DISSERTATION

QUANTITATIVE, TIME COURSE ANALYSES OF DENGUE-2 VIRUS REPLICATION AND DISSEMINATION IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JASON H. RICHARDSON ENTITLED ' QUANTITATIVE, TIME COURSE ANALYSES OF DENGUE-2 VIRUS REPLICATION AND DISSEMINATION IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)' BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

QUANTITATIVE, TIME COURSE ANALYSES OF DENGUE-2 VIRUS REPLICATION AND DISSEMINATION IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Aedes aegypti varies in its dengue virus (DENV) vector competence partially due to midgut infection (MIB) and escape barriers (MEB). The mechanisms of these barriers are unknown. Quantitative analyses of DENV infection may provide new insight to vector-virus interactions that impact the MIB and MEB. DENV-2 RNA was quantified during the extrinsic incubation period (EIP) in mosquito strains of varying DENV-2 susceptibility in order to better understand the dynamics of virus infection, replication, and dissemination.

A SYBR Green I based strand-specific, quantitative real-time RT-PCR assay was developed to quantify DENV-2 RNA from the midgut, heads and legs of individual *Ae. aegypti*. DENV-2 plus and minus strand RNA was quantified at each of 14 days post infectious blood meal (dpi) in a DENV-2 competent strain from Chetumal, Mexico. Amounts of positive and negative viral RNA strands were correlated throughout the EIP. Numbers of plaque forming units (PFU) were correlated with DENV-2 RNA copy number in both C6/36 cell cultures and mosquitoes. PFU were consistently lower than RNA copy number by 2--3 $\log_{10}$. Midgut levels of DENV-2 RNA peaked 8 dpi and fluctuated erratically between 6 and 9 dpi. Copies of DENV-2 RNA varied significantly among infected mosquitoes at each time-point.

The SYBR Green assay was used to quantify total DENV-2 RNA in individual *Ae. aegypti* from 3 strains (*Ibo 11*, *D2S3*, and *D2MEB*) previously selected to vary in midgut (MIR) and disseminated infection rates (DIR). A large proportion of *D2MEB* samples expressed a midgut escape barrier (MEB) and infected midguts of *D2MEB* had less DENV-2 RNA than *D2S3, Ibo 11,* and Chetumal. Mosquitoes with a disseminated infection had slightly higher levels of midgut DENV-2 RNA. The study of *D2MEB* supports a threshold model of vector competence and the hypothesis that reducing the rate of viral replication and/or destroying transcribed viral RNA in the midgut decreases overall vector competence.

The effect of mosquito midgut trypsins in DENV-2 infectivity was studied. Addition of soybean trypsin inhibitor (STI) in a DENV-2 infectious blood meal resulted in a 91–97% decrease in midgut DENV-2 RNA. STI treatment also resulted in slower DENV-2 replication in the midgut, less DENV-2 E protein expression, and decreased dissemination to the thorax and the head. A second uninfected blood meal, at 7 dpi, significantly increased DENV-2 replication in the midgut and recovered oogenesis, suggesting that the lower viral infection caused by STI was in part due to a nutritional effect. Mosquitoes fed DENV-2 digested *in vitro* with bovine trypsin (before STI addition) exhibited a transient increase in midgut DENV-2 at 4 dpi. Blood digestion and possibly DENV-2 proteolytic processing, mediated by midgut trypsins, influence the rate of DENV-2 infection, replication, and dissemination in *Ae. aegypti.*

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DEDICATION

This dissertation is dedicated to my father Jarrel H. Richardson who showed me the world. Among many things, he instilled in me a sense of adventure and a love of the outdoors. My guide and mentor, he taught me the meaning of commitment. I hold his memory dear and his examples of faith in facing adversity and dedication to the service of his country invaluable.

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CHAPTER 1: LITERATURE REVIEW

Introduction

Hematophagous arthropods, and the myriad disease agents they transmit, have plagued mankind from antiquity (Service 1978). The link between arthropods and pestilence was solidified in the late 19th century and man has attempted to disrupt these cycles ever since. Despite more than a century of research and experience in vector control, however, arthropod-borne diseases remain a leading cause of morbidity and mortality (Gubler 1998a). These pathogens, and their vectors, have had an unparalleled impact on the course of history (Zinsser 1935). There is an unmistakable causal relationship between human strife and infectious disease. Throughout history, natural disasters, famines, and armed conflicts have bred magnificent epidemics, many of them caused by insect, tick, and mite vectors. Multiple plague pandemics, epidemics of louse-borne typhus, yellow fever, malaria, and a host of other parasite and arthropod-borne virus (arbovirus) diseases have driven the destruction of armies, the fall of empires, and the death of kings. Arthropod-borne diseases continue to burden inhabitants of much of the developing world. High infant mortality rates and overall adult morbidity have impeded economic growth and forced a desperate cycle of poverty and strife in resource rich regions that should otherwise prosper.

Medical Entomology—a limited history. "*...and still it would seem, as if fatal, that the wisdom and experience of one generation should be forgotten by the next, that*

peace should extirpate the knowledge that had been gained in war..." (John Macculloch 1829). (Quoted in (Russell 1963)

There is no shortage of historical illustrations of the influence of vector-borne diseases on man. Even in the relatively short history of the settled Americas there are volumes paying tribute to the impact of these diseases on man. For our purposes, we will pick up the story in 1889 as the scourge of yellow fever and malaria, during and following the Spanish-American War, prompted a concentrated effort to control the diseases that threatened U. S. personnel. Here is a classic example of the connection between war, and/or colonialism, and tropical medicine and its vector biology component.

The occupation of foreign lands for military or colonial purposes has sped the advancement of tropical medicine, requiring governments to combat infectious diseases that jeopardized their interests (Cook 2003). As mentioned above, military might pales compared to that of the smallest vectors and even smaller pathogens they carry. Modern militaries have paid heed to this hard learned lesson by assembling vector research control teams to prevent, or at least minimize, the impact of vector-borne disease on immunologically naïve troops encamped on foreign soil (Schultz 1996). In the words of Hans Zinsser, "the wise general will do what the engineers and the sanitary officers let him" (Zinsser 1935).

The American occupation of Cuba, Puerto Rico, Guam, and the Philippines was no different. The high rate of disease related deaths among U.S. troops (more than five times the combat related deaths) led to the formation of the Yellow Fever Commission under the leadership of Walter Reed (Schultz 1996). Building on the early foundation of

medical entomology, Reed's team conclusively demonstrated the link between the mosquito, *Aedes aegypti*, and a "filter passing agent" that caused yellow fever (Reed et al. 1900). Reed thus joined the ranks of Patrick Manson (development of *Wuchereria bancrofti* in *Culex*), Ronald Ross and Giovanni Grassi (*Anopheles* spp. implicated in development and transmission of *Plasmodium*), Smith and Kilbourne (*Boophilus annulatus* transmission of *Babesia bigemina*), and many others who initiated an age of discovery in medical entomology (Philip and Rozeboom 1973, Service 1978).

By the beginning of World War I arthropods had been implicated in the transmission of myriad pathogens including causative agents of dengue fever, African sleeping sickness, Chagas disease, plague, Rocky Mountain spotted fever, louse-borne typhus, relapsing fever, and sand fly fever. The First World War brought staggering rates of malaria, particularly in Macedonia and in military training camps in the Southeastern United States, and louse-borne typhus in Serbia and Russia (Zinsser 1935, Bunn and Webb 1961, Pierce 1974, Hays 2000). For the first time the U.S. military commissioned trained entomologists to direct vector control and sanitation efforts (Reed et al. 1947, Pierce 1974, Schultz 1996).

The interwar period was marked by landmark research in arbovirology aided by the development of virus culture and isolation techniques using mice. This period saw the development of the 17-D attenuated yellow fever virus (YFV) vaccine and pioneering study of various mosquitoborne encephalitides in the Americas and viruses such as YFV, Murrray Valley, Rift Valley fever (RVFV), and Japanese encephalitis viruses (JEV) in the old world (Chamberlain 1982, 1987). The 1930s were also marked by the significant

invasion of *Anopheles gambiae* to Brazil, setting up a more efficient malaria transmission cycle than the continent had previously seen (Soper and Wilson 1943, Pierce 1974).

World War II ushered in a new series of advances in the field of medical entomology, as a growing contingent of entomologists was drafted to fight malaria as well as dengue fever, sand fly fever, scrub typhus, louse-borne typhus, and Q fever and their vectors across the globe (Reed et al. 1947, Bunn and Webb 1961, Schultz 1996, Hays 2000). As evident in the words of General Douglas MacArthur, "this will be a long war if for every division I have facing the enemy I must count on a second division in hospital with malaria and a third division convalescing from this debilitating disease," it was clear that military success was dependent on the ability to control malaria and louse-borne typhus (Russell 1963). The Surgeon General of the Army formed the Tropical Disease Control Division which included a Malaria Control Branch led by Paul F. Russell of the Rockefeller Foundation (Hays 2000). Additionally, the U.S. Department of Agriculture's Bureau of Entomology and Plant Quarantine was tasked to develop new pesticides and application techniques. With assistance from various agencies, including the Armed Services and the Rockefeller Foundation, hundreds of chemicals were tested for mosquitocidal and lousicidal properties. This led to the discovery of the public health applications of dichloro-diphenyl-trichlorethane (DDT) and the development of aerosol insecticide application for residual control of adult vectors and dusting of personnel to control body lice (Knipling 1945, 1948, Russell 1968). Through the use of nearly 3 million pounds of DDT per month by malaria survey and control units, malaria rates dropped significantly (Hays 2000).

The dramatic mosquito and louse control successes of WWII led to a post war euphoria and long held dreams of global eradication of disease agents and/or vector populations became realistic (Pierce 1974). Fred Soper's early field tests of DDT as a residual adulticide in Italy (1944) were expanded into a five year program, directed by Alberto Missiroli, and succeeded in eradicating malaria in Italy by 1949 (Russell 1968). The malaria eradication program in the United States, run by the U.S. Public Health Service's Office of Malaria Control in War Areas (renamed the Communicable Disease Center (CDC) in 1946), with considerable assistance from the Rockefeller Foundation and State Health departments, was successfully concluded in 1952 (Russell 1968, Philip and Rozeboom 1973).

These victories in the fight against malaria prompted the World Health Organization (WHO), in 1955, to begin a global malaria eradication program (Russell 1968). By 1967 the WHO Expert Committee on Malaria remarked that the number of persons at risk from malaria had been reduced by two-thirds as a result of global eradication efforts spearheaded by vector control programs employing the residual properties of DDT (Philip and Rozeboom 1973).

In addition to the advances in vector control that followed WWII, the field of medical entomology continued to progress as new vector-pathogen interactions were elucidated, in depth study of insect physiology flourished, and vector systematists took advantage of collections made during the war. The euphoric effect of the DDT age (along with the promises of vaccines and antibiotics) resulted in an over confidence and a premature declaration of victory against the age old foes. Soon it was "back to the drawing board" as DDT began to lose efficacy and, more importantly, public acceptance.

It would be a return to the common themes of vector biology—the need for development of new methods to control vectors of disease and the rebuilding of the public health infrastructure.

Resurgence and reemergence. The post WW II successes in the regional eradication of major vectors (and/or cases of disease—most notably, malaria and yellow fever) were largely temporary and unsustainable. Not only have these agents of disease and their vectors resurfaced, many have spread to new regions and others have emerged to be recognized for the first time as important causes of human illness (Gubler 1998a, b, Gratz 1999, Mackenzie et al. 2004). The need for alternative methods to control these pathogens, new and old, and their vectors has spawned a new cohort of research programs (and funding) under the banner of "emerging infectious diseases."

A number of factors continue to contribute to the observed pendulum swing as our ancient enemies cast off our efforts to destroy them. The relationships between vectors, pathogens, reservoirs, and their interactions in dynamic ecological settings is, in all cases complex, and in many cases poorly understood at best. Alterations in any of the components of the system can lead to new opportunities for disease emergence.

Among the most important contributors are the anthropogenic environmental and ecological changes of the late 20th century. Man's major contribution to these changes stem from: (1) agricultural practices such as dam construction, irrigation, and deforestation; (2) urbanization and the concentration of susceptible hosts; (3) population growth, the ensuing overflow of urban centers into rural surroundings, and the inability of sanitation and public health programs to keep pace with urban expansion; and (4) globalization and the increased frequency and speed of international travel and trade

(Morse 1995, 2004) (Figure 1.1). These changes often catalyze an increase in vector and/or reservoir densities and often result in new or more frequent human-vector contact (Morse 1995, Gubler 1998a, Gratz 1999, Childs 2004). Changes such as rapid urbanization and population growth can alter the epidemiology of enzootic disease cycles. Increased contact with vector or reservoir populations results in spill-over transmission where humans serve as incidental hosts. Emergence of Lyme disease in the United States, for example, has been linked to reforestation and the subsequent increase in deer and rodent populations, along with human migration to suburban areas where we are in more frequent contact with the tick vectors (Moore and Freier 2005). Similarly, human encroachment on forests has brought us in closer contact with myriad other pathogens--YFV and LaCrosse viruses (LACV) to name just two (Higgs and Beaty 2005).

Urbanization and over population have also impacted much of the world by placing an increasing burden on already struggling public health systems. With limited resources, many countries can no longer afford to maintain once successful vector control programs. Vectors, particularly peri-domestic species such as *Ae. aegypti*, have benefited from the combination of urban growth, increased larval habitat for container-breeders, and decreased vector control efforts. The interplay of these and other factors has contributed to the emergence of DF and DHF as the most important arboviral disease of man (McLean 1983, Gubler 2002b, Mackenzie et al. 2004).

In addition to local ecological disturbances that can lead to spill-over transmission events and trigger the emergence of "new" diseases, the expansion of international trade and tourism has opened the door to invasion by new vectors and rapid spread of

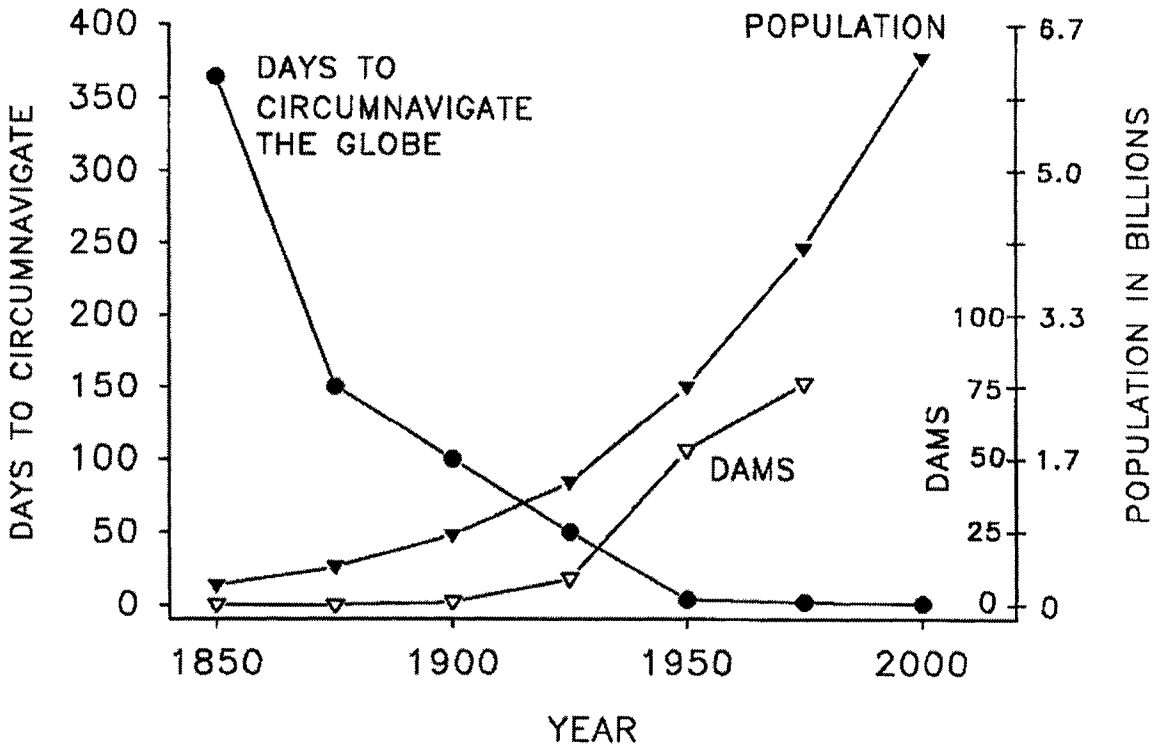

Figure 1.1. Historical trends and global changes that have enhanced the probability of the emergence of new virus diseases of humans and animals. The number of large dams refers to dams more than 75 meters high built in the United States, 1890 to 1975 (Nathanson 2001).

pathogens by infected hosts (Morse 1995, Lounibos 2002, Moore and Freier 2005). Although international trade has long been linked to the spread of vectors and pathogens, the globalization of trade and dramatic increases in the number of passengers and speed of flights between endemic and disease free regions has increased the likelihood of invasion by new diseases/vectors. We need only consider the 1999 invasion of the New World by West Nile virus (WNV) or the emergence and rapid spread of the human coronavirus and the severe acute respiratory syndrome (SARS) to illustrate the impact of global trafficking. Commerce and world travel have had an equally important impact on the epidemiology of dengue fever as discussed below.

Long term climatic changes also contribute to disease emergence and have been associated with a number of disease epidemics (Reeves et al. 1994, Gratz 1999, Linthicum et al. 1999, Githeko et al. 2000, Anyamba et al. 2001, Reiter 2001). Increased temperature can directly impact vectorial capacity by shortening both larval development and extrinsic incubation times (Reiter 2001). General warming trends can lead to increased precipitation and an increase in the availability of larval habitat for many species (Githeko et al. 2000). As mean temperatures rise there is also concern that regions currently too cold to support pathogen transmission may be warmed sufficiently for disease to emerge. Associations between disease emergence and climate change abound. Recent El Niño related temperature and precipitation changes, for example, have contributed to the emergence of hantavirus pulmonary syndrome in the United States (Engelthaler et al. 1999, Glass et al. 2002) and to malaria and Rift Valley fever epidemics in East Africa (Linthicum et al. 1999, Githeko et al. 2000).

Lastly, the exquisite ability of both pathogen and vector populations to quickly adapt to xenobiotics frequently leads to the increased range and prevalence of vector/pathogen populations that have developed resistance to control agents. The genetic plasticity of *Plasmodium* spp., for example, has led to rapid selection for drug resistant genotypes (Arav-Boger and Shapiro 2005). Combined with the ability of the anopheline vectors to rapidly develop insecticide resistance, this has significantly compromised antimalaria campaigns (Hemingway et al. 2002). The RNA viruses possess even greater mutability as will be described below.

Overview of arboviruses. The term arbovirus describes a group of taxonomically unrelated viruses that possess a common route of transmission via hematophagous arthropods--predominantly by mosquitoes and to a lesser extent by hard ticks, culicoid midges, and sand flies. The vast majority of arboviruses belong to just three families: the *Bunyaviridae*, *Flaviviridae*, and *Togaviridae*. Arboviruses have also been found in the *Reoviridae*, *Rhabdoviridae*, *Asfarviridae*, and *Orthomyxoviridae* (Gubler 2002a). The International Committee on Taxonomy of Viruses recognizes over 3,600 virus species and of these over 530 have been classified as arboviruses. With one exception, African swine fever virus (*Asfarviridae*), they possess an RNA genome which can be double-stranded (*Reoviridae*) or single stranded. The single stranded RNA viruses have either a positive (*Flavivirdae, Togaviridae*) or negative-sense (*Bunyaviridae, Rhabdoviridae, Orthomyxoviridae*) orientation.

Transmission cycles. As with the arboviruses themselves, the cycles by which they are transmitted are typified by diversity. By definition they share an arthropod vector and a vertebrate host (usually avian or mammalian). Transmission may

involve a single vector-host pair (e.g., DENV transmission—*Ae. aegypti*-man) or a complex set of vectors and hosts and transmission routes (e.g., LACV and WNV transmission) that may serve to maintain and/or amplify the virus. The epidemiology of arbovirus transmission is complicated further by various modes of vertical and horizontal transmission and the varying vectorial capacity of a given species at a given time and place (Weaver and Barrett 2004).

Arbovirus Diversity and Evolution. Beyond loose generalizations based on genome arrangement and conserved gene families, diversity is the norm for the arboviruses. Their genetic plasticity is coupled to an error prone RNA polymerase which lacks a proof reading function, short generation times, and large population sizes (Holmes 2003a). Consequently their genomes undergo constant mutation at a rate on the order of 10^{-3} to 10^{-5} misincorporations per nucleotide site and round of copying (Domingo et al. 1997). Consequently, each heterogeneous population consists of a random assortment of mutants, referred to as a quasispecies. This concept was originally developed by Manfred Eigen in 1977 and later adopted by Domingo and Holland to describe the RNA viruses (Eigen and Schuster 1977, Domingo and Holland 1997). As a virus attempts to attach, penetrate, and replicate within a new host cell, it explores the adaptive landscape. Haplotypes that are better able to replicate will undergo positive selection (Black and Salman 2005). In LACV, for example, high mutation rates are thought to permit efficient transmission of the virus between host systems (Borucki et al. 2002). Genetic drift and random associations with new host species or populations can lead to the selection of new variants through genetic bottle-necking and a founder effect. This is particularly relevant when host arbovirus titers are low and a feeding vector ingests very few viruses with

haplotypes that occupy a minute portion of the possible sequence space. In such cases a novel virus variant can be established by random chance. This was recently demonstrated in *Culicoides sonorensis* feeding on a bluetongue virus infected sheep (Bonneau et al. 2001).

Selection for new genotypes can have significant impacts on host cell specificity, pathogenicity, and overall disease epidemiology (Domingo and Holland 1997). Recent evidence points to a higher fitness of the viruses in the Southeast Asian genotype of DENV-2 relative to the less pathogenic viruses in the American genotype using both a human dendritic cell and mosquito assay to measure virus infection and replication rates (Cologna et al. 2005). With genetic drift and selection driving evolution of the quasispecies, arboviruses face the unique complication of alternating host landscapes. Many have hypothesized that this results in stabilizing selection and relatively low rates of evolution as compared to other RNA viruses (Weaver et al. 1991a, Weaver et al. 1992, Weaver et al. 1999, Chen et al. 2003, Diaz 2004). However, others have presented evidence contradicting this theory of evolutionary stasis imposed by host alternation (Novella 1999). Holmes (2003b) credits an unusually high deleterious mutation rate as the explanation for the relatively low long-term amino acid substitution rates despite high intra-host genetic variation observed in DENV.

Recombination and reassortment also enhance the evolutionary and epidemic potential of the arboviruses. Homologous recombination can occur in cells co-infected with more than one virus and is gaining recognition as a significant contributor to the genetic diversity of arboviruses (Holmes et al. 1999, Worobey et al. 1999, Chare et al. 2003). The impact of these events, relative to intramolecular point mutations, on

evolutionary potential differs among virus families. Western equine encephalitis virus provides a classical example of recombination occurring between two positive-sense alphaviruses, Sindbis and Eastern equine encephalitis viruses (Hahn et al. 1988). Whereas recombination appears to occur only infrequently in negative-sense RNA viruses (Chare et al. 2003), the phenomenon is more common in the flaviviruses (Twiddy and Holmes 2003) having been demonstrated in multiple species and genera including bovine viral diarrhea virus (BVDV) (*Pestivirus*) (Becher et al. 2001, Nagai et al. 2003), the four DENV serotypes (Holmes et al. 1999, Worobey et al. 1999, Tolou et al. 2001, Uzcategui et al. 2001, Diaz 2004), and Saint Louis and Japanese encephalitis viruses (Twiddy and Holmes 2003). Of the mosquito-borne flaviviruses studied, DEN viruses have shown the highest rates of recombination while no recombination has been detected in the tick-borne flavivirus sequences examined (Twiddy and Holmes 2003).

Reassortment has also been demonstrated to occur among arboviruses with multipartite genomes. Novel LACV genotypes have been observed following laboratory co-infection of *Ochlerotatus triseriatus* with two virus variants (Beaty et al. 1985). Reassortants have also been detected in LACV field isolates (Beaty and Bishop 1988). Reassortment of gene segments has been well studied in BTV. The group includes at least 24 serotypes and reassortment is thought to be a driving force behind the group's diversity (Oberst et al. 1987, Bonneau et al. 2001).

