MKYLAVLSVVVALVGLSVAISVAGKSTRKIDWSLVRPISEFAHVRQRIQSLTETK
SLMNQRIVGGQIASPGQIPYQAAILADIEDGSGLCGGVLISANYVLTAAVCVNGA
SEGTVILGAQNLQNENEDGQVRMDFTSSDVHVHEEYVEFIFRHNIAAIRLPQPVA
VTERIRPAVLPAATDSRTFAGMQATISGFGRTSDASTSFSDVLRYVSNPIMTNAD
CGAGYYGDLIDGQKMCLAYFNTRGPCIGDDGGPLTVQDAGQSLLVGIFSFGSVV
GCESQWPTVFVRITFYLDWIASHTDVVIGSH

A.a. SP2

MFTSTVVFASLMALASAFPSLDNGRVVNGQTATLGQFPFQVLLKVELSQGRALC
GGSLLSDQWVLTAGHCTDGAKSFEVTLGAVDFEDTTNDGRVVLTATEYHRHEK
YNPLFATNDVAVVKLPTPVEFNDRVQPVKLPTGSDTFTDREVVVSGWGLQKNG
GNVADKLQYAPLTVISNNECSKAYSPLVIKKSTLCAKGEHKESPCQGDSGGPLVL
EGENVQVGVVSFGHAVGCEQGYPGAFARLTSFVDWIKQKTGL

A.a. SP3

MFASTVIIASFVALSAAYPSIDQGRVVNGQTATLGQFPYQVLLRIQFAEGKALCG
GSLLSNQWVLTAGHCTDGAKSFEVTLGAVDFNSETDDGRVVLTATEYYRHEKY
NPLFATNDVAVVKLPQPVEFNDRVHPVELPSGPDSYANQEVVVSGWGLQKNGG
NVADKLQYAPLTVITNDECSQTYSPLVIKKTTLCAKGGNKESPCNGDSGGPLVLE
GSKVQVGVVSFGHAAGCELGYPGAFARVTSFVDWVKKKTGL

A.a. SP4

MASLFTVLAVASVLAITSQALPSRPHHLRSWSNPFMRSSGRIVGGYEVDIADVPF
QISLQHDFRHFCGGSIISPKWVLTAAHCAATSEDPQMNIRVGSTKHTQGGQLVAV
KRAVQHPEFDFATIDYDFALLELDEELQLDDGFYAVELPEQDESVEDGSCLQVSG
WGNTQSATESGVELRAAYVPAVNQEKCVEAYSSFGLVTPRMLCAGFDKGGKD
ACQGDSGGPLVEGSKLVGVVSWGKGCALEGYPGVYSRVAAVREWIKETSGV

A.a. SP5

MNQFLFVSFCALLDSAKVSAATLSSGRIVGGFQIDIAEVPHQVSLQRSGRHFCGG
SIISPRWVLTRAHCTTNTDPAAYTIRAGSTDRTNGGIIVKVKSVIPHPQYNGDTYN
YDFSLLELDESIGFSRSIEAIALPDASETVADGAMCTVSGWGDTKNVFEMNTLLR

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AVNVPSYNQAECAAALVNVVPVTEQMICAGYAAGGKDSCQGDSGGPLVSGDK
LVGVVSWGKGCALPNLPGVYARVSTVRQWIREVSEV

A.a. SP6

MVRIILLLTATFFACALGASTGGSHPLRPWWNALRSSGRIVGGFEVPVEEVPFQV
SLSRVGSSHFCGGSLLSERWVMTAGHCASSGQTNLQVRIGSSQHASGGQLFKVK
KVNRHPKYDEVTTDYDFALLELEETVTFSDSCAPVKLPQKDAPVNEGTCLQVSG
WGNTQNPAESSEVLRAAYVPAVSQKECHKAYLSFGGVTDRMVCAGFKEGGKD
SCQGDSGGPLVHDNTLVGVVSWGYGCAEAGYPGVYARVASVRDWVQEVSGL

A.a. SP7

MWFSVKISKLFLLFAASSVAASVDNDVKIIGGFPAQQSSTLHQVSIRQKSVDLALF
GSGHFCGGSLINDRTVLTAAHCLVNEEASYFRVVGGELNRLLQTQNTVIANVSK
VIIHESYNPNTFANDIGLLILDKPVESTHQTLRTIELATCRPIAGSICQTTGWGITNY
SHPMETVELMAVNVTIQPIESCNGTGSYNGDILDGMLCAGEITGGKDSCQGDSG
GPLVCGGFLAGIVSHGYGCGLASYPGIYSDVVHFREWIDKHMHTGGGENVKFTV
VGLVAVCMVHLVRAVWVV