Vectorial capacity. Having addressed the genetic diversity of arboviruses and their transmission cycles, we now turn to the broad concept involving the factors that affect the dynamics of vector-borne disease transmission—vectorial capacity. This field of vector biology was pioneered by malariologists attempting to distil the epidemiology

of malaria transmission into a single term—the number of potentially infective bites delivered to a single host in a day (Fine 1981). The term encompasses myriad variables including both biotic and abiotic factors that determine the ability of a specific vector to transmit a specific pathogen at a given place and time (Black and Moore 2005, Higgs and Beaty 2005). An adaptation of Macdonald's equation is commonly employed to model vectorial capacity (Garrett-Jones 1964):

$V = (ma^2p^nb)/-\log_e p$

where:

m = vector density in relation to the host

a = feeding frequency x host index (or the proportion of bloodmeals taken from this host species)

b = vector competence (the proportion of vectors that become infective following ingestion of an infective bloodmeal) (Hardy 1983)

p = probability vector will survive one day

n = length of the extrinsic incubation period (EIP) (in days)

$1/(-\log_e p)$ = vector's life span beyond EIP (in days)

While these contributing variables can be measured from field or laboratory studies and used to estimate V, in reality each parameter is dynamic and subject to environmental influence. This brings us back to the discussion of how social and environmental factors contribute to emergence/reemergence of vector-borne diseases. Changes in host population densities, for example, impact a and climatic changes are often manifest in altered values for $1/(-\log_e p)$, n, p, and a (Githeko et al. 2000, Reiter 2001). A fundamental consideration in measuring or predicting arbovirus transmission is

the fact that, in contrast to human malaria and DENV, most arboviruses are zoonoses with multiple potential host and vector species. The various vertebrate host populations are likely to differ in density, distribution and dispersal (spatial and seasonal), attractiveness to vectors, and virus susceptibility or immune status (DeFoliart et al. 1987). These differences can have a profound impact on the epidemiology of the disease.

The terms *m* and *a* (vector density in relation to the host and feeding frequency x host index, respectively) attempt to describe the dynamic relationship between host availability and preference. We have already mentioned how changes in host density can impact the epidemiology of a disease. It is intuitive that host preference and feeding habits have a profound impact on disease transmission. Historically, these vector specific, behavioral determinants of transmission were included as components of vector competence (Mitchell 1983, DeFoliart et al. 1987). However referring to McDonald's adapted equation, vectorial capacity encompasses these behavioral components. Therefore they will be discussed here and not in the ensuing discussion of vector competence where we will restrict our focus to intrinsic, physiological properties of the vector and its interaction with the pathogen.

It has become increasingly evident that vector behavior is a key component of vector-borne disease transmission (Mitchell 1991). This is perhaps most obvious in the few anthroponotic vector-borne diseases where variation for endophily and anthropophily, within and among vector species and populations, is fundamental to sustained transmission. Human malaria and dengue fever provide two classic examples and in both cases behavioral determinants of the primary vector's host preference and endophily have been shown to be under genetic control (Gillies 1964, Trpis and

Hausermann 1978). In addition to variation among species and populations, some vector populations are known to switch host preference during a single season. *Cx. tarsalis* and *Cx. nigripalpus*, for example, show variable bird:mammal feeding ratios during a single season (Tempelis et al. 1965, Edman and Taylor 1968).

Vector competence. In an early 1960s manuscript entitled *The Deadly Triangle*, William Dwight Pierce deals at length with the "*Principles Governing Insect Transmission of Disease*" (Pierce 1974). He asks the question that, forty years later, remains at the crux of vector biology-- "what becomes of an organism in the insect." As with vertebrate populations, vector species show significant spatial and temporal variability in their impact on disease transmission. Vector competence has been defined as "the ability...to become infected with, allow replication of, and transmit a virus to a susceptible host" (Kramer and Ebel 2003). In other words, it is the "intrinsic permissiveness of an arthropod" to infection, replication, and transmission of a pathogen (Black and Severson 2005).

The fact that all arthropod vectors are not created equal has been apparent since man began associating certain arthropods with disease. At the crudest level, man has long recognized that vector competence differs among arthropods in different phyla, classes, orders and families. Mosquitoes have long been associated with fevers (in fact the mosquito and malaria have a common name in Swahili, *mbu*), whereas other vector taxa were linked to other diseases: lice with typhus, tabanid flies with anthrax (Service 1978), ticks with relapsing fevers (associated by Livingstone in Africa and by Megnin 1882 in ancient Persia), the Tsetse fly and a "poisonous germ", black flies with river blindness, and mites with scabies and scrub typhus (Philip and Rozeboom 1973).

The next phase in our understanding of vector competence was the recognition that certain genera and species within a family were better suited to transmit certain pathogens. The Mandingo tribe in the Gambia have long differentiated between *Glossina palpalis* and *morsitans* as strong and weak vectors of sleeping sickness (Philip and Rozeboom 1973). Despite his poor grasp of entomology, Ronald Ross differentiated between the "dappled wing" and "brown" mosquitoes in his quest to understand plasmodium transmission (although serendipity would prevent him from understanding the differential competence of *Anopheles* and *Culex* species for various *Plasmodium* species (Desowitz 1991). Similarly, Patrick Manson distinguished between two mosquitoes prevalent in Amoy, China, focusing on the more common "dingy brown" *Culex* species for his work with filarial worm transmission (Pierce 1974). Carlos Finlay and members of the Yellow Fever Commission, including Reed and Lazear, made comparable observations that a single mosquito species was "the" vector of the YFV (at the time identified as *Cx. fasciatus*) (Reed et al. 1900). During the ensuing "Golden Age" of medical entomology vector biologists and arbovirologists quantified the relative vector competence of countless potential vectors for the medically important vector-borne pathogens. Aided by the concomitant advances in systematic studies and elucidation of vector species complexes, the notion of variable vector competence among species is now canonical.

The current phase of our understanding of vector competence involves that of intraspecific variability. It is now common knowledge that individuals within a species can show a range of oral susceptibility and/or ability to transmit an arbovirus. This has been the subject of many useful reviews (Gubler et al. 1982, Tabachnick et al. 1982,

Mitchell 1983, Hardy 1988, Houk and Hardy 1989, Gooding 1996, Beerntsen et al. 2000, Mellor 2000, Black et al. 2002, Kramer and Ebel 2003, Black and Severson 2005). The variability of *Ae. aegypti* and *Ae. albopictus* populations as vectors of DENV is well documented (Gubler and Rosen 1976b, Gubler et al. 1979, Tabachnick and Powell 1979, Tabachnick 1982, Tabachnick et al. 1985, Boromisa et al. 1987, Tardieux et al. 1990, Bosio et al. 1998, Vazeille-Falcoz et al. 1999, Bennett et al. 2002, Failloux et al. 2002, Armstrong and Rico-Hesse 2003, Bennett et al. 2005). The introduction of WNV to North America and the raised awareness regarding the impact of arbovirus introductions to the continent has resulted in renewed study of the vector competence of North American mosquito species for various arboviruses (Sardelis et al. 2002, Turell et al. 2002, Turell et al. 2005).

It is clear that intraspecific variation in vector competence is widespread. More elusive is an adequate understanding of the contributing factors. Ultimately, vector competence is determined by genetic (intrinsic) and environmental (extrinsic) factors that impinge on the physiological processes which condition: (a) the infection of, replication in, and escape from the midgut epithelium and (b) the infection of, replication in, and escape from the salivary glands (Hardy et al. 1983). Since the early 1950s when Chamberlain and colleagues began describing vector competence, vector biologists have attempted to answer Pierce's question. Although far from complete, we now have an understanding of the factors that modulate vector competence.

Extrinsic vector competence factors. The impact of extrinsic factors, such as temperature during the EIP, conditions of the larval habitat (including larval density, salinity, pH, temperature, and food availability) and the dose and strain of the

virus on vector competence, has been the focus of many laboratory studies (Chamberlain and Sudia 1955, Takahashi 1976, Baqar et al. 1980, Grimstad and Haramis 1984, Watts et al. 1987, Kay et al. 1989a, Grimstad and Walker 1991, Turell 1993, Nasci and Mitchell 1994, Reisen et al. 1997).

Arboviruses are uniquely adapted to survive in both ecto- and endothermic hosts. As a result they are able to develop under a range of temperatures. The rate of replication, however, differs within this temperature range and an inverse relationship between temperature and length of the EIP has been observed in various mosquito-virus pairs: *Haemogogus capricornii* and YFV (Bates and Roca-Garcia 1946), *Oc. triseriatus* and EEEV (Chamberlain and Sudia 1955), *Cx. pipiens quinquefasciatus* and SLEV (Hulburt 1973), *Cx. tritaeniorhynchus* and JEV (Takahashi 1976), *Cx. tarsalis* and WEEV (Kramer et al. 1983), both *Cx. pipiens* and *Ae. taeniorhynchus* and RVFV (Turell et al. 1985), *Ae. aegypti* and DENV-2 (Watts et al. 1987), *Cx. univittatus* and WNV (Cornel et al. 1993), *Cx. pipiens* and RVFV (Brubaker and Turell 1998), *Cx. pipiens* and WNV (Dohm et al. 2002). Others have shown the same temperature-EIP relationship in *Culicoides variipennis sonorensis* infected with the reoviruses BTV and AHSV (Mullens et al. 1995, Wellby et al. 1996). As usual with biological phenomena, exceptions have been observed. Ockelbo virus infection and dissemination rates do not differ in *Cx. torrentium* whether incubated at 10 or 24°C (Lundstrom et al. 1990). Western equine encephalomyelitis virus in *Culex tarsalis* has shown higher vector competence at 26 or 18°C than at 32°C (Kramer et al. 1983).

The majority of these experiments were conducted under artificial conditions in which vectors were held at fixed temperatures. A more relevant epidemiologic issue is

the relationship between daily and seasonal variations in temperature and rate of virus replication, dissemination, and transmission. In addition to affecting viral replication directly, the physiological changes affected by temperature (and for that matter day length and other environmental factors) can impact vector competence by depressing or accelerating host metabolic pathways (e.g., transcription and translation) required for virus replication. This is particularly relevant during the winter months and diapause when the EIP may become severely protracted as a result of incubation below a "zero development" temperature at which a virus will not multiply and may persist at low levels, below detection thresholds of traditional assays (Hardy 1988, Kay et al. 1989a, Mellor et al. 1998). African horse sickness virus (AHSV), for example, failed to replicate and became undetectable in *Culicoides spp.* held at 10°C for 13 days (Mellor et al. 1998). However, when the temperature was raised at 35 days post infection the infection rate rose to above 15%. Yellow fever virus also assumes this "dormant" posture when incubated at low temperature in *Haemogogus capricornii* and, upon temperature elevation, resumes replicative activity (Bates and Roca-Garcia 1946). Similarly, JEV infection remained localized in small foci in the midgut epithelium of *Cx. tritaeniorhynchus* kept under winter-like temperatures (Shichijo et al. 1972). When the temperature was raised to 18°C, foci of infection expanded and virus was able to disseminate to the hemocoel. The epidemiologic significance of this arboviral quiescence is exemplified by the recent establishment of endemic WNV in North America and the probable maintenance of virus in overwintering *Cx. pipiens* (Dohm and Turell 2001).

Larval habitat. Conditions under which larvae are reared affect both adult body size and energy reserves, which in turn have a significant impact on the vector

competence and vectorial capacity of certain arbovirus vectors (Nasci and Mitchell 1994, Takken et al. 1998, Zhou et al. 2004). Among the physical and chemical properties of the larval habitat that influence size, and thus vector competence, nutritional availability (relative to larval density) appears to be the most important (Takahashi 1976, Baqar et al. 1980, Grimstad and Haramis 1984, Kay et al. 1989a, Grimstad and Walker 1991, Turell 1993).

The link between larval nutrition and vector competence is unclear and is probably multifactorial. In some cases, smaller adult mosquitoes appear to be more competent as vectors. Adult female *Oc. triseriatus* that are nutritionally deprived as larvae tend to develop a thinner midgut basal lamina than well fed individuals (Grimstad and Walker 1991). The associated impact on virus escape from the midgut is a plausible explanation for the larval nutrition-vector competence connection. Malnourished females are also known to take multiple blood meals to initiate a gonotropic cycle which increases their vectorial capacity (Chadee and Beier 1997, Takken et al. 1998). Furthermore, *Ae. aegypti* females with low energy reserves ingested larger blood meals than females with high energy reserves (Mostowy and Foster 2004). In the laboratory, small *Ae. aegypti* and *Oc. triseriatus* ingested more Ross River virus and LACV particles, proportional to their size, than larger females and virus transmission was more common in small or starved *Oc. triseriatus* (Grimstad and Haramis 1984, Paulson and Hawley 1991, Nasci and Mitchell 1994). Nutritionally deprived *Cx. tritaeniorhyncus* females were more efficient at transmitting JEV (Takahashi 1976) and both larval crowding and malnourishment of the same species has been shown to affect WNV susceptibility (Baqar et al. 1980). Conversely, others have shown a lack of correlation between mosquito size

and vector competence in *Ae. vigilax*-RRV, *Cx. annulirostris*-Murray Valley encephalitis virus, and *Cx. tarsalis*-WEEV and SLEV studies (Kay et al. 1989b, Reisen et al. 1997, Jennings and Kay 1999).

Intrinsic vector competence factors. Ultimately both extrinsic environmental factors such as larval density, nutrient availability, and rearing temperature, and intrinsic genetic variability in both vector and virus work together to determine vector competence. For DENV-2, susceptible and refractory vector phenotypes have been associated with specific genetic loci but specific genes and precise mechanisms responsible for susceptibility and refractoriness remain unknown (Black et al. 2002, Kramer and Ebel 2003). These mechanisms are loosely and collectively referred to as infection and escape barriers (Figure 1.2). The barriers of concern in flavivirus transmission are limited to the midgut (although *Ae. aegypti* appears to have an ovarian barrier to DENV) while salivary gland barriers have been associated with LACV and WEEV infections (Kramer et al. 1981, Grimstad et al. 1985, Paulson et al. 1989). Mosquitoes that possess a midgut infection barrier (MIB) are refractory to viral infection and/or replication in the midgut epithelium. Mosquitoes with a midgut escape barrier (MEB) are capable of developing infected midgut cells but virus is unable to escape the midgut to cause a disseminated infection.

The cellular mechanisms that contribute to the MIB and MEB have yet to be demonstrated but potential explanations include the absence of cell surface receptors for the virus, cellular defenses such as RNA interference or apoptosis that create a nonpermissive environment (Murphy 1975, Blair et al. 2000, Adelman et al. 2001, Sanchez-Vargas et al. 2004, Travanty et al. 2004, Girard et al. 2005), and variability in

myriad host proteins involved in proteolytic cleavage (Ludwig et al. 1989, Ludwig et al. 1991, Mertens et al. 1996, Xu et al. 1997) uncoating, transcription, and translation of viral proteins and/or RNA (Black et al. 2002, Gomez-Machorro et al. 2004, Molina-Cruz et al. 2005). The MIB and MEB are critical determinants in the vector competence of *Ae aegypti* populations for flaviviruses (Gubler et al. 1979, Tabachnick et al. 1985, Schoepp et al. 1990, Bosio et al. 1998). Multiple genes or sets of genes appear to control each of these barriers (Miller and Mitchell 1991, Bosio et al. 1998, Bosio et al. 2000). At least five genomic regions have been associated with an *Ae. aegypti* MIB and three other loci have been linked to an MEB (Bosio et al. 1998, Bosio et al. 2000, Gomez-Machorro et al. 2004, Bennett et al. 2005).

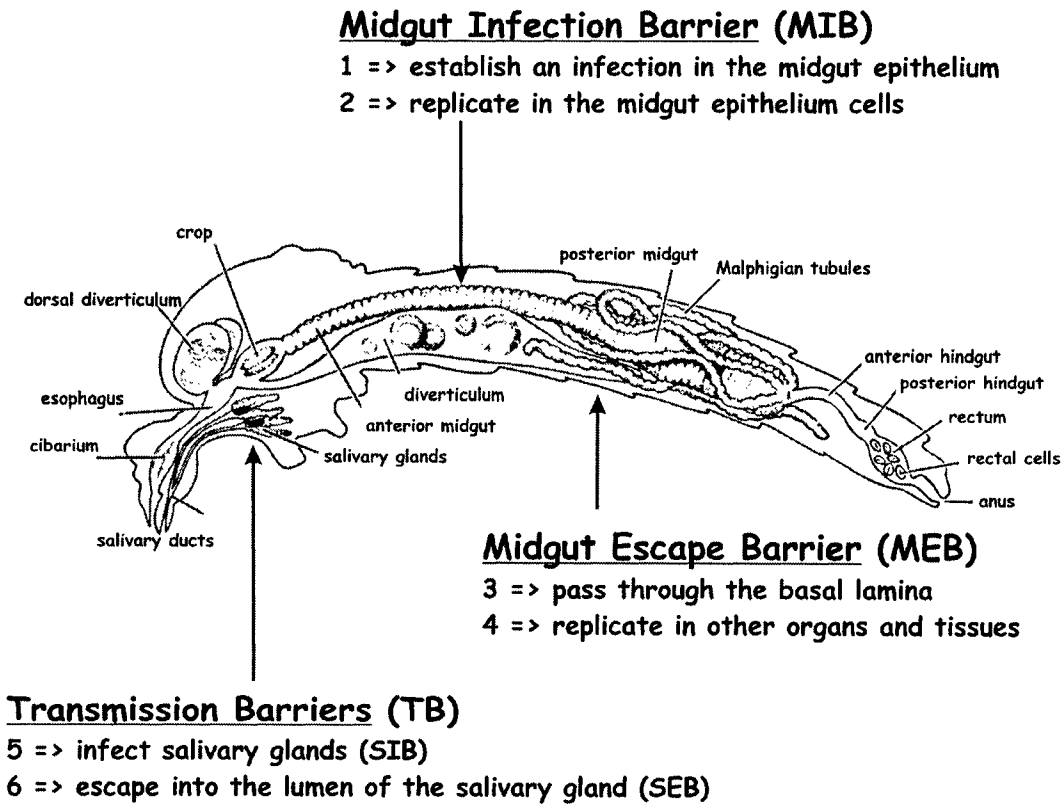

Figure 1.2. Arboviruses transmission barriers.

Qualitative and quantitative assessment of vector competence. Vector competence studies of DENV are typically qualitative in nature, involving the detection of viral antigens in the head or legs. The vector competence of the experimental group is then estimated as the proportion of tested mosquitoes that develop a disseminated infection. Early attempts to quantify vector potential were spearheaded, in part, by Roy W. Chamberlain and others at the CDC as part of an effort to implicate specific mosquito species as major vectors of American encephalitides (Chamberlain et al. 1954). This new approach involved a quantitative comparison of the infection and transmission rates of various suspected vector species allowed to feed upon high concentrations of an arbovirus. These studies were conducted under the assumption of an infection threshold, whereby mosquitoes exposed to a low concentration of virus would fail to develop an infection. This threshold was assumed to be species specific and biologically relevant as a determinant in the overall vector potential of a species. Species that demonstrated lower infection thresholds and higher infection and transmission rates were predicted to have a higher vector potential. While all three measures are important, even with a high rate of infection, a species that rarely transmits virus during feeding is an unlikely vector. Ultimately, the ability to pass infectious virus in the saliva determines the vector competence of a species. These early attempts to measure vector competence in the laboratory laid the foundation for future quantitative studies of vector competence. Unlike the alphaviruses assessed by Chamberlain and his colleagues in these early studies, DENV do not readily infect laboratory animals and thus estimation of transmission rates is difficult (Johnson and Roehrig 1999). Consequently, DENV dissemination is commonly used as a surrogate for actual transmission.

***Aedes (Stegomyia) aegypti* (L.).** *Ae. aegypti* exists as two subspecies: *Ae. aegypti formosus*, a black form found predominantly in sylvan habitats in sub-Saharan Africa and, *Ae. aegypti aegypti*, a pale, domestic form that is a pantropic container-breeder in urban habitats (Gubler et al. 1979, Tabachnick et al. 1985, Tabachnick 1991). The ancestral *Ae. aegypti* was likely a sylvan species inhabiting the forests of Africa which, under pressure from the drying of the Sahara region, evolved to the peridomestic form dependent on man's water storage container (Tabachnick 1991). As with two other recent invaders of North America, *Ae. albopictus* and *Oc. japonicus*, domestication and container breeding facilitated the spread of *Ae. aegypti* to the New World in the water stored in sailing ships (Christophers 1960, Lounibos 2002, Moore and Freier 2005). The continued expansion of *Ae. aegypti* populations in growing cities is a major contributing factor in the recent global resurgence of dengue fever (DF) and dengue hemorrhagic fever (DHF) in much of the tropics (Figure 1.3) (Gubler 2002a). *Aedes aegypti* populations show considerable variance in their competence as vectors of DENV (Gubler et al. 1979, Tabachnick and Powell 1979, Tabachnick et al. 1985, Tardieux et al. 1990, Bosio et al. 1998, Vazeille-Falcoz et al. 1999, Bennett et al. 2002, Failloux et al. 2002, Armstrong and Rico-Hesse 2003, Bennett et al. 2005).

Selected laboratory strains. The comparison of vector-virus interactions between susceptible and refractory populations may provide information on the molecular determinants of infection barriers and on overall ability of a species to transmit an arbovirus. Detailed study of these barriers has been facilitated by family based phenotypic selection to produce *Ae. aegypti* strains that are susceptible and resistant to infection or dissemination with DENV-2. Three such strains include a highly

Figure 1.3. Dengue: its current distribution, and countries with *Ae. aegypti* and at risk of introduction. (From Mackenzie et al. 2004)

susceptible strain, *D2S3* (Dengue 2 Susceptible on 3 chromosomes) selected from *Ae. ae. aegypti* (Puerto Rico strain) and *Ae. ae. formosus* (Ibo strain) parents to have a high midgut infection rate (MIR) (94-100%) and a high disseminated infection rate (DIR) (88-100%); a strain with a strong MEB, *D2MEB* (DENV-2 Midgut Escape Barrier) created from *D2S3* and *Ae. ae. aegypti* (Houston strain) parents to have a high MIR (74-88%) and a low DIR (13-33%); and a refractory strain, *Ibo 11*, with a low DIR (4-13%) selected from a laboratory strain of *Ae. ae. formosus* originally from Ibo, Nigeria (Ballinger-Crabtree et al. 1992, Bosio et al. 1998, Gomez-Machorro et al. 2004, Bennett et al. 2005). All strains were selected using a high passage DENV-2 strain, JAM1409, originally isolated in 1983 in Jamaica and subsequently passaged in C6/36 cells (Deubel et al. 1986).

Dengue viruses. Dengue viruses (family Flaviviridae, genus *Flavivirus*) include 4 antigenically distinct serotypes (DENV-1--4) transmitted both in an anthroponotic cycle by *Aedes (Stegomyia)* mosquitoes and in a zoonotic/sylvan cycle among monkeys. Each of the four DENV serotypes are subdivided into genotypes based primarily on sequence analysis of the E gene and/or an E/NS1 fragment (Rico-Hesse 2003). Human DENV strains are thought to have arisen within the last 1,500 years from an ancestor of sylvatic DENV isolates, identified in monkey populations in West Africa and Malaysia (Rudnick 1965, Wang et al. 2000, Diallo et al. 2003). Recent phylogenetic analyses indicate that the sustained human DENV transmission began between 125 and 320 years ago (Twiddy et al. 2003). Human DENV strains were recently shown to be better adapted to *Ae. aegypti* and *Ae. albopictus* than sylvan DENV strains, suggesting that the emergence of

urban, human viruses may have been facilitated by adaptation to anthropophilic mosquitoes concurrent with their domestication (Moncayo et al. 2004).

Dengue fever has been recognized for over 200 years but it was not associated with mosquito transmission until 1903, when Graham proved the connection (Graham 1903, Pierce 1974). Graham had used *Cx. quinquefasciatus* in his study and in 1906, Bancroft implicated *Ae. aegypti* as a vector, followed by identification of *Ae. albopictus* and *Ae. polynesiensis* as capable vectors (Gubler 1988). In 1944, a series of landmark studies were conducted at the Dengue Research Unit at the Rockefeller Research Institute for Medical research by Albert Sabin (Sabin 1952). Seven DENV human isolates were recovered from Hawaii, New Guinea, and India and inoculated into "volunteer" prisoners from the New Jersey State Prison. The ensuing studies provided the early vector biology, etiology, and symptomology of DENV and dengue fever as well as distinction of the four serotypes.

Epidemiology. Dengue virus infects an estimated 50-100 million people per year with approximately two fifths of the world's population at risk (Gubler 2002a, World Health Organization 2002). Clinical features are influenced by age and immune status and range from undifferentiated illness to dengue shock syndrome (World Health Organization 1997, Gubler 1998a). Classical dengue fever is referred to as a mild, self-limiting febrile illness, typically lasting 7-10 days and accompanied by severe headache, retroorbital pain, myalgia, and arthralgia (thus the name "break-bone" fever) (Nimmannitya 2003). Dengue fever may progress to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) with hemorrhagic manifestations characterized by vascular permeability, plasma leakage, and hypovolemic shock.

Pre World War II, dengue epidemiology consisted of infrequent epidemic cycles of classical dengue fever occurring throughout the tropics but with highest incidence in Asia. Dengue had limited social or economic impact until the 1950s when this pattern of disease was disrupted by the emergence of DHF/DSS and a rise in fatalities, particularly among untreated children. First recognized in the Phillipines in 1954, DHF/DSS has spread through Asia and in 1981 emerged in the New World, in Cuba (Guzman et al. 1988, Nimmannitya 2003). Dengue is now a major threat in Mexico and throughout Central and South America and DHF is now a problem in 17 countries in the Americas (Gubler and Kuno 1997). The number of cases has increased at an alarming rate (Figures 1.4 and 1.5). Over 609,000 dengue cases were reported in the Americas in 2001, including 15,000 DHF/DSS cases (World Health Organization 2002). This is more than double the number of cases reported in 1995. As of 2002, the WHO estimated an annual world-wide DHF hospitalization rate of 500,000. Case fatality rate averages ~ 2.5% with a range of ~ 1 to 20% depending on level of care (World Health Organization 2002).

As discussed above, various factors have contributed to the increase in DENV transmission. The factors that have resulted in larger vector populations, urbanization, and global virus trafficking can explain the increase in dengue cases (Figures 1.1, 1.3--1.7). However, this does not explain the changing epidemiology and emergence of DHF/DSS. The rise of DHF/DSS has been associated with two main factors: co-circulation of multiple DENV serotypes, leading to antibody dependent enhancement from sequential infection with heterologous viruses, and emergence of virulent virus

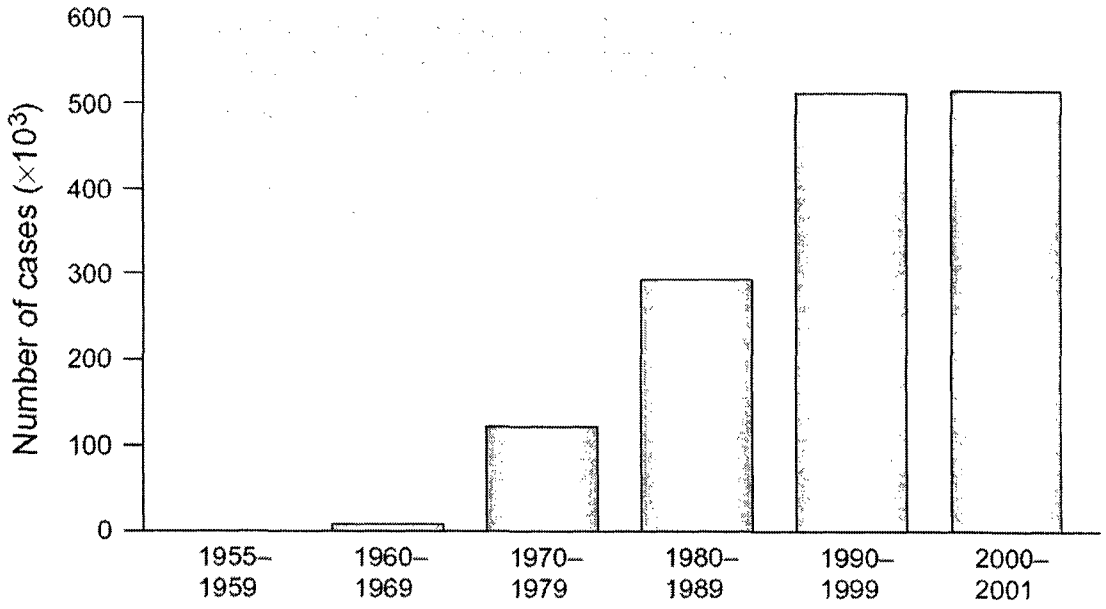

Figure 1.4. Dengue/dengue hemorrhagic fever, average annual number of cases reported to WHO, 1955–2001. (From Mackenzie et al. 2004)

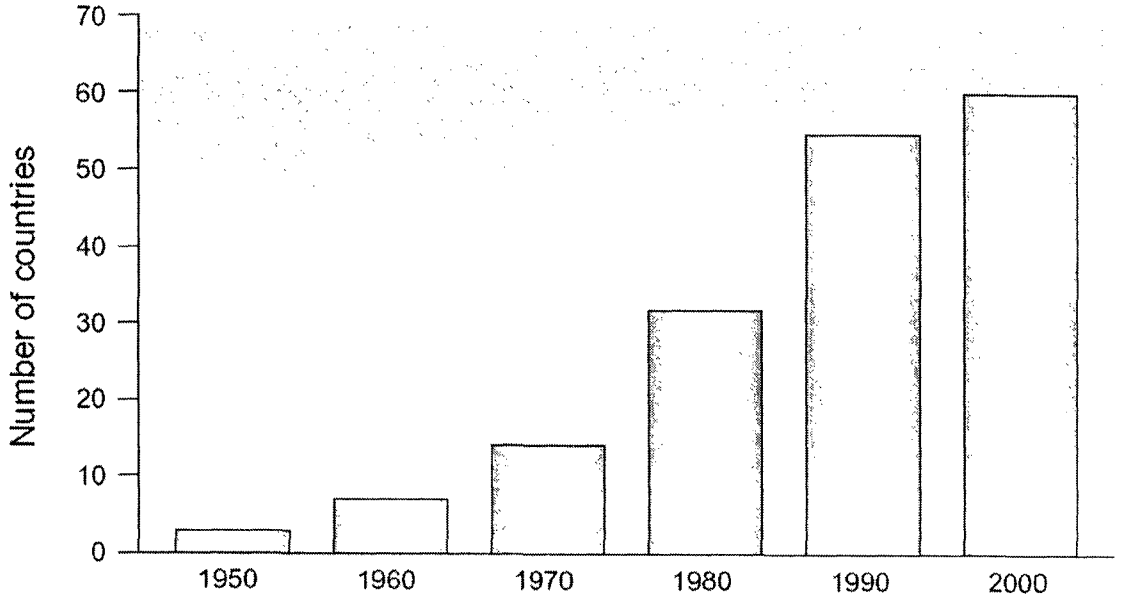

Figure 1.5. Countries in the world reporting DHF cases, 1950–2000 (cumulative) (From Mackenzie et al. 2004)

genotypes with greater epidemic potential (Figures 1.6 and 1.7) (Gubler 1988, Halstead 1988, Rico-Hesse et al. 1997, Gubler 2002a, Halstead 2003). Immune enhancement during secondary heterlogous infection is clearly involved and secondary infection is the leading risk factor for DHF (Halstead 1988). Primary infection can also develop into DHF, supporting the virulent virus theory. In the Americas, DENV-2 DHF has been associated with the southeast Asian genotype (Rico-Hesse 1990, Rico-Hesse et al. 1997).

Viral replication. Dengue viruses are single stranded, positive sense (+) RNA viruses. The replication of dengue viruses involves two distinct RNA species: plus strand or genomic RNAs and partially double stranded (ds) replicative intermediates (Chambers et al. 1990). The replicative intermediates are RNAs made up of antisense strands bound to an average of five complementary, nascent positive sense RNA chains (Vaughan et al. 2002). These partially dsRNA molecules serve as transcriptional templates for semi-conservative replication. The dengue virus replication complex is comprised of most of the seven nonstructural proteins (NS1 to NS5), in close association with the dsRNA template, contained within virus induced membrane-bound vesicles (Mackenzie et al. 1998, Khromykh et al. 2000). These vesicles serve as virus production "factories" that are readily visible by immunofluorescent staining. Conserved complementary cyclization sequences in the 5' and 3' untranslated regions are thought to facilitate replication of full length viral RNA molecules (Khromykh et al. 2001).

Positive and negative stranded RNA molecules can be quantified independently. This is useful in proving that virus is replicating RNA in the samples whereas the presence of genomic RNA does equate to a replicating virus (Vaughan et al. 2002).

Asymmetrical replication results in a 1 - 2 $\log_{10}$ excess of genomic RNA over RI-RNA (Pogue et al. 1994, Wang et al. 2002, Molina-Cruz et al. 2005, Richardson et al. 2005).

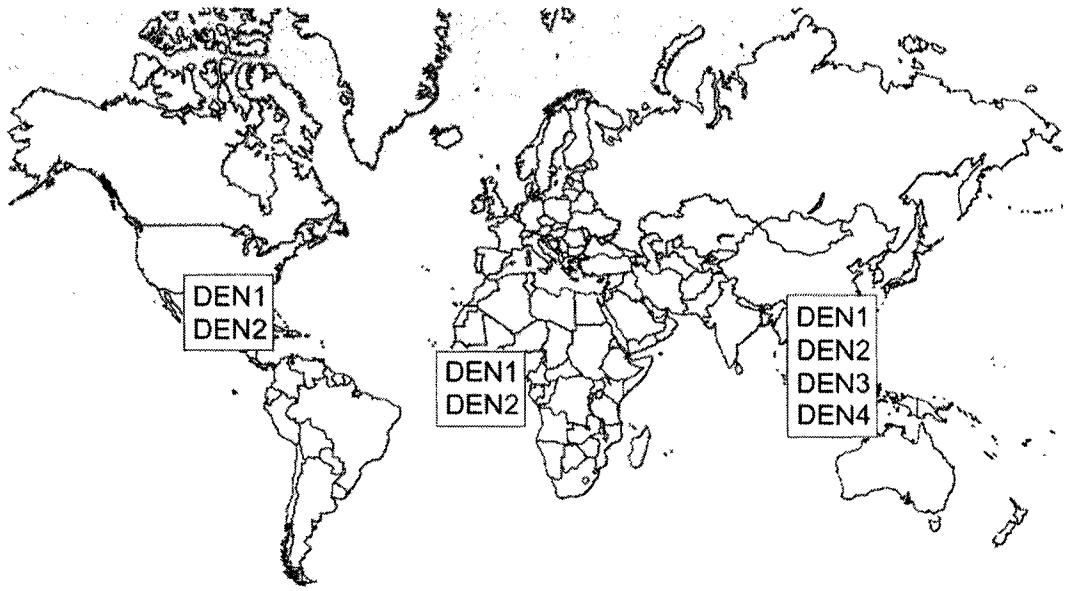

Figure 1.6. Global distribution of dengue virus serotypes, 1970. (From Mackenzie et al. 2004)

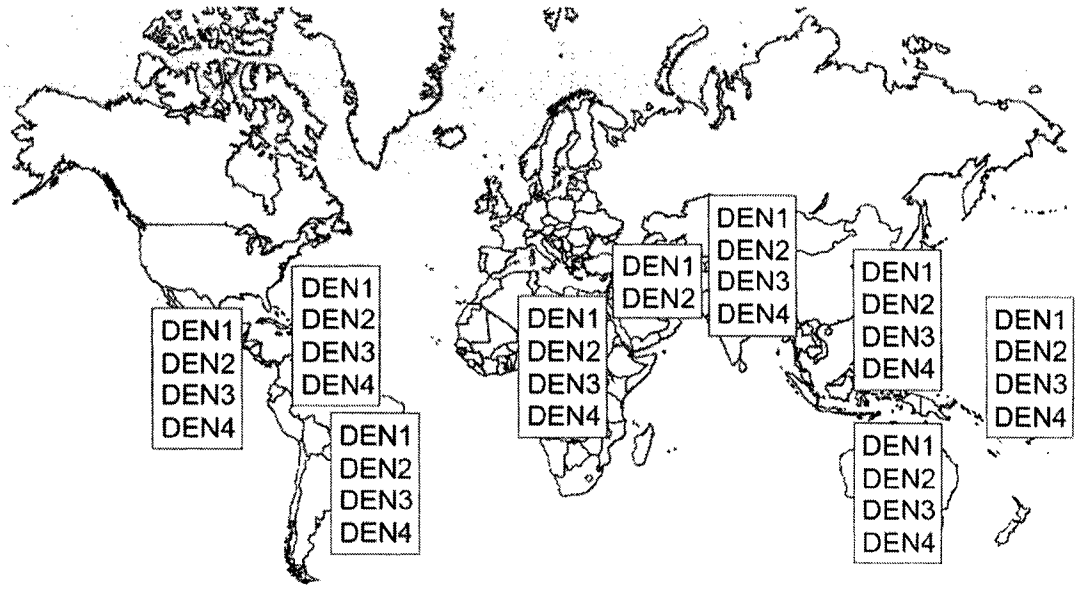

Figure 1.7. Global distribution of dengue virus serotypes, 2004. (From Mackenzie et al. 2004)

Dengue virus detection. Since the introduction of the first quantitative DENV assays in the early 1970s, the plaque assay (in various mammalian cell lines) and end-point titrations (in adult mosquitoes and mosquito cell lines) have been reliable standards for quantifying infectious DENV (Whitehead et al. 1971, Rosen and Gubler 1974, Kuberski and Rosen 1977, Tesh 1979, Schoepp and Beaty 1984). These assays are laborious and time consuming and take a week or more before results are available (Yamada et al. 2002, Bae et al. 2003). This limits the throughput of these assays and thus their utility in robust quantitative studies of virus replication and vector competence. Plaque formation from DENV infection is also restricted by the phenotype and passage history of the virus strain and the type and lot of cells employed (Rosen and Gubler 1974, Armstrong and Rico-Hesse 2001). Consequently, the relationship between plaque formation and infectious particles is known to vary as viruses become adapted to *in vitro* conditions (Mangada and Igarashi 1998). Nucleic acid detection assays provide a useful complementary method of estimating viral load by overcoming some of these limitations.

Real-time RT-PCR. Real-time RT-PCR protocols have gained acceptance as rapid, specific, and sensitive tools to study arbovirus-vector interactions (Lanciotti et al. 2000, Armstrong and Rico-Hesse 2001, Nasci et al. 2002, Armstrong and Rico-Hesse 2003, Bae et al. 2003, Kauffman et al. 2003, Lambert et al. 2003, Johnson et al. 2004, Vanlandingham et al. 2004, Farajollahi et al. 2005, Lambert et al. 2005, Molina-Cruz et al. 2005). A variety of fluorophores is now available for real-time RT-PCR. Of these, the dsDNA binding dye, SYBR Green I (Molecular Probes) is the most flexible and economical. When bound to dsDNA, the fluorescence of SYBR Green I is increased approximately 1000-fold providing the sensitivity required for assays of low starting

copy number targets (Morrison et al. 1998, Cosa et al. 2001). Unlike real-time assays that utilize TaqMan, hybridization probes, or molecular beacons, SYBR Green I is a nonspecific dsDNA binding dye which requires only two standard PCR oligonucleotide primers to measure fluorescence emission. This significantly reduces the cost of assay design and allows one to rapidly adapt protocols to new templates of interest without the requirement to design and purchase probes for each target sequence. SYBR Green I is ideal for assays targeting rapidly evolving RNA viruses since a single point mutation in the target region can reduce target detection by 47% in assays based on sequence specific probes (Papin et al. 2004). The nonspecific nature of SYBR Green I intercalation frequently results in low level background fluorescence from primer-dimers and other non-specific amplicons. A melting curve analysis of each sample facilitates verification of amplicon identity.

Specific aims. The overall objective of this study was to assess the utility of a quantitative real-time RT-PCR assay to study vector competence and the DENV-2-*Ae. aegypti* interaction. The ultimate goal was to understand the mechanisms that impact infection and escape from the mosquito midgut.

The specific aims were:

1. To develop and validate a SYBR Green assay for DENV-2, to compare the level of DENV-2 RNA to number of PFU, to quantify viral replication and dissemination during a 14 day EIP, and to evaluate the relationship between plus and minus strand DENV-2 RNA.

2. To characterize quantitative changes in DENV-2 in the midgut and in the head throughout the 14 day extrinsic incubation in *D2S3, D2MEB* and *Ibo11 Ae. aegypti*

strains. A secondary goal of this study was to test the hypothesis that increased transcription associated with bloodmeal digestion would result in increased midgut DENV-2 RNA levels.

3. To test the role of trypsins in DENV-2 infection, replication, and dissemination. The objectives of this aim were: (1) to evaluate the effect of inhibiting mosquito midgut trypsins with STI on DENV infectivity to *Ae. aegypti* during the digestion of a DENV-2 infected blood meal and (2) to assess whether *in vitro* digestion of DENV-2 in cell culture with bovine trypsin recovers infectivity to STI-treated mosquitoes.

CHAPTER 2

QUANTITATIVE ANALYSIS OF DENGUE-2 VIRUS RNA DURING THE EXTRINSIC INCUBATION PERIOD IN INDIVIDUAL *AEDES AEGYPTI* (CHETUMAL STRAIN) USING REAL-TIME RT-PCR

Introduction

Dengue viruses (genus *Flavivirus*, family Flaviviridae) include 4 antigenically distinct serotypes (DENV-1--4) transmitted among humans by *Aedes* mosquitoes, primarily *Ae. aegypti*. The changing epidemiology of DENV has spurred a global resurgence of both dengue fever (DF) and dengue hemorrhagic fever (DHF) with DF incidence estimated at 50--100 million cases per year and DHF ranking as a leading cause of pediatric hospitalization and death (Gubler 2002a).

Vector competence refers to the intrinsic ability of an arthropod to become orally infected and to support virus replication, dissemination, and transmission (Hardy et al. 1983). Understanding the relative vector competence of mosquitoes at the species, population, and individual level is critical to the study of vector biology and the success of future vector-borne disease control programs. *Aedes aegypti* populations vary in their competence for DENV (Gubler et al. 1979, Tabachnick and Powell 1979, Tabachnick et al. 1985, Tardieux et al. 1990, Bosio et al. 1998, Vazeille-Falcoz et al. 1999, Bennett et al. 2002, Failloux et al. 2002, Armstrong and Rico-Hesse 2003, Bennett et al. 2005). This is due, in part, to the presence or absence of midgut infection and/or midgut escape barriers (MIB, MEB) (Bosio et al. 1998, Bennett et al. 2002, Black et al. 2002). Vector

competence studies of DENV are typically qualitative in nature involving the detection of viral antigens in the head or legs. The vector competence of the experimental group is then estimated as the proportion of tested mosquitoes that develop a disseminated infection.

Dengue viruses have a single stranded, positive sense RNA genome. Replication involves three distinct RNA species: genomic (+) RNA, the antisense (-) copy of the genome, and partially double stranded RNA replicative intermediates (Cleaves et al. 1981, Chu and Westaway 1985, Chambers et al. 1990). The latter two species are only present while the virus is replicating (Vaughan et al. 2002). Asymmetrical strand replication results in a 1--2 $\log_{10}$ excess of (+) RNA compared to (-) RNA (Pogue et al. 1994, Wang et al. 2002). Quantification of (+) and (-) RNA during the course of infection is useful in assessing the kinetics of viral infection and replication. This approach has been used to quantify DENV (-) RNA in human peripheral blood mononuclear cells in which the amount of DENV (-) RNA correlates with plasma viral load (Wang et al. 2002).

Plaque assays and end-point titrations are the most accurate methods for quantifying infectious DENV virus (Whitehead et al. 1971, Rosen and Gubler 1974, Kuberski 1979, Tesh 1979). However, these assays are laborious and time consuming and require at least a week for completion (Yamada et al. 2002, Bae et al. 2003). Plaque formation from DENV infection can also be restricted by the phenotype and passage history of the virus strain and by the type and lot of cells employed in the assay (Rosen and Gubler 1974, Armstrong and Rico-Hesse 2003). Rapid advances in molecular biology have facilitated new approaches to evaluate quantitative aspects of vector

competence. In particular, quantitative real-time RT-PCR based assays provide the sensitivity, speed, and statistical power to conduct high throughput experiments. The precise quantitation of viral RNA from a sample of only 5 μL allows for the study of viral infection, replication, and dissemination in specific tissues in individual mosquitoes on a scale that was previously impractical.

Real-time RT-PCR assays have only recently been adapted to study virus-vector interactions (Lanciotti et al. 2000, Armstrong and Rico-Hesse 2001, Nasci et al. 2002, Armstrong and Rico-Hesse 2003, Bae et al. 2003, Kauffman et al. 2003, Lambert et al. 2003, Johnson et al. 2004, Vanlandingham et al. 2004, Farajollahi et al. 2005, Lambert et al. 2005, Molina-Cruz et al. 2005). Here we report the quantitation of DENV-2 RNA during the 14 day extrinsic incubation period in individual *Ae. aegypti*. A SYBR Green I based strand-specific, quantitative real-time RT-PCR assay was developed to quantify DENV-2 RNA from individual midgut and leg samples of *Ae. aegypti*. To test the validity of our assay, parallel plaque and real-time RT-PCR assays were conducted on infected C6/36 cell cultures and 3 groups of infected *Ae. aegypti*.

Materials and Methods

***Aedes aegypti* rearing and virus preparation.** Eggs from the F_5 generation of an *Ae. aegypti* collection from Chetumal, Mexico were hatched and reared in a controlled environment at 27°C, 80% relative humidity, and 12 hour photoperiod. An initial assessment showed that 85% of mosquitoes exposed *per os* to DENV-2 develop a disseminated infection. The *D2S3* strain, previously selected for high susceptibility to DENV-2, was used for the assay comparison experiments. Typically 95-100% of *D2S3* mosquitoes develop a disseminated infection (Bennett et al. 2005). Adults were kept in 1

liter waxed cardboard containers with water and sucrose. A high passage Jamaica 1409 strain of DENV-2 (Deubel et al. 1986) was grown in the *Ae. albopictus* C6/36 cell line (Bennett et al. 2002). In assessing the correlation between PFUs and DENV-2 RNA amounts, four additional DENV-2 genotypes were compared: American/Asian (Yuc14757), Cosmopolitan 96Mer (BC17), Asian 2 97 Acap (C932), American 94QRoo (BC-139). Strains, sources, and passage history of these viruses are listed in Table 2.1.

TABLE 2.1. Source of the DENV-2 strains used in this study.

Genotype	ID	Source	Year	Passage history
American	94 QRoo	Human isolate (BC-139) from Mexico. Acquired by the Centers of Disease Control and Prevention.	1994	Patient→ C6/36 → 2 (C6/36)
American/ Asian	Yuc 14757	Human isolate (14757) from the Centro de Investigaciones Hideyo Noguchi, Merida, Yucatan, Mexico.	2002	Patient → C6/36 → BHK → C6/36
Asian 2	97 Acap	Human isolate (C932) from the Instituto Nacional de Salud Publica, Cuernavaca, Mor., Mexico.	1997	Patient→ C6/36 → 3 (C6/36)
Cosmopolitan	96 Mer	Human isolate (BC-17) from Mexico. Acquired by the Centers of Disease Control and Prevention.	1996	Patient→ C6/36 → 2 (C6/36)
Prototypic	Jam 1409	Laboratory prototypic DENV-2 strain initially isolated from a patient in Jamaica.	1983	High passage

The passage history includes the original source of the virus and the number of times it was passaged in a specific cell line prior to this study. Phylogenetic analysis of genotypes was based on the prM-E viral region (Lorono-Pino et al. 2004). The DENV-2 Yuc 14757 strain was a new isolate not included in the original phylogenetic analysis.

Mosquito infections. Sucrose and water were removed 36 hours prior to oral challenge. Water-jacketed membrane feeders were covered with hog intestine and placed on containers with 3 to 4 day old mosquitoes. Defibrinated sheep blood was mixed with the virus suspension (1:1) and ATP was added at a final concentration of 1 mM. Mosquitoes were allowed to feed for 45--60 min on a 37°C blood meal with final

infectious virus titer of $10^{9.5}$ tissue culture infectious dose 50% ($TCID_{50}$)/mL (Reed and Muench 1938). Mosquitoes that failed to feed to repletion were discarded.

Mosquito dissection and RNA extraction. Ten *Ae. aegypti* were collected daily, chilled and dissected live in 20 μL of Tris buffered saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl) on a chill table at ~4°C. Midguts and legs were placed in separate 1.5 mL microcentrifuge tubes containing 100 μL of a denaturing guanidine isothiocyanate buffer (RLT lysis buffer, Qiagen, Valencia, CA) to inactivate RNAses. Samples were snap frozen and stored at -80°C. RNA was extracted using RNeasy total RNA extraction kits (Qiagen) and eluted in 50 μL of RNAase free water.