A.a. SP8

MWFSVKISKLFLLFAASSVAASVDNDVKIIGGFPAQQSSTLHQVSIRQKSVDLALF
GSGHFCGGSLINDRTVLTAAHCLVNEEASYFRVVGGELNRLLQTQNTVIANVSK
VIIHESYNPNTFANDIGLLILDKPVESTHQTLRTIELATCRPIAGSICQTTGWGITNY
SHPMETVELMAVNVTIQPIESCNGTGSYNGDILDGMLCAGEITGGKDSCQGDSG
GPLVCGGFLAGIVSHGYGCGLASYPGIYSDVVHFREWIDKHMHTGGGENVKFTV
VGLVAVCMVHLVRAVWVV

A.a. SP9

MASLFTVLAIASVLAITSQALPNRPQHLRSWSIPFMRSSGRIVGGYEVDIADVPFQI
SLQHGFGHFCGGSIISPKWVLTAGHCAGSSEDPQMNIRVGSTLHTQGGQLVAVK
RVVQHPEYDSTTIDYDFALLELDEKLQLNDEFYAVELPEQDEPVEDGSCLQVSG
WGNTQSATESGVELRAAYVPAVNQEKCVEAYSSFGLVTPRMLCAGFDKGGKD
ACQGDSGGPLVEGSKLVGVVSWGKGCAEEGYPGVYSRVAAVREWIKETSGV

A.a. SP10

MLRTLAAAVTLCAVVVYGSYTARSKPGQSGGRIVGGIAVNISDYPYQVSLQRNQ
HFCGGSVLNDRWILTAAHCTKGITNASVLKIRAGSTEVRSGGILAQVRDIYFHPK
QNSWSNYDFSLLELKEPLKLSKGIQPISLPTHGDSFEDGTLCEVSGWGNTRNANE
SSLSLRAASVPLFNQEKCSTVYKEYGGVSESMICAGYEEGGKDSCQGDSGGPLV

A.a. SP11

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MWFSVKISKLLLLFAASSVAASVDNDVFPAQQSSTRHQVSIRRKSVDLALFGSGH
FCGGSLINNRTVLTAAHCLVNEEGKRRVASYFRVVGGGLNRLLQTQNTVIANVS
KVIIHESYNPNTFANDIGLLILDKPVESSHQTLRTIELATCRPIAGSICQTTGWGTT
KYDLPMATVELMAVNVTIQPIESCNGTGSYNGTILDGMLCAGEITGEKDSCEGDS
GGPLVCGGFLAGIVSHGKRCGSSSYPEIYSDVVHFREWIDKHMYTSGGENVKFT
VVGLVSVCMVHLVRAVWFV

A.a. SP12

MWFSVKISKLLLLFAASSVAASVDNDMKIIGGFPAQQNSTRHQVSIRQKSVDLAL
FGSGHICGGSLINDRTVLTAAHCLVNEEASYFRVVGGELNRLLQTQNTVIANVSK
VIIHESFDLKTKANDIGLLILDKPVESSHQTLRTIELATCRPIAGSICQTTGWGTTEY
DLPMVTVELMAVNVTIQPIESCNGTESYNGTILDGMLCAGEITGGKDSCQGDSG
GPLVCGGFLAGIVSHGEGCGWASYPGLYSDVVHFREWIDKHMHTSGGENVKFT
VVGLVAVCMVHLVKAVWVV

A.a. SP13

MWFSVKISKLFLLFAASSVAASVDNDIIGGFPAQQSSTLHQVSIRQKSVDLALFGS
GHICGGSLINDRTVLTAAHCLVNEEASYFRVVGGGLNRLLQTQNTVIANVSKVII
HESYNPNTFANDIGLLILDKPVESSHQTLRTIELVTCRPIAGSICQTTGWGTTKYGL
PMVTVELMAVNVTIQPIESCNGTGSYNGTILDDMLCAGEI

A.a. SP14

QPVSLPSGSDSYADRKVIVSGWGLQQNGGTVAQKLQFAPLKVITNLKCMKTYSP
LVIKKTTVCAQGGEKQSPCNGDSGGPLVLEGGFVQVGVVSFGHGTGCERGLPGA
FARVTSFVDWIKQKTGL

A.a. SP15

MIASIVIISSLVALSATFPSADIARIVNGQTAAVGQFPYQALLKIQLPQGRALCGGS
LINAQWVLTAGHCTQGATSFEITLGAVDMEQQSEDGRVVLVASEFYRHEKYNPL
FASNDVAVVKLPNPVQFNERIQPIQPTGSDSYADRTVVVSGWGLQKSGGNVAPK
LQFAPLKVITNSKCMKTYNPLVIKKTTICAQGGEKQSPCNGDSGGPLVLEGSNVQ
VGVVSFGHASGCDRGLPGAFARLTSFSDWIKQKTGM

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