Quantitation of total RNA. The RiboGreen RNA-Specific Quantitation Assay (Molecular Probes, Eugene, OR) was used to quantify total RNA extracted from each mosquito to assess variation in amounts of starting tissue and in RNA extraction efficiency (Jones et al. 1998). The published protocol was modified to enable RNA quantitation using the Opticon Monitor fluorescence detection platform (MJ Research, Waltham, MA) that was also used in the real-time RT-PCR assay (Hashimoto et al. 2004). The Opticon Monitor was calibrated for a 10X RiboGreen dye solution (60 μL per well) prepared with 1X TE and the 3200X DMSO stock solution provided by the manufacturer. Sample RNA was treated with the DNAase provided with the RiboGreen kit. A baseline was generated for each run by detecting background fluorescence in wells with 51 μL per well of 10X dye. The program was set to 60 s at 37°C, followed by 5 cycles for 20s at 37°C each, followed by a fluorescence reading. The detection protocol was paused, 9 μL of the DNAase treated RNA samples and standards were added to 51 μL of 10X RiboGreen in triplicate, and the run was completed with 10, 20 s cycles at

37°C each followed by a fluorescence reading. Data were exported into Microsoft Excel where baseline values were subtracted from sample readings. Fluorescence of the RNA standards was regressed on RNA amounts to derive a standard curve for quantification of RNA in mosquito tissues.

cDNA synthesis. DENV-2 specific primers targeted a 177 bp region of the NS5 gene and have proven reliable in real-time RT-PCR studies of DENV-2 (Laue et al. 1999, Molina-Cruz et al. 2005). This gene codes for the RNA dependent RNA polymerase, the most conserved of the DENV proteins (Chambers et al. 1990). Primer sequences were: DENV-2 NS5 forward (5'ACAAGTCGAACAACCTGGTCCAT 3') and DENV-2 NS5 reverse (5' GCCGCACCATTGGTCTTCTC 3'). The mfold web server (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/)(Zuker 2003) was used to identify potential secondary structures that might reduce primer binding. First strand synthesis reactions contained 0.625μM either forward, reverse, or paired primers and 0.20mM dNTP mix, 5 μL of template RNA, and DEPC treated H_2O to a final volume of 10.9 μL. To minimize inhibition of SYBR Green I, DTT was excluded (Lekanne Deprez et al. 2002). Reactions were heated to denature dsRNA complexes (86°C for 15 min) and brought to 0°C on an Eppendorf PCR-Cooler (Eppendorf, Hamburg, Germany). A total of 4 μL 5X First-Strand Buffer and 0.1 μL SuperScript II Reverse Transcriptase (RT) (Invitrogen, Carlsbad, CA) were added to bring the reaction volume to 20μL. cDNA was synthesized at 42°C for 50 min. To quantify samples for DENV-2 (-) RNA, only the forward primer was added to synthesize cDNA from the (-) RNA strands. Conversely, only the reverse primer was used during the RT step for samples to be measured for (+) RNA. Both primers were then used in the subsequent PCR reactions to amplify the

cDNA pools generated from a single primer. The RT was inactivated with a 15 min incubation at 95°C. Controls without primers were included to confirm RT inactivation.

Preparation of DENV-2 Standards. The NS5 fragment of DENV-2 (JAM1409) was amplified and cloned into the pCR 2.1 plasmid using the TA cloning kit (Invitrogen). Inserts were sequenced to confirm presence of the target fragment. The HiSpeed Plasmid Midi Kit (Qiagen) was used for large-scale preparation and purification of the DENV-2 NS5 plasmid. Following quantification by UV spectrophotometry, the plasmid was diluted to 10^8 plasmids/μL for use in a 10-fold dilution series. Aliquots were stored at -80°C with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO).

DENV-2 quantitative real-time RT-PCR. The PCR mixture contained 1X DyNAzyme buffer (Finnzymes, Espoo, Finland), 0.2 mM dNTP mix, 0.5X SYBR Green I, 2.5 mM $MgCl_2$, 0.25 μM forward and reverse primers, 0.4U DyNAzyme II Recombinant DNA Polymerase (Finzymmes), and 2 μL cDNA in a final volume of 20 μL. SYBR Green I was obtained in a 10,000X stock concentration, diluted in DMSO to a working stock of 100X, and stored at -20°C in 15 μL aliquots to minimize exposure to light and freeze-thaw cycles.

Reactions were run in Microseal 96 Microplates covered with optically clear caps (MJ Research) and centrifuged at 3000 rpm for 5 min. Opticon 2 thermal cycling settings were: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec, 64°C for 20 sec, 72°C for 30 sec, and 84°C for 1 sec for fluorescence measurement. After a final extension at 72°C for 10 min, a melting curve was obtained with the program: 70°C to 95°C, 0.2°C/read, 1 sec hold and the negative first derivative of the dissociation rate (-dF/dT) was used to confirm product specificity. This protocol was used to measure both (+) and

(-) DENV-2 RNA. Standard curves were generated on each plate by analyzing 2 x 10^2--2 x 10^8 copies/reaction of DENV-2 plasmid standards. All cDNA samples and plasmid standards were assayed in triplicate.

Fluorescence generally followed a logistic curve increasing with each PCR cycle. The point at which fluorescence had increased ~50% over background is the fluorescence threshold. This was subsequently set at 0.040 (-1.40 $\log_{10}$) for all assays. The cycle threshold (C_T) is the number of cycles along the abscissa at which fluorescence crosses this threshold. Amplification efficiency (E) was calculated from the slope of the linear regression of C_T versus the $\log_{10}$ template copy number where:

$$E = 10^{(-1/\text{slope})} - 1.$$

An E of 1 corresponds to 100% efficiency. E was derived using a 10-fold dilution series of sample cDNA and DNA standards. Assay repeatability was estimated using the coefficient of variation ($CV = (\sigma_x / \bar{x}) \times 100$) among triplicate samples within a 96-well plate and among the same standards run on 10 different plates. To assess variation in the efficiency of cDNA synthesis, we estimated variation in the reverse transcription and PCR reactions separately (Freeman et al. 1999). Triplicate cDNA synthesis reactions on DENV-2 positive and negative control mosquito RNA samples were performed followed by quantitative real-time PCR reactions in triplicate for each cDNA pool.

Plaque assays. Plaque and real-time RT-PCR assays were performed in parallel on DENV-2 infected *Ae. aegypti* (*D2S3*) and C6/36 cells to assess the relationship between DENV-2 RNA copy number and numbers of infectious virus particles. Mosquitoes were infected with the high passage, JAM1409 strain and the C6/36 cells were infected with one of five DENV-2 strains: JAM 1409, Yuc14757, 96Mer, 97 Acap,

94QRoo (Table 2.1). Frozen mosquitoes were thawed, triturated individually in 750--1000 μL of minimal essential medium (MEM) containing 2% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), centrifuged at 14,000 rpm for 3 min, and the supernatant was filtered through a 0.22 μm syringe filter. RNA was extracted from an aliquot of each sample using the QIAamp viral RNA extraction kit (Qiagen) and a separate aliquot was used for the plaque assay. Confluent cultures of LLC-MK2 cells were grown in MEM with Earl's salts and supplemented with 10% FBS, 2 mM L-glutamine (Invitrogen, GIBCO Products), 1X nonessential amino acids solution (Cellgro) (Mediatech, Herndon, VA), 100 U/mL of penicillin and 100 μg/mL of streptomycin, and maintained in a CO_2 incubator at 37°C. Ten-fold dilutions of each sample were incubated for 1 h on LLC-MK2 cell monolayers in 6-well plates with constant rocking. The first overlay was then added (1% agar, 1X Earl's balanced salts solution, 0.066% yeast extract, 0.33% lactoalbumin hydrolysate, 2% FBS, 0.22% sodium bicarbonate, 50 μg/mL of gentamycin, and 2 μg/mL of fungizone). Following solidification of the initial overlay, 6-well plates were inverted and incubated for 7 days at 37°C in a CO_2 incubator. A second overlay was then added containing 0.125% neutral red and plaques were counted after 48 hrs of incubation.

Results

DENV-2 quantitative real-time RT-PCR. Figure 2.1A is output from the Opticon 2 monitor software version 2.02 that shows fluorescence increasing as the number of RT-PCR cycles proceeds. Each curve corresponds to an individual well containing from 20 - 2 x 10^8 copies of plasmids. The increase in fluorescence with increasing numbers of cycles follows a logistic curve. The point at which fluorescence

had increased ~50% over background was the fluorescence threshold and was set at 0.040 (-1.40 $\log_{10}$) for all assays (Figure 2.1A). C_T is the number of cycles along the abscissa at which fluorescence crosses the threshold. Figure 2.1B is the regression of C_T against starting concentration of plasmid standards. Note that the regression is straight over a 7 $\log_{10}$ dynamic range.

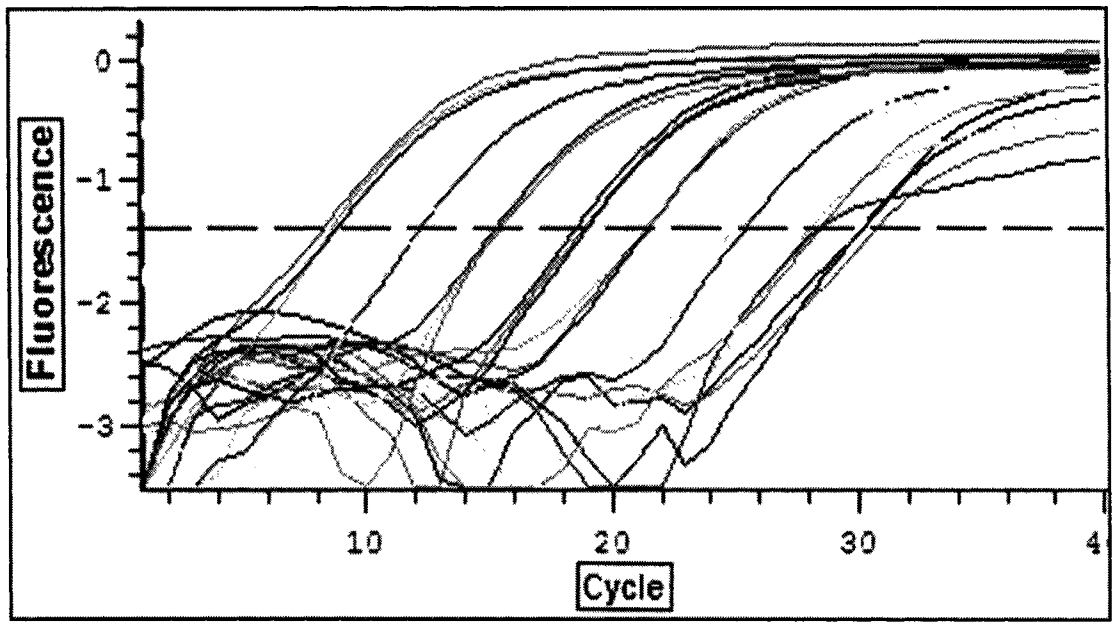

Figure 2.1. A Fluorescence with increasing RT-PCR cycles in individual wells containing 2 x 10^1--2 x 10^8 plasmids as standards.

Melting curve analyses are presented in Figure 2.2. In these analyses, fluorescence decreases as double stranded DNA melts with increasing temperature. Melting peaks are plotted by calculating the negative first derivative of the change in fluorescence over time (-dF/dT) (Ririe et al. 1997). Figure 2.2A shows both the melting profile and the -dF/dT curves of RT-PCR products arising from 10 different positive midguts at 1 dpi. A single -dF/dT peak appears at ~86°C. To more completely define the shape of the melting

curve, the software also calculates half the height of the -dF/dT peak and then measures the width of the -dF/dT peak at this height (~1.6°C). Figure 2.2B shows the -dF/dT profiles of primer-dimers that arise when $\leq$20 plasmid copies are used. The peaks are broader (~4.5°C) and occur well below ~86°C. Figure 2.2C is the profile of two negative control RNA samples alongside a product from a DENV-2 positive control.

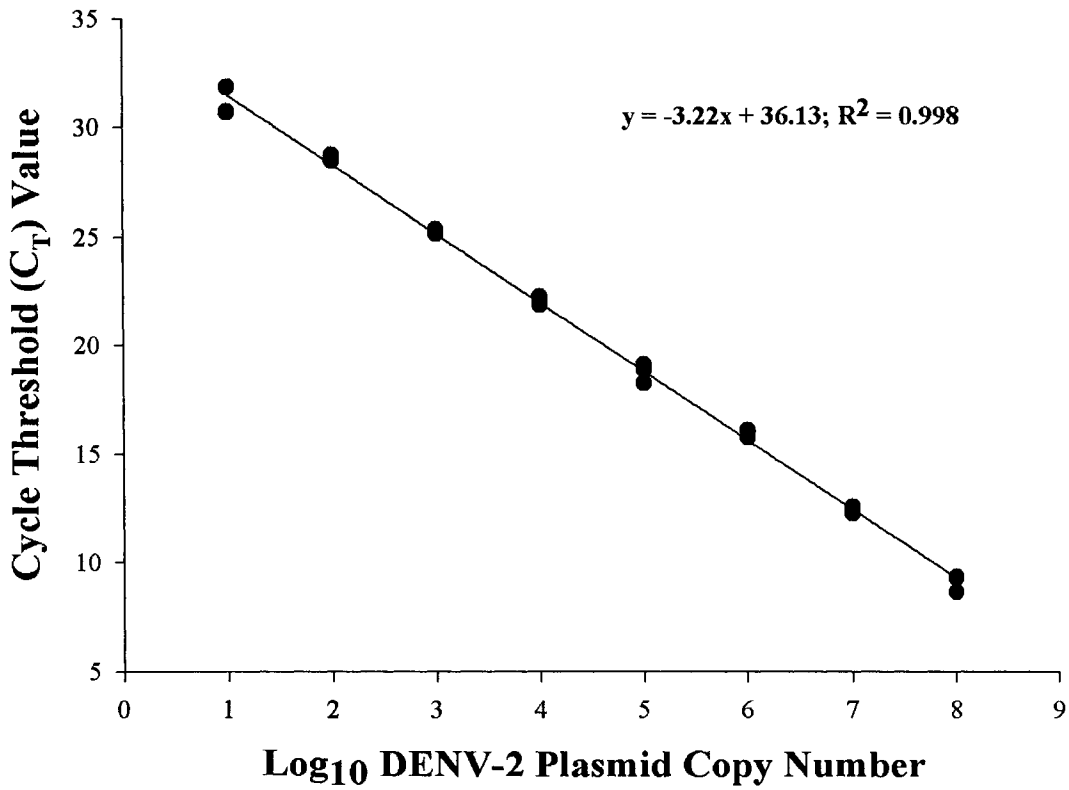

Figure 2.1. B Linear regression analysis of C_T as a function of $\log_{10}$ 2 x 10^1-2 x 10^8 plasmids.

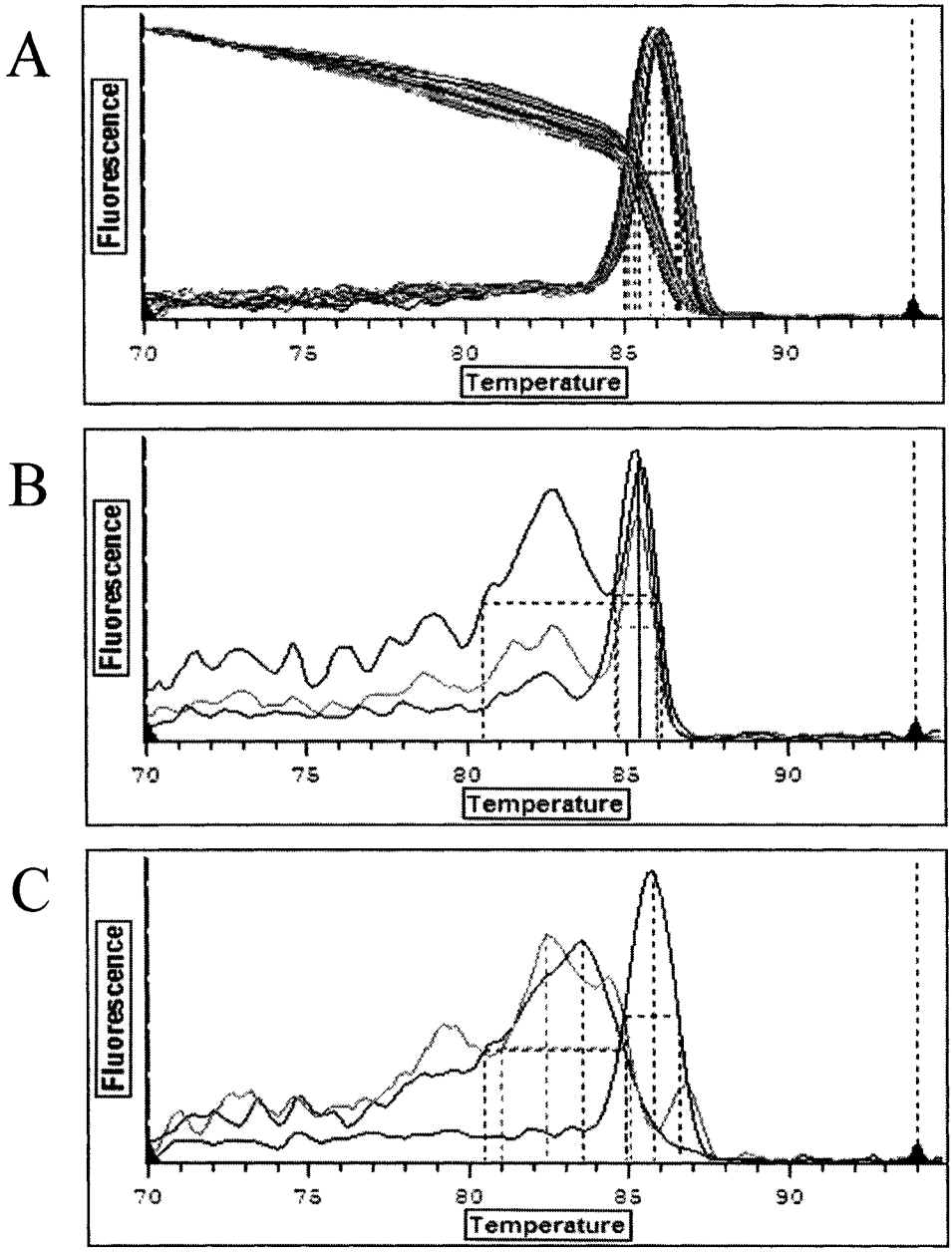

FIGURE 2.2. Melting Curve Analyses. Fluorescence decreases as double stranded DNA melts with increasing temperature. These plots represent negative first derivative of the dissociation curves to test for product specificity. A) Peaks of 10 positive midguts at 1 dpi. B) Melting profiles of primer-dimers between 80--84°C that arise when 2 x 10^1 plasmid standards are used. C) Two negative control RNA samples alongside a peak at 86°C arising from a DEN2 positive control.

Estimates of the variation in C_T values attributed to the cDNA synthesis and PCR steps are summarized in Table 2.2. Repeatability in C_T values was compared among cDNA syntheses starting with RNA from 3 infected mosquitoes. Among three real-time PCR reactions all prepared from a single cDNA synthesis of an infected mosquito, the CV averaged 2.78% (range = 1.71--3.59, SD = 0.58). Among, three independent cDNA syntheses from a single mosquito, the CV averaged 2.1% (range = 1.96--2.35, SD = 0.21). The CVs of the two steps did not differ significantly ($p = 0.087$). The high C_T values from negative and no RNA controls was due to nonspecific fluorescence (likely primer-dimers) commonly observed with SYBR Green based assays.

The quantity of DENV-2 (JAM1409) NS5 plasmids contained in a 10-fold serial dilution was compared with the relative amount of cDNA contained in a 10-fold serial dilution as estimated by real-time PCR. This was done to test for non-linearity when assessing quantity of DENV-2 (JAM1409) NS5 with plasmid standards as compared with cDNA. The slopes of plasmid amounts (-3.47 ± 0.16) and cDNA amounts (-3.28 ± 0.10) were not significantly different. The mean E of 0.94 for the DENV-2 DNA plasmid standards (n = 30, 10^2--10^8) was similar to 1.02 for the cDNA samples (n = 3, undiluted--10^{-4}).

The reproducibility of quantitative, real-time PCR assays was assessed by calculating the CV of three C_T estimates among plasmid standards loaded and analyzed on the same plate. These CVs were then compared with the CVs of C_T among the same plasmid standards but loaded and analyzed on different plates on different days. The CVs estimates were plotted against $\log_{10}$ plasmid copies/20 μL reaction (Figure 2.3).

TABLE 2.2. Comparison of variation in cDNA synthesis and amplification steps.

Mosquito #[a]	RNA aliquot	C_T[b]	qPCR CV[c]	C_T[d]	RT CV
1	A	20.32 ± 0.48	2.38%	20.26 ± 0.48	2.35%
	B	20.70 ± 0.62	3.02%		
	C	19.75 ± 0.47	2.39%		
2	A	18.80 ± 0.68	3.59%	19.21 ± 0.38	1.96%
	B	19.28 ± 0.60	3.12%		
	C	19.54 ± 0.64	3.25%		
3	A	20.27 ± 0.50	2.47%	20.72 ± 0.42	2.03%
	B	20.79 ± 0.36	1.71%		
	C	21.11 ± 0.65	3.09%		
Mean[e]			2.78 ± 0.58%		2.11 ± 0.21%
Neg control	A	34.24 ± 1.53	4.46%	32.61 ± 1.41	4.31%
	B	31.84 ± 0.31	0.96%		
	C	31.77 ± 0.04	0.11%		
No RNA	A	33.92 ± 1.24	3.66%	33.10 ± 0.73	2.21%
	B	32.52 ± 0.06	0.20%		
	C	32.85 ± 1.16	3.53%		

[a] Numbers represent individual infected mosquitoes. Letters represent aliquots of RNA used in the cDNA synthesis step.
[b] Mean cycle threshold values ± standard deviation of three wells with the same cDNA sample.
[c] Coefficient of variation.
[d] Mean cycle threshold values ± standard deviation of three different cDNA samples from the same mosquito. Fluorescent signals from negative and no RNA controls were nonspecific (likely primer-dimers) according to melting curve analyses.
[e] ANOVA of CV values of the two steps was not significant ($p = 0.087$)

The accuracy of copy number estimates was reduced in samples with <100 copies of DENV-2 plasmid and non-specific peaks were revealed in the melting curves of the 10^1 standard. The intra-plate CV was 5.4% with 100 plasmid copies but then fell to ~1%

with 2 x 10^3--2 x 10^7 copies. Among plates CVs were slightly higher than CVs within-plates. These results are comparable to other published real-time RT-PCR flavivirus assays (Yang et al. 2002, Kauffman et al. 2003, Letellier and Kerkhofs 2003, Castelain et al. 2004).

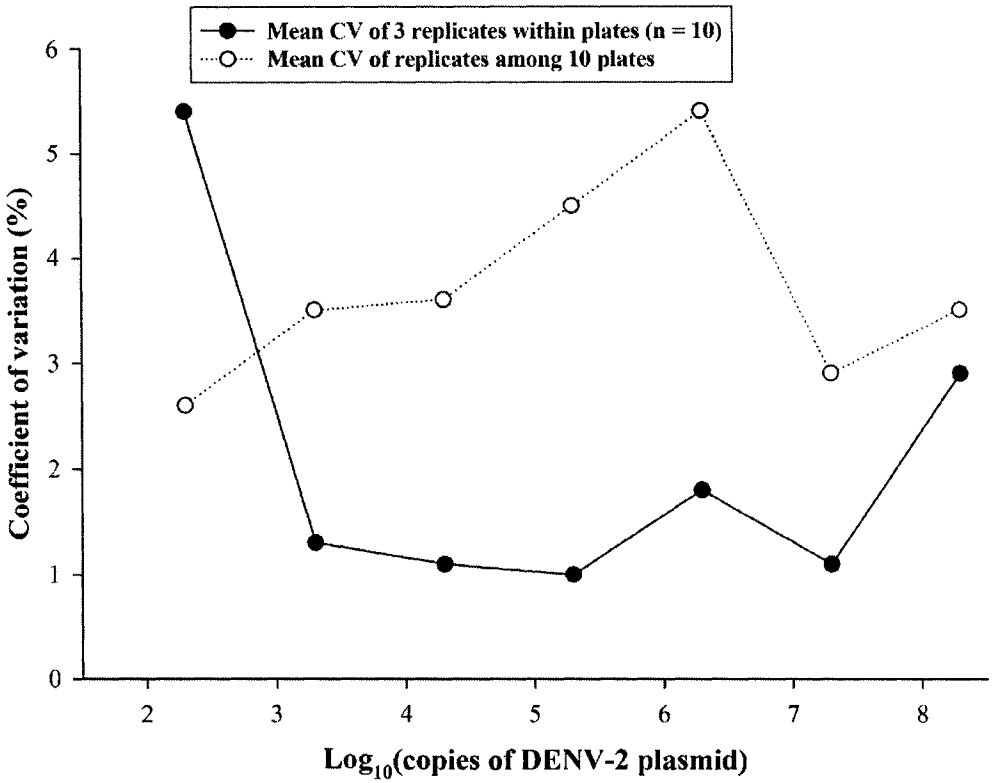

FIGURE 2.3. Reproducibility of DENV-2 quantitative, real-time RT-PCR assays. The CV of three C_T estimates was calculated among plasmid standards loaded and analyzed on the same plate. These CVs were then compared with the CVs of C_T among the same plasmid standards but loaded and analyzed on different plates on different days.

RNA quantitation assay. Mosquitoes differed significantly in total midgut RNA levels within each timepoint and between daily means (ANOVA, $p < 0.0001$) (Figure 2.4). Total RNA levels in midguts over the 14 dpi ranged four fold from ~ 500--2000 ng/midgut. Total RNA from midguts presented a cyclical pattern with alternating peaks

(1, 8 and 14 dpi) and drops (0, 2--3 and 11--12 dpi). This fluctuating pattern suggests major metabolic changes in the midgut. Transcription during the first days is likely associated with blood digestion. Mosquitoes were not given a second blood meal nor was gonotrophic status recorded in individual females. The relationship of physiological processes to fluctuations in total RNA amounts are therefore unknown.

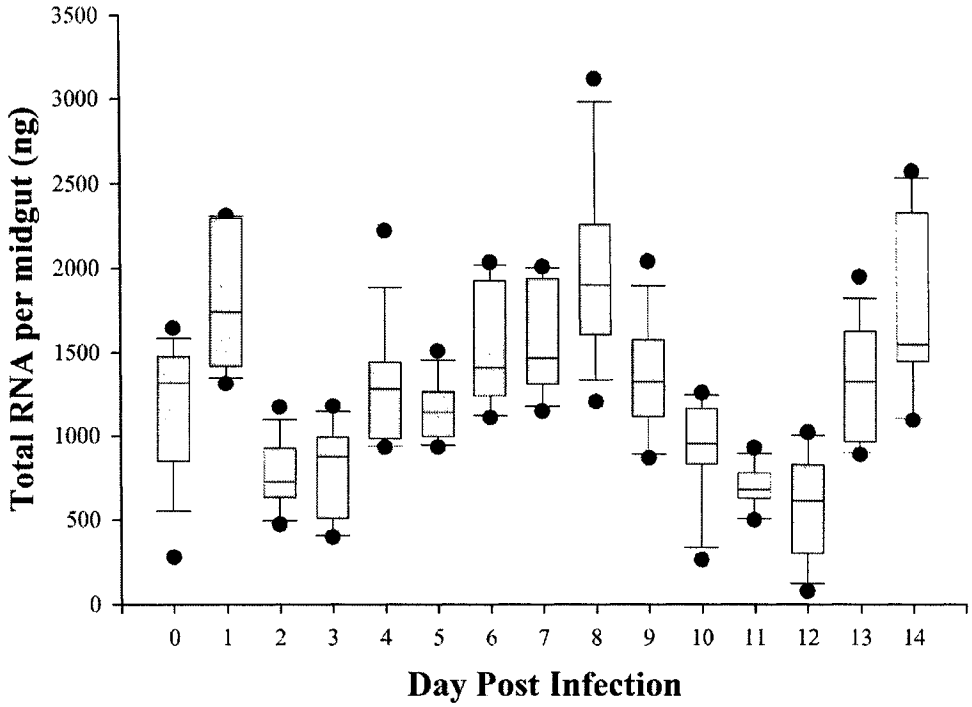

FIGURE 2.4. Total mosquito RNA estimated with the RiboGreen assay. RNA from 10 midguts was quantified on each day. Whisker caps represent 10^{th} and 90^{th} percentiles. Box limits represent 25^{th} and 75^{th} percentiles. Lines within the boxes mark the median. Circles indicate the 5^{th} and 95^{th} percentiles. Day 0 corresponds to mosquitoes dissected immediately after they blood fed.

Real-time RT-PCR and plaque assays. Plaque and real-time RT-PCR assays were performed in parallel on DENV-2 infected C6/36 cells and *Ae. aegypti* to assess the

relationship between DENV-2 RNA and numbers of infectious virus particles. Among the 5 DENV-2 genotypes the average rate of DENV-2 RNA copies/PFU was 1,592 with a range of 135--4,019. Figure 2.5 shows the $\log_{10}$ of the number of DENV-2 RNA copies as determined by real-time RT-PCR regressed on PFU. The regression curve lies above the RNA copy = PFU line (shown in the lower right hand side of the graph) and the *y*-intercept was significantly > 0 ($p \leq 0.0001$). In general, viral RNA levels overestimated PFUs by ~ 2--3 logs. The slope of the untransformed data (ΔRNA/ΔPFU) was 80.0 suggesting that among the 5 DENV-2 genotypes there were 80 viral RNA copies/PFU or that 80 in 81 copies (98.8%) were not detected as infectious particles in the plaque assay.

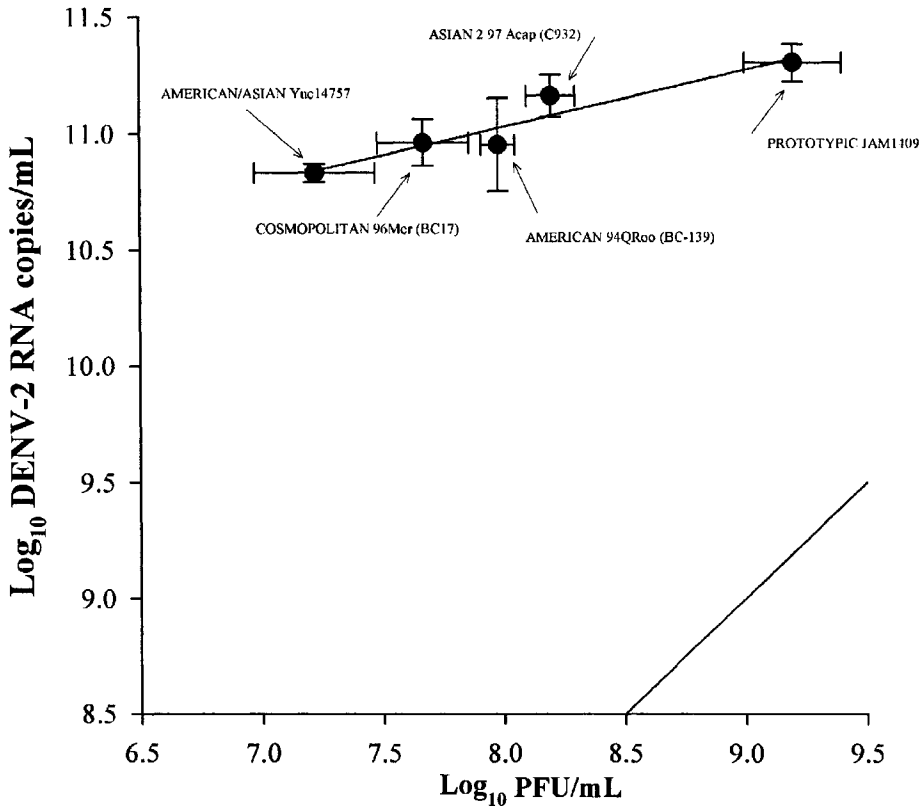

FIGURE 2.5. Plaque assay vs. DENV-2 RNA copies with 5 different DENV-2 genotypes grown in C6/36 cells. The regression equation is DENV-2 copies = 0.242 PFU + 9.09 ($R^2 = 0.91$). Both slope and intercept are significantly greater than 0.

Figure 2.6 shows a similar regression in RNA collected from DENV-2 infected *D2S3 Ae. aegypti* and fed upon DENV-2 preparations from two different dates. Again, the regression curves lie above the RNA copy = PFU line and the *y*-intercepts were significantly > 0 ($p \leq 0.0001$). The slopes and *y*-intercepts were not significantly different between DENV-2 virus preparations. In the first infection, the average rate of DENV-2 RNA copies/PFU was 367 with a range of 72--2,147 while in the second infection, the average rate of DENV-2 RNA copies/PFU was 12,227 with a range of 2,294--69,063. The slope of the untransformed data (ΔRNA/ΔPFU) varied from 59--

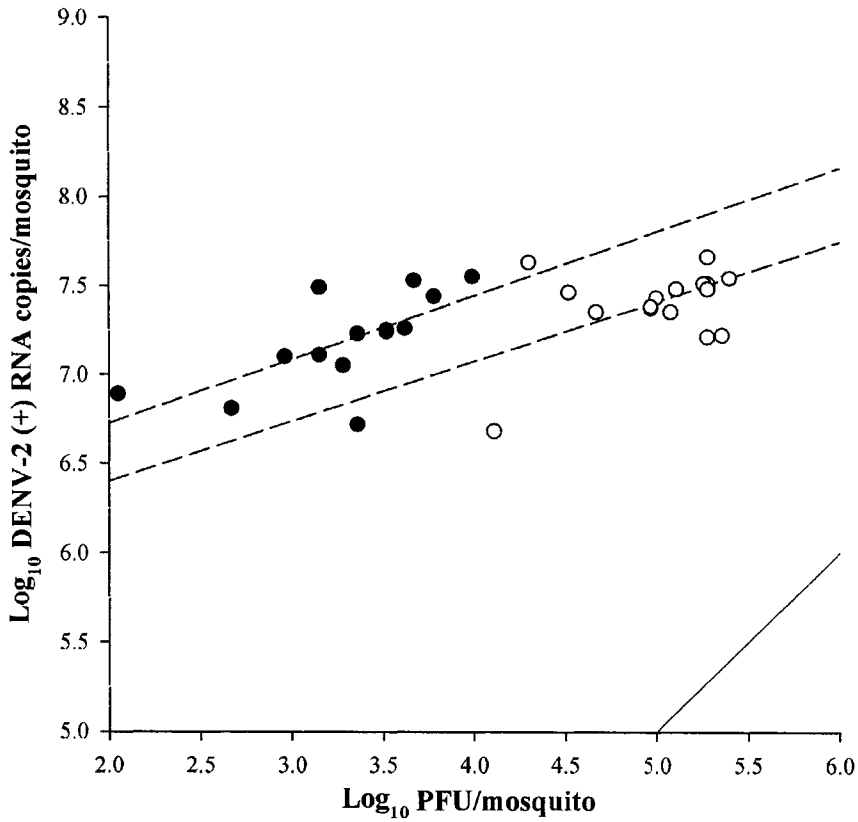

FIGURE 2.6. Plaque assay vs. DENV-2 RNA copies in 2 replicate groups of *D2S3 Ae. aegypti* mosquitoes. The regression equations are: DENV-2 copies = 0.358 PFU + 6.01 ($R^2 = 0.45$) and DENV-2 copies = 0.335 PFU + 5.73 ($R^2 = 0.36$).

2,954 RNA copies/PFU suggesting that ~ 98.3--99.9% of the viral RNA measured by real-time RT-PCR was not detected as infectious particles in the plaque assay.

Dengue virus extrinsic growth kinetics and strand-specific detection. The amount of DENV-2 RNA (+) and (-) strands in midgets over the course of the extrinsic incubation period are shown in Figure 2.7 and in legs in Figure 2.8. DENV-2 RNA levels in midguts over the 14 dpi ranged 3 orders of magnitude from ~ 10^3--10^6 copies/midgut. In comparison, total RNA varied only 4 fold (Figure 2.4) thus adjusting for total RNA had an imperceptible effect and in the remaining analyses, neither DENV-2 RNA levels nor PFU are standardized on total RNA.

DENV-2 RNA (+) and (-) in the midgut decreased sharply during the "eclipse" phase of infection between 0 and 2 dpi. The detection of (-) DENV-2 RNA during this period probably reflects replicative intermediates in the C6/36 cell suspension used for the infectious blood meal. A replication phase with steadily increasing viral RNA levels was observed from 2--6 dpi. At this point DENV-2 RNA replication in the midgut fluctuated from 6--9 dpi. Viral RNA copy number then steadily increased from 10--14 dpi. Viral RNA copy number varied significantly among mosquitoes sampled at each time-point (Table 2.3). There was a consistent relationship between (+) and (-) RNA over the 14 days ($r = 0.87$--0.99 between 3--14 dpi) (Table 2.3).

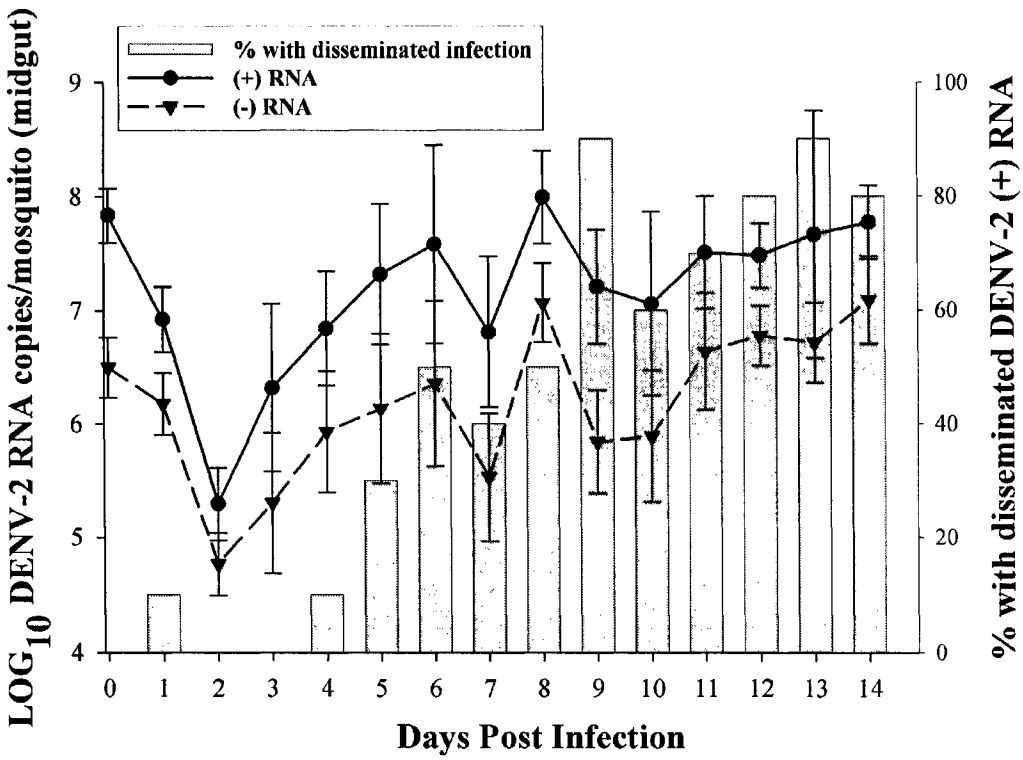

FIGURE 2.7. Quantitative analysis of DEN2 replication in *Aedes aegypti* (Chetumal strain) midguts. One percent of a midgut was used per reaction. Y-error bars represent the standard deviation of DENV-2 RNA copies in the mosquitoes testing positive on each day. Bars indicate percentage of mosquitoes with a disseminated infection (DENV-2 RNA detected in legs). Refer to Table 2.3 for number of positive midguts at each timepoint. Day 0 corresponds to mosquitoes dissected immediately after they blood fed.

In the disseminated infection (leg) assay (Figure 2.8), 1 of 10 mosquitoes tested positive for both strands on day 1. At 4 dpi, a single mosquito demonstrated (+) but not (-) RNA (Table 2.3). After 4 dpi the number of positive samples and the level of (+) RNA increased gradually. In comparison with (+) RNA, detection of (-) RNA was delayed and the rate of increase in (-) RNA was lower. There was a significant positive correlation between (+) and (-) RNA detected in legs after 8 dpi (Table 2.3). Prior to day

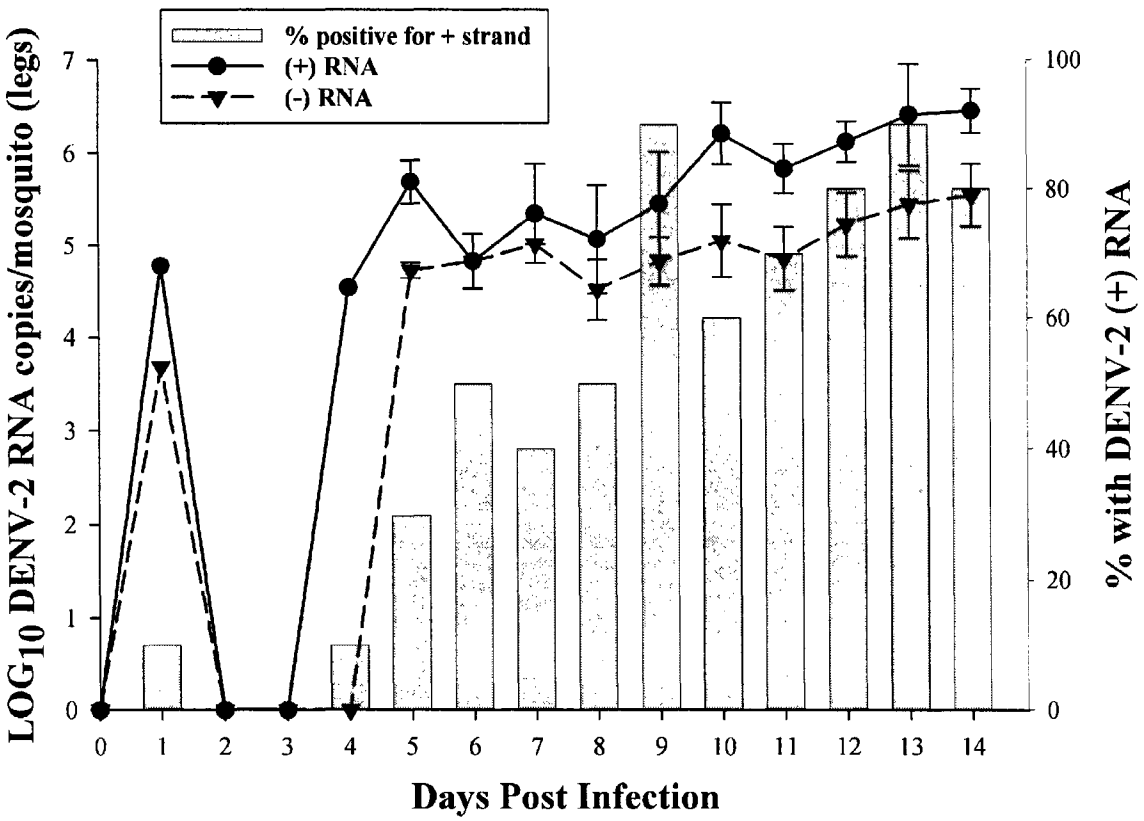

FIGURE 2.8. Quantitative analysis of DENV-2 replication in leg tissue from individual *Aedes aegypti* (Chetumal strain). Y-error bars represent the standard deviation of DENV-2 RNA copies in the mosquitoes testing positive for each day. Bars indicate percentage of mosquitoes with DENV-2 (+) RNA in legs. Refer to Table 2.3 for the number of positive mosquitoes at each time point.

8, there were not enough infected leg samples to test for a correlation. In contrast to midgut samples, (-) RNA was detected in a few of the (+) RNA positive legs until 12 dpi.

The amount of DENV-2 (+) RNA in midguts and legs over the course of the extrinsic incubation period are plotted in parallel in Figure 2.9. The amount of DENV-2 (+) RNA in legs was correlated with the dpi ($r = 0.82$, $p \leq 0.001$). In contrast, the amount of DENV-2 (+) RNA in midguts was not correlated with the dpi ($r = 0.48$, $p \geq 0.05$) and was only weakly correlated with DENV-2 RNA (+) strands in legs ($r = 0.55$, $p \leq 0.05$).

Furthermore, DENV-2 (+) RNA in midguts and legs were independent if data from 0--3 dpi were removed ($r = 0.29$, $p \geq 0.05$).

TABLE 2.3. Summarized results of real-time RT-PCR quantification of (+) and (-) DENV-2 RNA in midgut and leg samples of individual mosquitoes over a 14 day extrinsic incubation period.

	Midgut samples			Leg samples		
	(+) Strand Assay	(–) Strand Assay		(+) Strand Assay	(–) Strand Assay	
Day pi	Number (% positive) p-value[a]	Number (% positive) p-value	Strand Correlation (r)[b]	Number (% positive) p-value	Number (% positive) p-value	Strand Correlation (r)
0	10 (100)****	10 (100)****	0.83	0 (0) NA	0 (0) NA	NA
1	10 (100)****	10 (100)****	0.71	1 (10) NA	1 (10) NA	NA
2	9 (90) ****	9 (90) ****	0.64	0 (0) NA	0 (0) NA	NA
3	10 (100) ****	10 (100) ****	0.93	0 (0) NA	0 (0) NA	NA
4	8 (80) ****	8 (80) ****	0.99	1 (10) NA	0 (0) NA	NA
5	8 (80) ****	8 (80) ****	0.97	3 (30) **	2 (20)	NA
6	10 (100) ****	10 (100) ****	0.96	5 (50) *	1 (10) NA	NA
7	10 (100) ****	10 (100) ****	0.87	4 (40) ****	2 (20)	NA
8	9 (90) ****	9 (90) ****	1.00	5 (50) ****	3 (30) **	0.87
9	10 (100) ****	10 (100) ****	0.95	9 (90) ****	7 (70) **	0.91
10	9 (90) ****	9 (90) ****	0.98	6 (60) ****	6 (60) ****	0.90
11	10 (100) ****	10 (100) ****	0.92	7 (70) ****	7 (70) ****	0.96
12	8 (80) ****	8 (80) ****	1.00	8 (80) ****	8 (80) ****	0.94
13	10 (100) ****	10 (100) ****	0.99	9 (90) ****	9 (90) ****	0.95
14	8 (80) ****	8 (80) ****	0.99	8 (80) ****	8 (80) ****	0.97

[a] p values, ANOVA of DENV-2 RNA copy number of positive mosquitoes samples for each day. Note: day 0 samples were taken immediately after the blood meal.
NA – no correlation could be calculated due to < 3 positive mosquitoes
* $p \leq 0.05$
** $p \leq 0.01$
*** $p \leq 0.001$
**** $p \leq 0.0001$

[b] Pearson correlation coefficient between (+) and (–) strand DENV-2 RNA copy number within individual mosquitoes.

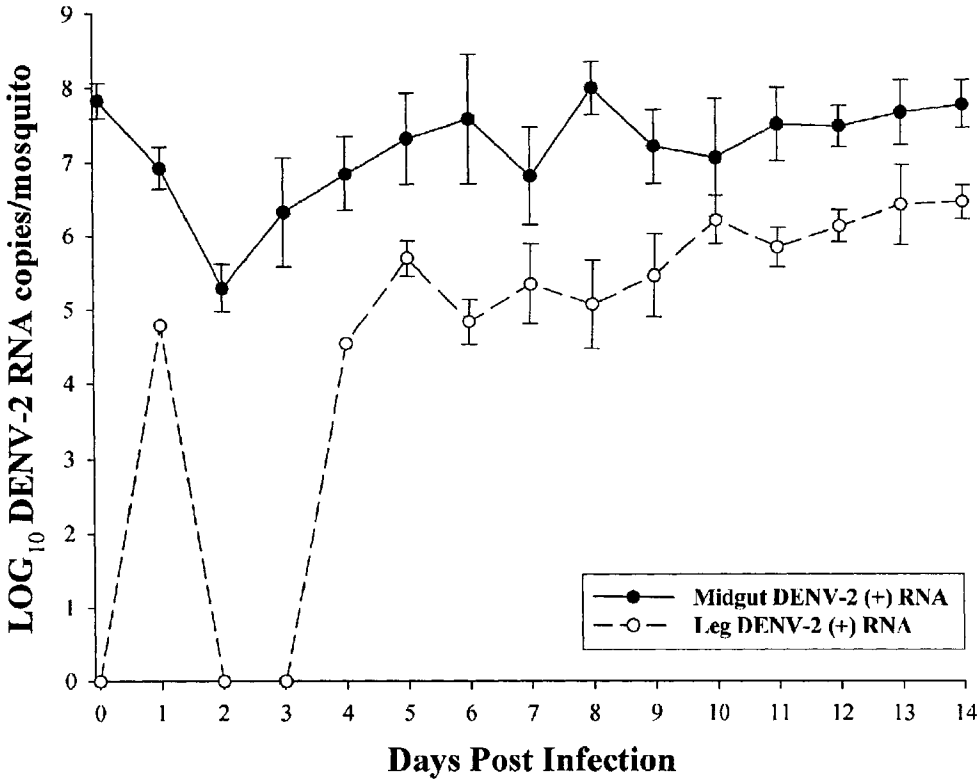

FIGURE 2.9. Comparison of DENV-2 (+) RNA in midguts and legs over the course of the extrinsic incubation period.

Discussion

SYBR Green I is a nonspecific dsDNA binding dye that only requires design of conserved oligonucleotide primers to measure rates of increase in fluorescence during PCR. SYBR Green I based assays therefore facilitate development of protocols to detect novel arboviral genotypes, serotypes or species, in which information on target sequences may be limited. When bound to dsDNA, the fluorescence of SYBR Green I is increased ~1000-fold providing the sensitivity required for assays of nucleic acids with low starting copy number (Morrison et al. 1998, Cosa et al. 2001). The assay reported herein has a lower detection limit of 20 RNA copies and a lower quantitation limit of ~200 RNA

copies. Lowered specificity is of potential concern with SYBR Green because it is not sequence specific. Low-level background fluorescence can arise from primer-dimers and other non-specific amplicons. A melting curve analysis and agarose gel electrophoresis help to assess amplicon identity.

Quantification of housekeeping genes is commonly used to standardize the number of copies detected among samples that vary in overall RNA amounts (e.g. legs and midguts). This is problematic because expression of housekeeping genes varies among tissues, developmental and physiological states, and experimental treatment (Thellin et al. 1999, Radonic et al. 2004, Lindblom et al. 2005). Furthermore, during blood feeding and vitellogenesis, dramatic changes in overall transcription occur among many housekeeping genes. We measured total RNA to avoid specific expression profiles among housekeeping genes. At any timepoint, viral RNA represented a small fraction (<1%) of the total RNA, however, vector RNA varied only 4 fold over the course of the extrinsic incubation period while viral RNA fluctuated by three orders of magnitude. Therefore, adjustments of viral RNA relative to total RNA imperceptibly changed viral growth curves.

Among mean values from mosquitoes processed on each day, total RNA (Figure 2.4) and DENV-2 (+) RNA in the midgut (Figure 2.7) were correlated between days 2--10 post infection ($r = 0.80$, $p = 0.009$). Furthermore, in individual midguts over all days ($n=139$), total RNA was correlated with DENV-2 (+) RNA ($r = 0.31$, $p = 0.0001$) and DENV-2 (-) RNA ($r = 0.26$, $p = 0.0022$). This suggests the possibility that metabolic changes in the midgut (reflected in changes in total RNA) also affect DENV-2 virus replication. For example, both total and DENV-2 (+) RNA peak on day 8 (Figures 2.4

and 2.7). This supports previous findings that suggest that the midgut has less nutrient availability following 8 dpi and this may lead to a decrease in virus replication (Molina-Cruz et al. 2005). However, when individual midguts were analyzed by day, total and DENV-2 (+) RNA in individual mosquitoes were only correlated on days 0, 12, and 13. This loss of correlation suggests that variation measured among individual mosquitoes on a given day was much larger than the variation among means on different days.

Positive correlations between DENV-2 (+) RNA and numbers of PFU were observed in both C6/36 cells and *Ae. aegypti* (Figures 2.5 and 2.6). The large ratio of RNA copies to PFUs reflects the complex and dynamic relationship between a replicating virus and the cells it infects. The variability of infectivity estimates arises due either to defective, immature, or inactivated virus particles, or to free viral RNA from within cells harboring an infection. Other factors could include numbers of freeze-thaw cycles or fluctuating pH among samples. The RNA copy:PFU ratios published here are comparable to the ratios observed for YFV in *Ae. aegypti* (1000--5000 RNA copies/PFU) (Bae et al. 2003), those reported for West Nile virus in saliva of various mosquito species (80--1,134,649 genome equivalents/PFU) (Colton et al. 2005), and those reported for DENV-2 in peripheral blood mononuclear cells (10--200 RNA copies/PFU) (Wang et al. 2002).

Studies of vector competence have generally reported the proportion of exposed mosquitoes that develop midgut or disseminated infections or transmit the virus to suckling mice (Gubler and Rosen 1976a, b, Miller et al. 1982, Bosio et al. 2000, Gomez-Machorro et al. 2004, Bennett et al. 2005). This is the first study to examine fluctuations in DENV-2 RNA copy number in the midgut and legs among individual mosquitoes over

the 14 day course of the extrinsic incubation period. A typical eclipse phase associated with decrease in infectious virus levels during digestion of the blood meal was seen on days 0--2. During the eclipse phase, there was a poor correlation between amounts of (+) and (-) strands from 0 to 3 dpi. This probably reflects variation in the constitution of virus in the blood meal. Analyses of DENV-2 (+) and (-) RNA in five independently prepared blood meals revealed significant variation in the (+) to (-) strand ratio (1.26--1.55) with no correlation between the amount of the two strands ($r = 0.0014$).

One mosquito developed a disseminated infection during the eclipse phase. This has been observed in earlier studies and the prevailing hypotheses involve either a "leaky midgut" and/or tracheal cell conduits that enable rapid viral escape from the midgut (Houk and Hardy 1979, Hardy et al. 1983, Weaver et al. 1991b, Engelhard et al. 1994, Bowers et al. 1995, Chandler et al. 1998, Romoser et al. 2004). This could also arise from variation in tracheal cell density, location, or degree of basal lamina penetration (Romoser et al. 2004). Dissemination rates first reached 50% at 6 dpi. Detection of (+) RNA in legs preceded detection of (-) RNA and the number of mosquitoes testing positive for (+) RNA exceeded those with (-) RNA until 10 dpi (Table 2.3). Our strand specific assay thus detected early dissemination of virus prior to initiation of replication. The steady increase in viral RNA between 2--6 dpi suggests that there is no abrupt increase in virus when midgut epithelial cells become infected and replication begins. Antiviral or other mosquito cell responses might have caused the pronounced and rapid changes in viral RNA copy number observed in the midgut between 6 and 9 dpi. A similar decrease in DENV-2 levels in *Ae. albopictus* (whole bodies, 10 dpi) has been observed (Gubler and Rosen 1977).

This is the first documentation of pronounced fluctuations of DENV-2 in individual midguts. The standard errors around estimates of DENV-2 RNA amounts were large, reflecting the variation in DENV-2 RNA levels among mosquitoes. Nevertheless, between 6--10 dpi, day-by-day comparison of the amounts of DENV-2 RNA were significant (Duncan's Multiple Range and Student-Newman-Keuls tests: data not shown). During 6--10 dpi, the percentage of mosquitoes with disseminated infections was also increasing but with many fluctuations (Figure 2.7). It is possible that the same mechanisms affecting DENV-2 RNA levels may also condition midgut escape or dissemination barriers. This would be especially true if viral dissemination from the midgut is dose dependent with a mass exodus of mature virus occurring once a threshold is reached. The rebound in DENV-2 RNA seen on day 8 may be a viral response to a putative antiviral response (Figure 2.7). Accumulation of dsRNA or viral proteins that modulate RNAi or apoptosis at certain levels may facilitate this rebound (Ahlquist et al. 2003, Irusta et al. 2004, Sanchez-Vargas et al. 2004). In addition, cellular defenses might only be successful against a portion of the DENV-2 quasispecies. Any mutant viral haplotypes more successful at circumventing host defenses would dominate and rapidly replicate. Mutant sequences may code for proteins that suppress host apoptotic or RNAi pathways. Mutations in the 3' and 5' untranslated regions, or other critical components of the replication complex, could facilitate faster replication (Ahlquist et al. 2003).

Real-time RT-PCR appears to be a practical and reliable method that may provide new insights into virus-vector interactions during the extrinsic incubation period. We are in the process of repeating this study in lines of *Ae. aegypti* that have been selected for susceptibility (*D2S3*) (Bennett et al. 2005) a low midgut infection rate (*Ibo 11*) (Gomez-

Machorro et al. 2004), a low dissemination rate (*D2MEB*) (Bennett et al. 2002). The precise quantitation of viral RNA during infection, replication, and dissemination may help elucidate how DENV refractory *Ae. aegypti* reduce or eliminate DENV infections.

CHAPTER 3

QUANTITATIVE ANALYSIS OF DENGUE-2 VIRUS REPLICATION IN THREE *AEDES AEGYPTI* STRAINS WITH DIFFERING SUSCEPTIBILITY

Introduction

The dengue viruses (family Flaviviridae, genus *Flavivirus*) consist of 4 distinct serotypes (DENV-1--4) most commonly transmitted among humans by *Aedes aegypti* and *Ae. albopictus*. *Aedes aegypti* populations differ in vector competence with regard to DENV (Gubler et al. 1979, Tabachnick and Powell 1979, Tabachnick et al. 1985, Tardieux et al. 1990, Bosio et al. 1998, Vazeille-Falcoz et al. 1999, Bennett et al. 2002, Failloux et al. 2002, Armstrong and Rico-Hesse 2003, Bennett et al. 2005). The species exists as two subspecies: *Ae. aegypti formosus*, a black form that is relatively refractory to infection with flaviviruses and is found predominantly in sylvan habitats in sub-Saharan Africa, and *Ae. aegypti aegypti*, the pale, domestic form that is relatively susceptible to infection with flaviviruses and is pantropic in urban habitats (Gubler et al. 1979, Tabachnick et al. 1985, Tabachnick 1991, Bosio et al. 1998). In certain regions of Africa a predominance of *Ae. aegypti formosus* is thought to contribute to the relatively low rate of apparent DF and DHF cases.

The intrinsic permissiveness of a vector to infection, replication, and transmission of a virus is referred to as vector competence (Hardy 1988, Higgs and Beaty 2005). It is a quantitative trait, that responds to artificial selection, and is a function of midgut and salivary gland infection and escape barriers (Bosio et al. 1998). Mosquitoes that possess

a midgut infection barrier (MIB) are refractory to viral infection and/or replication in the midgut epithelium. Mosquitoes with a midgut escape barrier (MEB) are capable of developing infected midgut cells but virus is unable to escape the midgut to cause a disseminated infection. The MIB and MEB are critical determinants in the vector competence of *Ae. aegypti* populations for flaviviruses (Gubler et al. 1979, Tabachnick et al. 1985, Schoepp et al. 1990, Bosio et al. 1998). The cellular mechanism(s) that contribute to the MIB and MEB have yet to be demonstrated but potential barrier mechanisms include the absence of cell surface receptors for the virus, cellular defenses such as RNA interference or apoptosis that create a nonpermissive environment (Murphy 1975, Blair et al. 2000, Adelman et al. 2001, Sanchez-Vargas et al. 2004, Travanty et al. 2004, Girard et al. 2005), and variability in myriad host proteins involved in proteolytic cleavage (Ludwig et al. 1989, Ludwig et al. 1991, Mertens et al. 1996, Xu et al. 1997) uncoating, transcription, and translation of viral proteins and/or RNA (Black et al. 2002, Gomez-Machorro et al. 2004, Molina-Cruz et al. 2005).

Studies of DENV susceptibility are typically qualitative in , involving the detection of the presence or absence of viral antigens in midgut, head or leg tissues. Qualitative assays have been used to identify at least 5 *Ae. aegypti* genetic loci associated with a DENV-2 MIB and three regions associated with a MEB (Bosio et al. 1998, Bosio et al. 2000, Gomez-Machorro et al. 2004). However, the MIB and MEB appear to have quantitative aspects to them as well. Arboviral infections of mosquito midguts are dependent on a threshold effect, whereby a bloodmeal with a sufficiently high viral titer must be ingested to overcome a threshold and thus mosquitoes ingesting a bloodmeal with low virus titers are less likely to become infected (Chamberlain et al. 1954, Gubler

et al. 1979, Miller et al. 1982, Houk and Hardy 1989, Bennett et al. 2002). In addition, tissues positive for DENV-2 may vary in the amount of DENV-2. Bosio et al. (1998) showed, in strains of both *Ae. aegypti aegypti* and *Ae. aegypti formosus,* that the amount of DENV-2 in the midgut was independent of DENV-2 amount in the head as measured by end-point titration. This indicates that a threshold model does not seem to apply to the MEB. Quantitative analyses using real-time RT-PCR have provided insights into the infection, replication, and dissemination of DENV-2 JAM1409 in *Ae. aegypti* (Molina-Cruz et al. 2005, Richardson et al. 2005). These studies, made on a susceptible, recently colonized field strain from Chetumal, Mexico, demonstrated that midgut levels of DENV-2 RNA vary significantly among infected mosquitoes and fluctuate erratically between 6 and 9 days post infection (dpi).

The primary goal of this study was to characterize quantitative changes in DENV-2 in the midgut and in the head throughout the 14 day extrinsic incubation in *D2S3, D2MEB* and *Ibo11 Ae. aegypti* strains. While the midgut infection and disseminated infection rates have been described in these strains, the amounts of virus produced in tissues throughout the extrinsic incubation period were unknown. In addition, these 3 selected strains are more genetically uniform; thus quantitive differences among strains associated with a MIB and MEB should be more apparent than among a genetically heterogeneous group of mosquitoes with a mixture of MIBs and MEBs, as existed in the recently colonized and outbred Chetumal, Mexico strain used earlier (Richardson et al., 2005). A secondary aim of this study was to test the hypothesis that increased transcription associated with bloodmeal digestion would result in increased midgut DENV-2 RNA levels. The long-term goal of this research is to identify the physiological

and genetic determinants that impact DENV-2 midgut infection and escape barriers and ultimately to manipulate these barriers as part of future vector control programs.

Materials and Methods

Mosquito strains and rearing protocol. Three laboratory strains of *Ae. aegypti* were used in this study. A highly susceptible strain, *D2S3* (Dengue 2 Susceptible on 3 chromosomes); a strain with a strong MEB, *D2MEB* (DENV-2 Midgut Escape Barrier); and a refractory strain, *Ibo 11*, selected from an *Ae. aegypti formosus* strain originally from Ibo, Nigeria (Ballinger-Crabtree et al. 1992, Bosio et al. 1998, Gomez-Machorro et al. 2004, Bennett et al. 2005). Strains were all selected using a high passage DENV-2 strain, JAM1409, originally isolated in 1983 in Jamaica and subsequently passaged in C6/36 cells (Deubel et al. 1986). Eggs were hatched and reared (~100 larvae per liter) in a controlled environment at 27°C, 80% relative humidity, and 12 hour photoperiod. Adults were kept in 1.9 L waxed cardboard containers with water and sucrose.

Virus preparation and mosquito infections. High passage DENV-2 JAM1409 was cultured in the *Ae. albopictus* C6/36 cell line as previously described (Bennett et al. 2002) with two modifications: virus was inoculated at an MOI of 0.001 and cultured for 12 days with a medium change at day 7. Sucrose and water were removed from mosquito cages 48 and 24 hours, respectively, prior to oral challenge. Water-jacketed membrane feeders were covered with hog intestine and placed on containers with ~250, 3 to 4 day old female mosquitoes. Defibrinated sheep blood was mixed with the virus suspension (1:1) and ATP was added at a final concentration of 1 mM. Mosquitoes were allowed to feed for 45--60 min on a 37°C bloodmeal with final infectious virus titer of $10^{6.8}$ plaque forming units (PFU)/mL. Mosquitoes were chilled briefly and mosquitoes

that failed to feed to repletion were discarded. Mosquitoes from all strains were fed simultaneously with aliquots of a single virus preparation. All fed mosquitoes were maintained in the controlled environment as described above. At day 14 post infection half of the remaining mosquitoes from each strain were provided with a noninfectious bloodmeal. Engorged females were sorted and retained.

Mosquito dissection and RNA extraction. Ten to 15 *Ae. aegypti* were collected daily, submerged intact in 50 μL of RNAlater (Qiagen, Valencia, CA) and frozen at −80°C for later processing. RNAlater preserved samples were dissected in 20 μL of Tris buffered saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl). The head was first removed followed by removal of the complete midgut. Tissues were placed in separate 1.5 mL microcentrifuge tubes containing 100 μL of a denaturing guanidine isothiocyanate buffer (RLT lysis buffer, Qiagen, Valencia, CA). RNA was extracted using RNeasy total RNA extraction kits (Qiagen) and eluted in 50 μL of RNAase free water.

DENV-2 RNA quantitation. Total DENV-2 RNA was measured in individual midguts and heads by quantitative, real-time, one-step RT-PCR (QuantiTect™ SYBR® Green RT-PCR kit, Qiagen) using primers targeting a 177 bp region of the NS5 gene as previously described (Richardson et al., 2005). Reactions were set up according to the manufacturer's instructions (0.25 μM primer final concentration, 2 μL of template, final volume of 20 μL) with the following thermal profile: cDNA synthesis at 42°C for 30 minutes, reverse transcriptase inactivatation at 95°C for 15 minutes, 40 amplification cycles of 10 seconds at 94°C, 15 seconds at 64°C, and 20 seconds at 72°C. Fluorescence readings were taken at 78°C after each cycle. A final extension at 72°C for 5 minutes was completed before deriving a melting curve (70--90°C, 0.2°C/read, 1 sec hold) to confirm

the identity of the PCR product. All samples and standards were quantified in triplicate. Standard curves were generated on each plate by analyzing 2 x 10^2--2 x 10^7 copies/reaction of pCR 2.1 plasmid (Invitrogen, Carlsbad, CA) standards containing the NS5 fragment of DENV-2 JAM1409. Negative control mosquito RNA samples were included on each plate.

Calculating infection rates and scoring vector competence. In addition to quantitative assessments, qualitative scoring of vector competence was performed. The MIR and DIR for each strain was calculated as the number of positive midguts divided by the number of mosquitoes tested and the number positive heads divided by the number of positive midguts, respectively. Each strain was also scored for MIB and MEB proportions (MIB = 1–MIR; MEB = 1–DIR). Overall susceptibility or VC of each strain was calculated as the number of mosquitoes exhibiting a DI divided by the total number of mosquitoes tested. Mosquitoes were not assayed for salivary gland infection or virus transmission.

Results

Infection rates. Midgut infection and dissemination rates are presented in Table 3.1 and Figures 3.1 and 3.2. Cumulative MIRs and DIRs are presented for each day throughout the extrinsic incubation period in Figures 3.3 and 3.4. The cumulative MIR in *D2S3* was 54%, 45% in *D2MEB* and 48% in *Ibo 11* while the corresponding DIRs were 44%, 21%, and 38%, respectively. *D2MEB* mosquitoes had a stronger MEB as compared with the other strains (χ^2 [2 d.f,] = 12.63, p = 0.0018). Cumulative DIRs were not different between *D2S3* and *Ibo 11* (χ^2 [1 d.f.]= 0.74, p = 0.39).

TABLE 3.1. Midgut and head infection rates for three strains assessed by real-time RT-PCR.

	Midgut Infections (MI)			Disseminated Infections (DI)			Vector Competence (VC)[c]		
	MIR[a] (number positive)			DIR[b] (number positive)					
Day pi	*D2S3*	*D2MEB*	*Ibo 11*	*D2S3*	*D2MEB*	*Ibo 11*	*D2S3*	*D2MEB*	*Ibo 11*
0	100 (10)	100 (4)	100 (5)	--	--	--	--	--	--
2	30 (3)	40 (4)	70 (7)	0 (0)	25 (1)	0 (0)	0	10	0
4	70 (7)	40 (4)	47 (7)	0 (0)	0 (0)	14 (1)	0	0	7
6	50 (5)	70 (7)	53 (8)	20 (1)	0 (0)	25 (2)	10	0	13
7	30 (3)	80 (8)	40 (6)	0 (0)	0 (0)	66 (4)	0	0	27
8	50 (5)	40 (4)	40 (6)	100 (5)	50 (2)	33 (2)	50	20	13
9	50 (5)	20 (2)	33 (5)	80 (4)	100 (2)	60 (3)	40	20	20
10	70 (7)	20 (2)	53 (8)	86 (6)	50 (1)	50 (4)	60	10	27
12	20 (2)	50 (5)	47 (7)	100 (2)	60 (3)	57 (4)	20	30	27
14	70 (7)	20 (2)	36 (5)	86 (6)	0 (0)	80 (4)	60	0	29
17A[d]	70 (7)	60 (6)	40 (4)	--	--	--	--	--	--
17B[e]	90 (9)	50 (5)	30 (3)	--	--	--	--	--	--
21A	90 (9)	--	36 (5)	--	--	--	--	--	--
21B	90 (9)	--	58 (7)	--	--	--	--	--	--

[a] MIR = Midgut infection rate = (MI/n)x100. The number in parenthesis is the number of positive samples.
[b] DIR = Disseminated infection rate = (DI/MI)x100. The number in parenthesis is the number of positive samples.
[c] VC = (DI/n) x 100
[d] Day A samples received a noninfectious blood meal on day 14 post infection
[e] Day B samples were not refed
Note: day 0 samples were taken immediately after the blood meal.

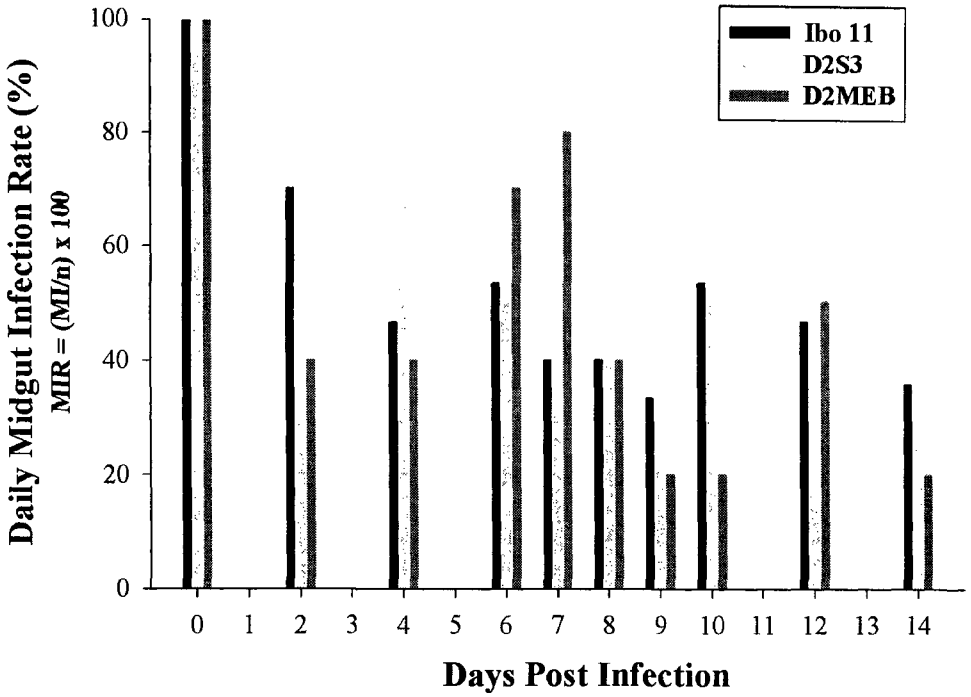

FIGURE 3.1. Midgut infection rates for three strains by day. Day 0 corresponds to mosquitoes dissected immediately after they blood fed.

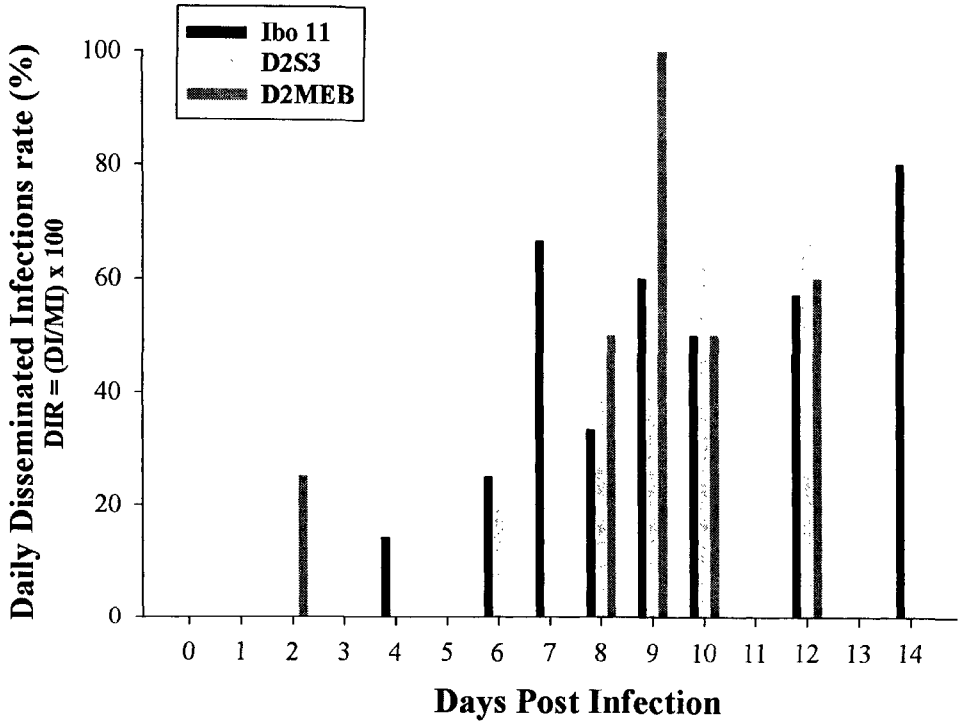

FIGURE 3.2. Head infection rates for three strains by day.

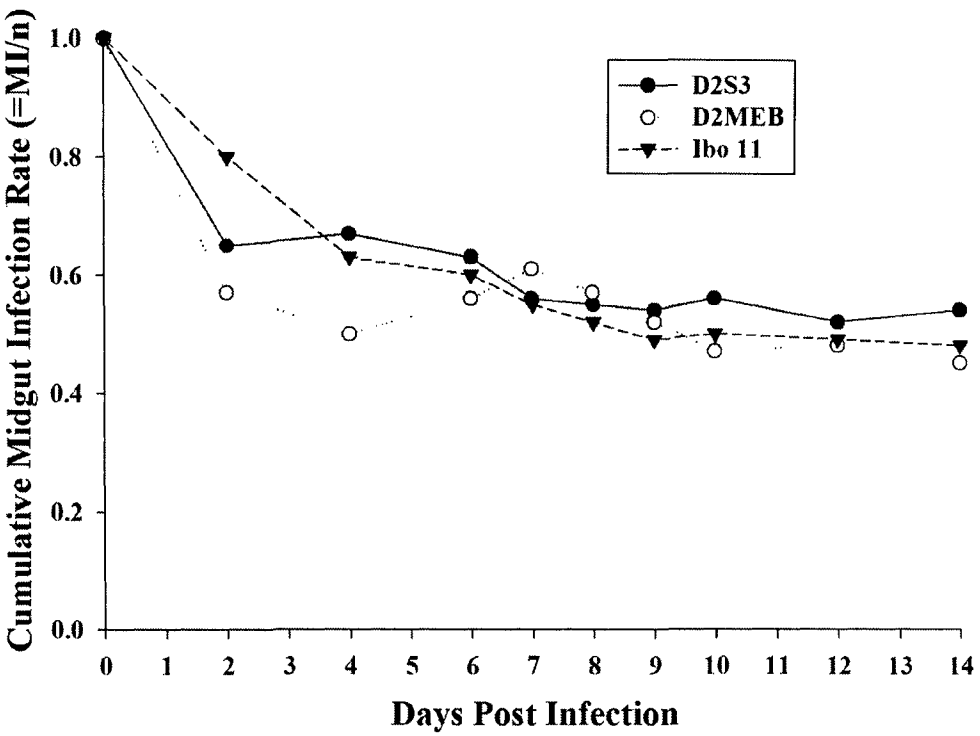

FIGURE 3.3. Cumulative rate of midgut infections during the 14 day EIP.

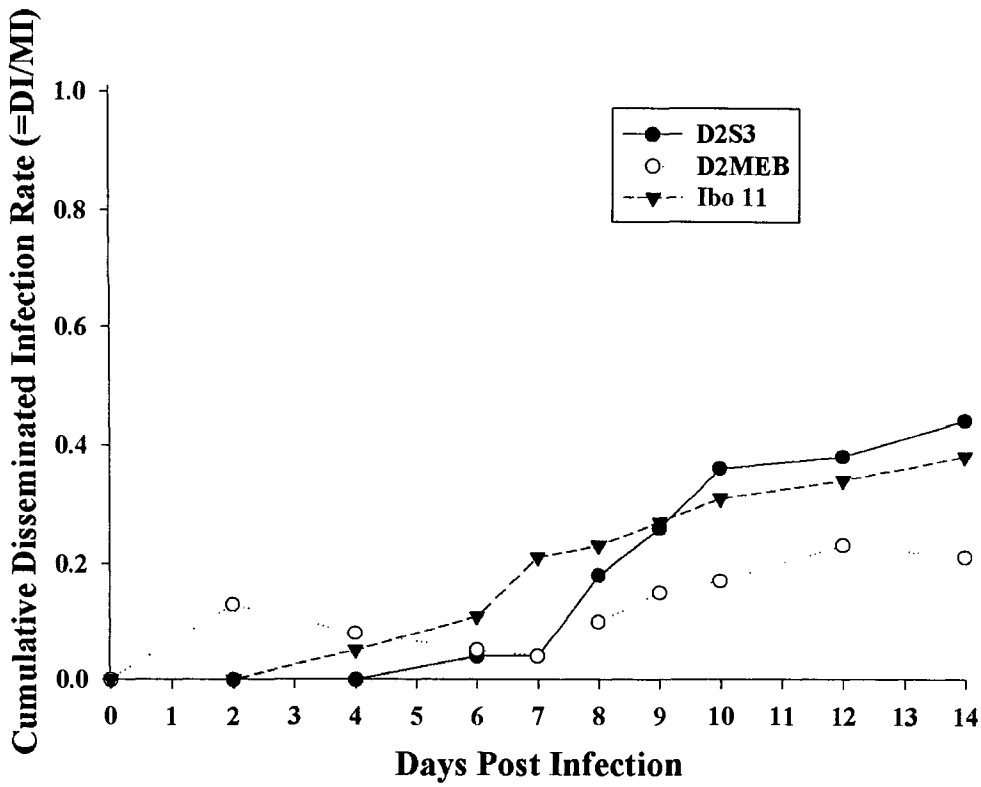

FIGURE 3.4. Cumulative rate of disseminatef (head) infections during the 14 day EIP.

Midgut DENV-2 RNA levels. Melting curve analyses were completed following real-time RT-PCR amplification to assess the specificity of amplified products. Presence of DENV-2 RNA was confirmed by a single -dF/dT peak at ~80°C. The levels of midgut DENV-2 RNA over the course of the EIP are plotted both as means of infected samples (Figure 3.5A) and as mean values for all individuals (Figure 3.5B) (Table 3.2). Figure 3.5A includes previously reported data (from *Ae aegypti,* Chetumal infected with DENV-2 JAM1409) as a reference (Richardson et al. 2005). The variation in DENV RNA levels within *D2S3, D2MEB,* and *Ibo 11* mosquitoes from each day was not significantly different from the variation previously observed for the outbred Chetumal strain ($p = 0.17$).

The viral growth curves in *D2S3* and *Ibo 11* midguts were similar to those derived from the *Ae. aegypti* Chetumal strain (Figure 3.5A) (Molina-Cruz et al. 2005, Richardson et al. 2005). However, the amounts of DENV-2 RNA detected in midguts at day 0 in *D2S3* and *Ibo 11* were almost 2 $\log_{10}$ lower than in the Chetumal strain. As seen in previous studies (Molina-Cruz et al. 2005, Richardson et al. 2005), midgut viral RNA levels become erratic from 7--10 dpi. Notably, at 10 dpi the midut levels of DENV-2 RNA were approximately equal among all strains (~7 $\log_{10}$). Following day 10, midgut DENV-2 RNA amount in *D2S3* and *D2MEB* decreased while it increased in *Ibo 11.* The viral RNA levels in *D2S3* midguts returned to ~7 $\log_{10}$ by day 17 while the *D2MEB* levels remained ~1 $\log_{10}$ lower (Figure 3.6). With the exception of the eclipse phase (0--2 dpi), the viral RNA curve in *D2MEB* is different from *Chetumal, D2S3,* and *Ibo11.* Replication of DENV-2 RNA was considerably slower in the midgut of these mosquitoes.

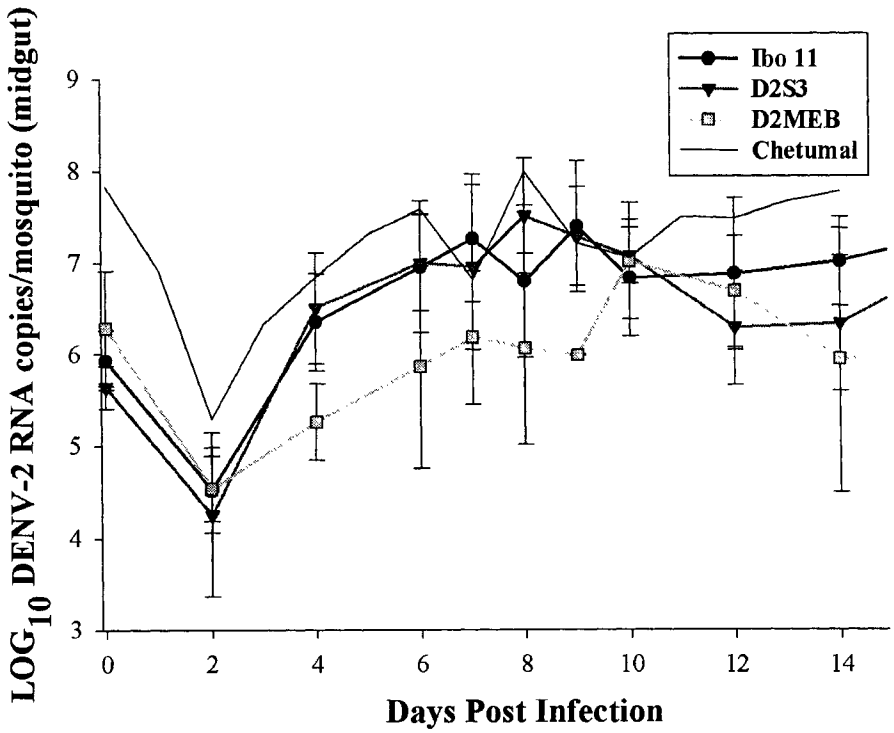

FIGURE 3.5A. Daily mean quantity of DENV-2 RNA among positive midguts sampled at a given timepoint. Previous findings from *Ae. aegypti* Chetumal are plotted for reference. Y error bars represent the coefficient of variation of DENV-2 RNA copies in the mosquitoes testing positive on each day. Points without error bars had a sample size of 1.

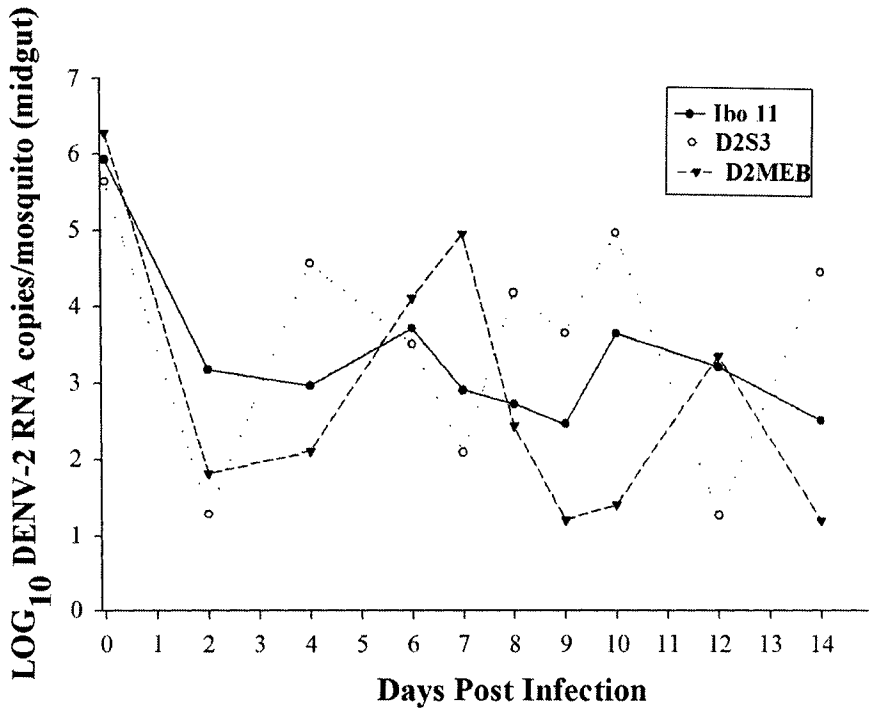

FIGURE 3.5B. Daily mean quantity of DENV-2 RNA among all midguts sampled at a given timepoint (includes non infected mosquitoes).

TABLE 3.2 Real-time RT-PCR results: DENV-2 RNA levels in midgut samples.

	D2S3		D2MEB		Ibo 11	
	All Midguts	Positive Midguts	All Midguts	Positive Midguts	All Midguts	Positive Midguts
Day	Log_{10} copies per midgut ± CV	Log_{10} copies per midgut ± CV	Log_{10} copies per midgut ± CV	Log_{10} copies per midgut ± CV	Log_{10} copies per midgut ± CV	Log_{10} copies per midgut ± CV
0	5.64 ± 0.04	5.64 ± 0.04	6.28 ± 0.10	6.28 ± 0.10	5.93 ± 0.05	5.93 ± 0.05
2	1.28 ± 1.64	4.26 ± 0.21	1.81 ± 1.30	4.54 ± 0.08	3.17 ± 0.70	4.53 ± 0.10
4	4.55 ± 0.70	6.50 ± 0.09	2.10 ± 1.30	5.26 ± 0.08	2.96 ± 1.11	6.35 ± 0.08
6	3.50 ± 1.06	7.00 ± 0.08	4.10 ± 0.72	5.86 ± 0.19	3.71 ± 0.98	6.95 ± 0.10
7	2.08 ± 1.62	6.95 ± 0.13	4.94 ± 0.54	6.18 ± 0.12	2.90 ± 1.28	7.26 ± 0.10
8	4.17 ± 0.95	7.51 ± 0.08	2.43 ± 1.31	6.06 ± 0.17	2.72 ± 1.28	6.79 ± 0.12
9	3.64 ± 1.06	7.28 ± 0.07	1.20 ± 2.11	5.98 ± 0.00	2.46 ± 1.47	7.39 ± 0.10
10	4.95 ± 0.69	7.07 ± 0.04	1.40 ± 2.11	7.01 ± 0.09	3.64 ± 0.98	6.83 ± 0.09
12	1.26 ± 2.11	6.28 ± 0.10	3.34 ± 1.06	6.68 ± 0.09	3.20 ± 1.12	6.87 ± 0.12
14	4.43 ± 0.70	6.33 ± 0.12	1.19 ± 2.15	5.94 ± 0.24	2.50 ± 1.40	7.01 ± 0.07
17A	4.93 ± 0.70	7.04 ± 0.07	3.47 ± 0.88	5.79 ± 0.14	2.93 ± 1.29	7.28 ± 0.06
17B	6.50 ± 0.36	7.22 ± 0.08	3.02 ± 1.06	6.04 ± 0.05	2.23 ± 1.62	7.42 ± 0.08
21A	6.52 ± 0.36	7.24 ± 0.06			2.49 ± 1.39	6.96 ± 0.06
21B	6.65 ± 0.36	7.39 ± 0.06			3.97 ± 0.90	6.81 ± 0.13

When all positive midguts (n = 236) were compared, DENV-2 RNA quantities differed significantly among strains ($p < 0.0001$). Mean levels of DENV-2 RNA in the midgut of *D2MEB* mosquitoes were significantly lower than those in *D2S3* ($p = 0.007$) or *Ibo 11* ($p = 0.002$) (Table 3.3). Between 4 and 10 dpi *D2MEB* midguts had 0.73--1.41 $\log_{10}$ DENV-2 less RNA than *Ibo 11* and 0.77--1.51 less RNA than *D2S3* (Figure 3.5A).

Analysis of the DENV-2 RNA levels in the midgut immediately following the bloodmeal (day 0) indicated that mosquitoes from the *D2S3* strain ingested less virus than the other strains (Figure 3.7, Table 3.2). The difference was significant between *D2S3* and *D2MEB* mosquitoes ($p = 0.01$). No significant differences were detected in the other pair-wise comparisons although the small sample size from each strain limited the power of the test. Behavioral differences in feeding were also observed between the strains. *D2MEB* did not feed well--only ~50% of females fed to repletion, while ~90% of both *Ibo 11* and *D2S3* females took a complete bloodmeal.

Midgut DENV-2 RNA levels were compared between mosquitoes that developed a disseminated infection with those that did not to test for evidence of a threshold titer of DENV-2 RNA associated with a MEB. Mosquitoes that developed a disseminated infection between 6 --10 dpi had, on average 0.4 $\log_{10}$ more DENV-2 RNA in the midgut than those in which virus failed to disseminate ($p = 0.03$) (Table 3.4). Midgut escape barrier may therefore be somewhat dependent on viral RNA levels in the midgut.

At 14 dpi a subset of each strain was fed a noninfectious bloodmeal and midgut DENV-2 RNA levels were compared to unfed individuals. DENV-2 RNA levels were unaffected by a second bloodmeal ($p > 0.5$) (Figure 3.6, Table 3.5).

Table 3.3. DENV-2 RNA levels in the midgut—comparison of positive midguts among strains days (0-14 dpi).

Strain	N	DENV-2 RNA[a]		p-value
Ibo 11	64	6.57 ± 1.02		
D2S3	54	6.50 ± 0.93		0.0049
D2MEB	42	5.96 ± 0.88		
			Ibo 11 vs. D2S3	0.7058
			Ibo 11 vs. D2MEB	0.0023
			D2S3 vs. D2MEB	0.0069

[a] Mean Log_{10} DENV-2 RNA level ± variance

Table 3.4. DENV-2 RNA levels in the midgut—comparison of mosquitoes with and without a DI.

Time period (dpi)[a]	3 Strains Combined		
	Head (-)[b]	Head (+)	p value
4—14	6.47±0.71 (72)	6.94±0.53 (55)	0.001
6—14	6.58±0.75 (54)	6.94±0.53 (55)	0.02
6—10	6.63±0.70 (45)	7.03±0.62 (36)	0.03

[a] dpi = days post infection, midgut samples within this time period were included in the analysis.
[b] Mean Log_{10} DENV-2 RNA level in the midgut ± variance (n).

Table 3.5. DENV-2 RNA levels in the midgut--comparison of positive midguts at 17 dpi with and without ingestion of a second, non infectious blood meal on day 14.

Strain	Second meal	n	DENV-2 RNA[a]	p-value
Ibo 11	Yes	4	7.28 ± 0.21	0.75
	No	3	7.42 ± 0.40	
D2S3	Yes	7	7.04 ± 0.26	0.55
	No	9	7.22 ± 0.37	
D2MEB	Yes	6	5.79 ± 0.66	0.53
	No	5	6.04 ± 0.09	

[a] Mean Log_{10} DENV-2 RNA level ± variance.

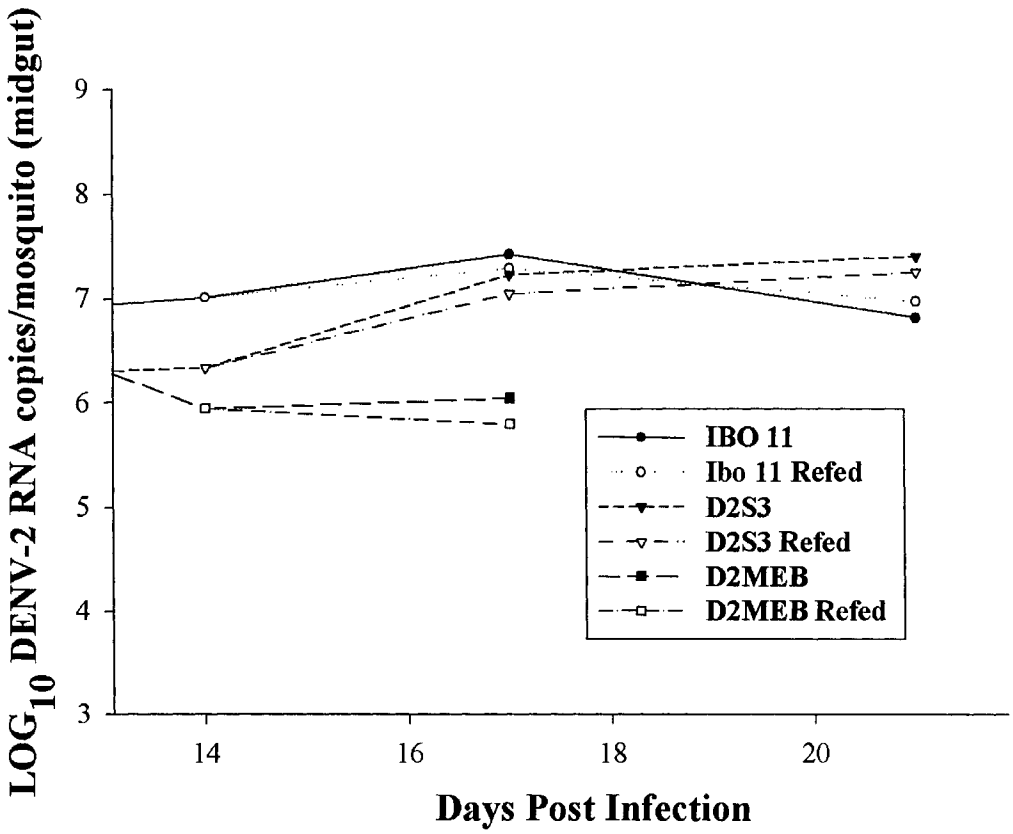

FIGURE 3.6. Mean quantity of DENV-2 RNA, from 14—21 dpi. Mosquitoes fed a second, non-infectious bloodmeal at day 14 are compared to controls.

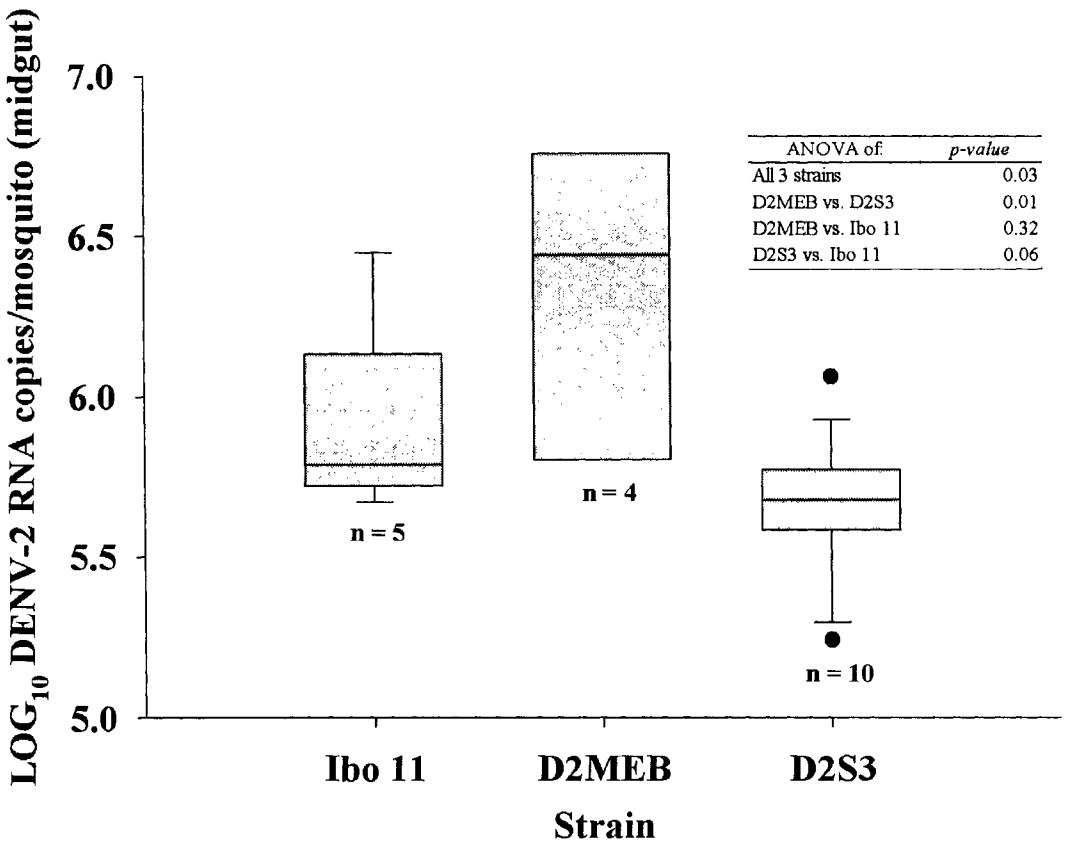

FIGURE 3.7. Mean quantity of DENV-2 RNA in mosquitoes immediately following the bloodmeal (0 dpi). Whisker caps represent 10th and 90th percentiles. Box limits represent 25th and 75th percentiles. Lines within the boxes mark the median. Circles indicate the 5th and 95th percentiles.

Head DENV-2 RNA levels. The quantity of viral RNA in heads is presented both as means of positive samples (Figure 3.8A) and as means of all individuals (Figure 3.8B) (Table 3.6). Figure 3.8A includes previously reported data from Chetumal for reference (Richardson et al. 2005). Although viral RNA in the heads of *D2MEB* were ~0.5 $\log_{10}$ lower than those detected in the other strains, the strains did not differ significantly in mean DENV-2 RNA levels among mosquitoes with a disseminated infection ($p = 0.35$) (Table 3.7). During the initial 2--7 days of the EIP, virus dissemination was rare (Figure 3.2) and viral RNA levels were erratic (Figure 3.8A). Beginning on day 8, plots of mean DENV-2 RNA among infected heads were similar among strains (Figure 3.8A) but when uninfected mosquitoes were included (Figure 3.8B), a significant difference was detected between the *D2MEB* heads and the other two strains.

Compared to midguts, heads consistently had ~ 0.5 $\log_{10}$ lower DENV-2 RNA concentration ($p = 0.00037$) (Table 3.8) and no significant correlations were detected between the concentration of viral RNA in the midgut and the head (Figure 3.9). The two measurements were clearly independent in *D2S3* ($n = 24$, $r = 0.11$, $p = 0.61$), *Ibo 11* ($n = 24$, $r = 0.03$, $p = 0.88$) and *D2MEB* mosquitoes ($n = 9$, $r = 0.61$, $p = 0.08$).

Discussion

The cumulative MIRs in the present study (*D2S3* = 54%, *D2MEB* = 45%, *Ibo 11* = 48%) were markedly different from those reported in earlier studies in which the MIR typically ranged between 94 --100% for *D2S3* and from 74--88% for *D2MEB* (Gomez-Machorro et al. 2004, Bennett et al. 2005), while < 10% of *Ibo 11* mosquitoes had infected midguts. Furthermore there was no difference in the cumulative MIR between

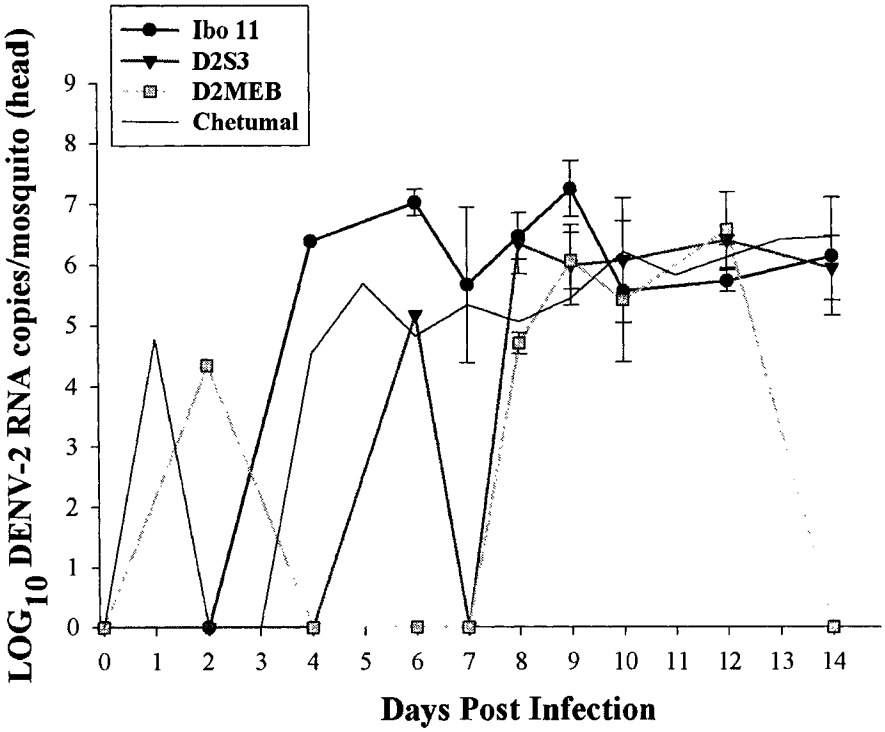

FIGURE 3.8A. Daily mean quantity of DENV-2 RNA among positive. Only heads from mosquitoes with a midgut infection were tested. Previous findings from *Ae. aegypti* Chetumal are plotted for reference. Y error bars represent the coefficient of variation of DENV-2 RNA copies in the mosquitoes testing positive on each day. Points without error bars had a sample size of 1.

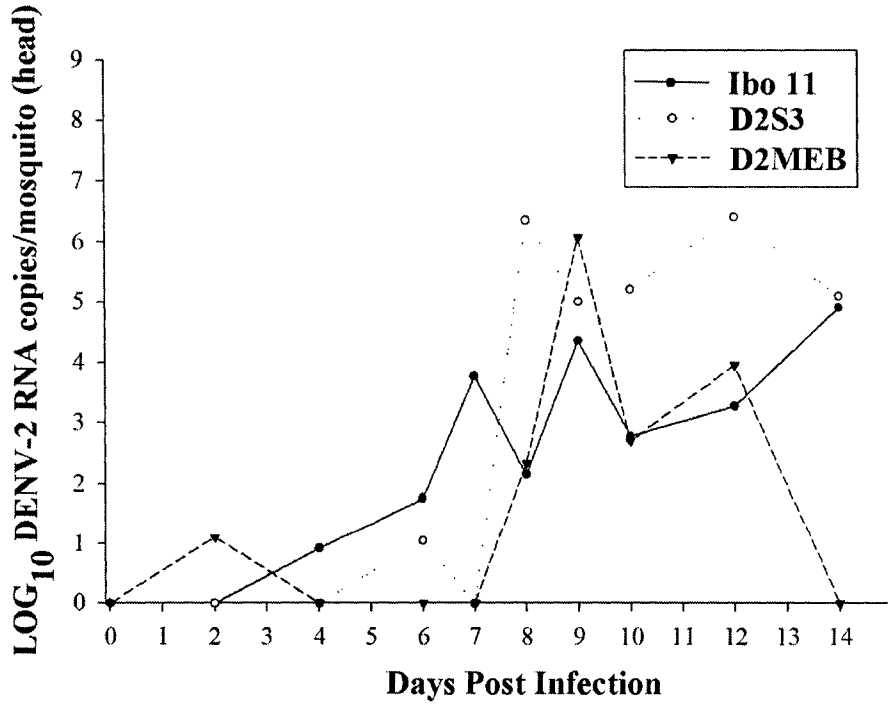

FIGURE 3.8B. Daily mean quantity of DENV-2 RNA among all heads sampled. Only heads from mosquitoes with a midgut infection were tested.

TABLE 3.6. Real-time RT-PCR results: DENV-2 RNA levels in head samples.

	D2S3		D2MEB		Ibo 11	
	All heads	Positive Heads	All heads	Positive Heads	All Heads	Positive Heads
Day	Log_{10} copies per head ± CV	Log_{10} copies per head ± CV	Log_{10} copies per head ± CV	Log_{10} copies per head ± CV	Log_{10} copies per head ± CV	Log_{10} copies per head ± CV
0	0.00	0.00	0.00	0.00	0.00	0.00
2	0.00	0.00	1.09 ± 2.00	4.34 ± NA	0.00	0.00
4	0.00	0.00	0.00	0.00	0.91 ± NA	6.38 ± NA
6	1.03 ± 2.24	5.17 ± NA	0.00	0.00	1.76 ± 1.84	7.02 ± 0.03
7	0.00	0.00	0.00	0.00	3.77 ± 0.82	5.66 ± 0.23
8	6.34 ± 0.08	6.34 ± 0.08	2.35 ± 1.16	4.70 ± 0.04	2.16 ± 1.55	6.46 ± 0.06
9	4.99 ± 0.50	5.98 ± 0.11	6.05 ± 0.08	6.05 ± 0.08	4.35 ± 0.92	7.24 ± 0.06
10	5.19 ± 0.48	6.06 ± 0.17	2.71 ± 1.41	5.40 ± NA	2.78 ± 1.10	5.55 ± 0.21
12	6.39 ± 0.01	6.39 ± 0.01	3.94 ± 0.92	6.56 ± 0.10	3.27 ± 0.94	5.72 ± 0.03
14	5.08 ± 0.45	5.93 ± 0.09	0.00	0.00	4.90 ± 0.58	6.13 ± 0.16

Table 3.7. DENV-2 RNA levels in the head—comparison of positive heads among strains.

Strain	N	DENV-2 RNA[a]	p-value
Ibo 11	24	6.14 ± 0.92	
D2S3	24	6.06 ± 0.46	0.35
D2MEB	9	5.66 ± 0.92	
		Ibo 11 vs. D2S3	0.78
		Ibo 11 vs. D2MEB	0.21
		D2S3 vs. D2MEB	0.18

[a] Mean Log_{10} DENV-2 RNA level ± variance.
Note the low sample size of positive D2MEB heads likely contributed to the lack of a significant difference.

Table 3.8. DENV-2 RNA levels—comparison of viral RNA levels in positive midguts to that of heads.

Tissue	N	DENV-2 RNA[a]	p-value
Midgut	224	6.55±0.94	0.00037
Head	50	6.01±0.67	

[a] Mean Log_{10} DENV-2 RNA level ± variance.
Note that the 0.49 log difference here is less than that between Ibo 11 and D2MEB head levels above with similar variances.

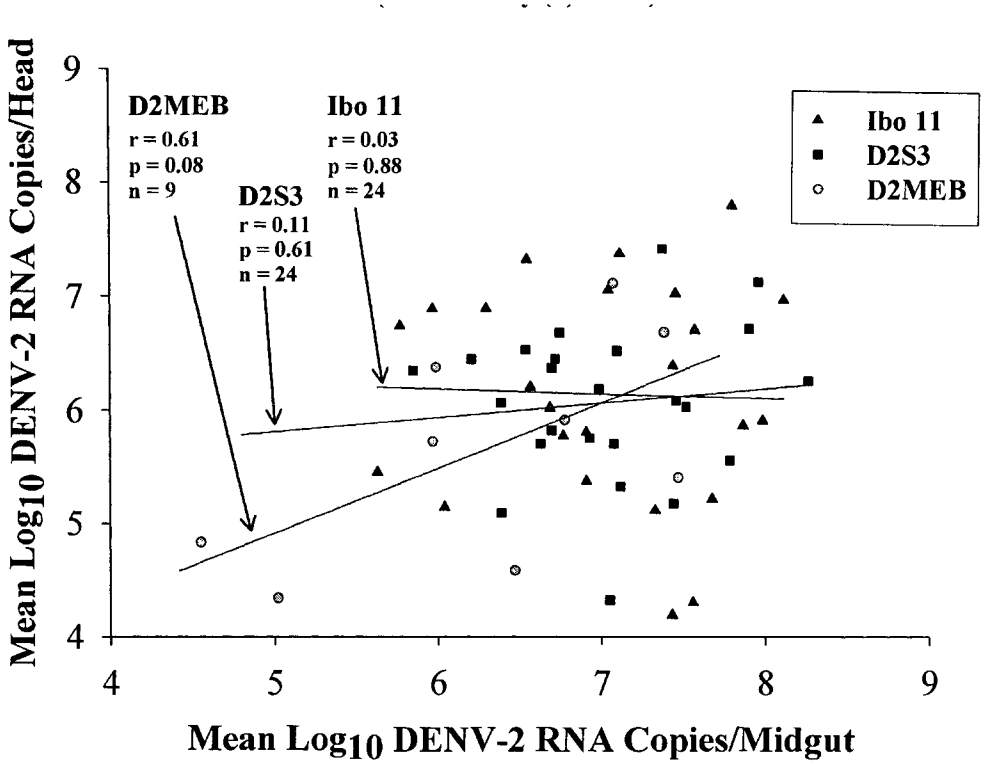

FIGURE 3.9. Plot of midgut versus head DENV-2 RNA quantity from individual mosquitoes that developed a disseminated infection

the three strains at 14 dpi (χ^2 [2 d.f.] = 1.68, p = 0.43), indicating that *Ibo 11* mosquitoes did not express a stronger MIB than the other strains. Midgut infection by DENV-2 has been shown to be directly correlated with virus titer in the bloodmeal (Gubler and Rosen 1976b, Bennett et al. 2002) and the lower than expected MIRs in the present study could be associated with a relatively low titer of virus in the bloodmeal ($10^{6.8}$ PFU/mL). Compared to the Chetumal strain infected with $10^{9.5}$ $TCID_{50}$/mL, the *D2S3, D2MEB, and Ibo 11* midguts had ~ 2 $\log_{10}$ less DENV-2 RNA at day 0. However, if a low titer bloodmeal alone caused lower infection rates in *D2S3* and *D2MEB*, a similar effect should have been seen in *Ibo11*. Instead, the *Ibo11* MIR was higher than expected. Gomez-Machorro et al. (2004) presented evidence that the *Ibo11* MIB QTL on chromosome II is linked to deleterious or lethal alleles. It is therefore possible that the MIB alleles may have been removed by selection in the 5 generations that had passed since Gomez-Machorro et al. (2004) mapped the MIB QTL in *Ibo11*. In contrast, in *D2MEB,* QTL were detected at 31 cM on Chromosome I, at 32 cM on Chromosome II, and between 44-52 cM on Chromosome III. Alleles at these QTL were additive or dominant in determining rates of dengue-2 dissemination and accounted for ~45% of the phenotypic variance (Bennett et al. 2005). This advanced intercross line is in the F_{15} generation and the MEB phenotype remains stable.

In general, the dynamics of viral RNA levels in *D2S3* and *Ibo 11* were similar to those previously demonstrated for the *Ae. aegypti* Chetumal strain (Figure 3.5A) (Molina-Cruz et al. 2005, Richardson et al. 2005). Despite being selected for their infection barriers, the inbred strains were similar to the Chetumal strain in the wide range of midgut viral RNA levels observed between individuals within the daily collections.

DENV-2 RNA midgut levels in *D2MEB* were clearly lower than other strains between 2 and 10 dpi. Either viral replication was inhibited, viral RNA was being degraded after it was transcribed, or both events were occurring. These patterns are consistent with a threshold model of vector competence and the hypothesis that reducing the rate of viral replication and/or destroying transcribed viral RNA in the midgut decreases overall vector competence. Under this model, intracellular processes that inhibit viral replication or destroy viral RNA (e.g., RNAi, apoptosis, or reduced rates of transcription and translation) could have a profound impact on viral dissemination. The fact that mosquitoes with a disseminated infection had more viral RNA in the midgut than those that lacked viral RNA in the head further supports this model. As was noted earlier by Bosio and colleagues (1998), correlations between midgut and disseminated DENV-2 titers were insignificant.

Differences in YFV replication rates between susceptible and refectory *Ae. aegypti* strains have been reported (Miller and Mitchell 1991). As only whole mosquitoes were assayed, the authors were forced to speculate on whether virus replication differences were occurring internal or external to the midgut. They concluded that replication occurred in the midguts of both lines at equal levels and that differences in overall virus levels resulted from events outside of the midgut. Reduced midgut levels of Western equine encephalitis virus were also noted in *Culex tarsalis* strains that possessed a MEB (Kramer et al. 1981).

In all samples tested, DENV-2 RNA levels were unaffected by a second bloodmeal ($p > 0.5$) (Figure 3.6, Table 3.5). Synthesis of viral RNA was not limited by a lack of nutrients at days 17 or 21 post infection. Future studies should focus on the

impact of nutrient availability in *D2MEB* during the primary dissemination window 6--10 dpi.

The strong MEB in *D2MEB* resulted in small sample sizes in heads. Although levels of viral RNA in the heads of *D2MEB* were ~0.5 $\log_{10}$ lower than those detected in the other strains, the strains did not differ significantly in mean DENV-2 RNA levels among samples with a disseminated infection ($p = 0.35$) (Table 3.7). Similarly, the test for correlation between the level of viral RNA in the midgut and the head indicated a weak but insignificant association in *D2MEB* samples (Figure 3.9).

In conclusion, this study demonstrated that DENV-2 RNA levels are lower in the midgut of *Ae. aegypti* with a MEB (*D2MEB*) than they are in *Ibo 11* or *D2S3* midguts. It is unclear whether this was a result of mechanisms that inhibit viral replication or post-transcriptional degradation of viral RNA. Small sample size obscured our ability to determine the downstream effect of reduced midgut levels of viral RNA on rate of dissemination and level of viral RNA in the head. Future studies should be conducted to clarify the effects of midgut virus replication rates on these qualitative and quantitative aspects of virus dissemination in *D2MEB*.

CHAPTER 4

EFFECT OF MOSQUITO MIDGUT TRYPSIN ACTIVITY ON DENGUE-2 VIRUS INFECTION AND DISSEMINATION IN AEDES AEGYPTI

(Note: The research reported in this chapter was the result of collaboration with others and has been published by Molina-Cruz, A., L. Gupta, J. Richardson, K. Bennett, W. C. Black, IV, and C. Barillas-Mury, 2005. The author of this dissertation developed the real-time RT-PCR assays; assisted in mosquito rearing, infection, maintenance, and processing; performed much of the RNA extraction and quantitation; and made significant contribution to the preparation of the manuscript).

Introduction

Aedes aegypti is a container-breeding mosquito with a cosmopolitan distribution in tropical and subtropical regions of the world and on a global basis is a common vector of yellow fever and dengue fever flaviviruses (DENV) (Gubler 2002a). Since the demise of mosquito-control programs beginning in the late 1960s, *Ae. aegypti* reestablished itself throughout tropical and subtropical areas of the Americas. Despite the widespread availability of an effective and safe vaccine, yellow fever remains an important public health problem in much of Africa and South America (Barrett and Monath 2003). *Aedes aegypti* is also the most prevalent vector of DENV (serotypes 1–4) in a human-mosquito cycle. Dengue fever is one of the most rapidly expanding diseases in the tropics, with more than 2 billion people at risk. All four serotypes of the virus are now circulating in the Americas, and an estimated 100 million human infections occur annually.

Vector competence refers to the intrinsic permissiveness of an arthropod vector to infection, replication, and transmission of a virus (Hardy 1988, Woodring et al. 1996). The midgut is the first organ that an arbovirus encounters; it can prevent the invasion and replication of the viruses (midgut infection barrier) or the dissemination to other tissues (midgut escape barrier). The molecular nature of these barriers is unknown, but they have been found to be major determinants of vector competence to DENV during experimental infections. The barriers also vary in prevalence in natural populations, leading to large intraspecific variation of *Ae. aegypti* vector competence to DENV (Gubler et al. 1979, Tabachnick et al. 1985, Bennett et al. 2002).

Dengue viruses are (+) RNA viruses that for replication synthesize a complementary (–) RNA (Chambers et al. 1990). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) has been shown to be a rapid and sensitive method to quantify DENV (Houng et al. 2000, Armstrong and Rico-Hesse 2001, Callahan et al. 2001, Drosten et al. 2002, Wang et al. 2002, Armstrong and Rico-Hesse 2003).

The early events of flavivirus midgut infection are not well understood. They presumably involve receptor binding and some midgut cell penetration mechanism. DENV have been shown to penetrate C6/36 mosquito cells in culture by membrane fusion and sometimes by receptor-mediated endocytosis (Hase et al. 1989, Barth and Schatzmayr 1992). Other arboviruses (bunya- and orbiviruses) have been shown to have increased affinity for the midgut after proteolytic processing of virion surface proteins (Ludwig et al. 1989, Ludwig et al. 1991, Mertens et al. 1996, Xu et al. 1997). With this precedent, we decided to test the involvement of midgut trypsins in determining DENV infectivity in *Ae. aegypti*.

Midgut trypsins play a central role during blood digestion in *Aedes aegypti* (Noriega and Wells 1999). This mosquito synthesizes early trypsin and late trypsin proteins *de novo* upon blood feeding. Early trypsin activity peaks 3 hours after blood feeding and then drops within a few hours. Early trypsin activity regulates late trypsin mRNA synthesis, which reaches a maximum level 24 hours after feeding, followed by an increase in late trypsin protein, which reaches 4--6 μg/midgut. Late trypsin accounts for most of the endoproteolytic activity during blood digestion in the *Ae. aegypti* midgut (Noriega and Wells 1999). Addition of an excess of soybean trypsin inhibitor (STI) in the blood meal inhibits blood digestion (Klowden 1987) by inhibiting early trypsin activity and the expression of late trypsin (Barillas-Mury et al. 1995). We evaluated the effect of inhibiting mosquito midgut trypsins with STI on DENV infectivity to *Ae. aegypti* during the digestion of a DENV-2 infected blood meal. We also assessed whether *in vitro* digestion of DENV-2 in cell culture with bovine trypsin recovers infectivity to STI-treated mosquitoes.

Materials and Methods

***Aedes aegypti* rearing.** Mosquitoes used were the F5 generation of a wild population from Chetumal, Mexico. This population is highly susceptible to DENV-2 (Bennett et al. 2002). Eggs were hatched and mosquitoes were raised at a constant temperature of 27°C and 80% relative humidity in an insectary with a 12:12 hour photoperiod. Eggs were collected in water cups containing paper filters.

DENV-2 mosquito infections. The Jamaica 1409 strain of DENV-2 (Deubel et al. 1986) was amplified in C6/36 cells (Schoepp et al. 1990, Bennett et al. 2002). Briefly, 0.5 mL aliquots of virus stock were used to infect 75 cm^2 flasks of confluent C6/36 cells

at a multiplicity of infection of 1.5 infectious virus particles/cell. Infected cells were incubated for 14 days at 28°C in L15 medium supplemented with 2% heat-inactivated fetal bovine serum, penicillin (1%), streptomycin (1%), and L-glutamine (1%). Medium was changed on day 7. Virus and cells were harvested on day 14 with a cell scraper. The virus suspension was mixed 1:1 with defibrinated sheep blood. ATP was added to a final concentration of 1 mM, STI was added to a final concentration of 2 mg/mL as previously used (Barillas-Mury et al. 1995), and the blood meal was incubated at 38°C for 15 minutes. The blood meal was placed in membrane feeders covered with hog gut. Blood meal virus titers were determined by inoculating serial 10-fold dilutions of the respective meal onto C6/36 cells in the wells of a 96-well plate. Cells were assayed by immunofluoresence, and 50% tissue culture infectious dose ($TCID_{50}$) titers were determined (Schoepp et al. 1990). The final infectious blood meal virus titer was $10^{9.5}$ $TCID_{50}$/mL in the sample without STI and $10^{9.2}$ $TCID_{50}$/mL in the sample with STI.

For mosquito infections, 400–500 mosquitoes/4-L carton were starved of sucrose and deprived of water for 36 hours prior to blood feeding. Blood meals were maintained at a constant temperature of 37°C. Mosquitoes that were 3--4 days old were allowed to feed for 45--60 minutes. Fully engorged mosquitoes were selected and held in the insectary. Untreated control mosquitoes were provided with 10% protease-free sucrose (Sigma, St. Louis, MO) while the sucrose in the STI-treated mosquitoes was laced with 2 mg/mL STI. Samples of 20 mosquitoes were collected 1, 2, 4, 6, 7, 11, and 14 days after feeding. Mosquitoes were frozen in dry-ice ethanol and kept at –70°C awaiting further analysis.

***In vitro* digestion with bovine trypsin.** One volume of 14-day DENV-2 culture in C6/36 cells was digested with 0.1 mg/mL bovine trypsin (12,700 units/mg, Sigma T-1426) for 15 minutes with shaking at room temperature. The reaction was stopped by adding STI to a final concentration of 8 mg/mL and incubating for another 15 minutes with shaking at room temperature. Afterwards, the digested viral culture was diluted with 1 volume of 14-day-old C6/36 and 2 volumes of defibrinated sheep blood (Treatment 3; Table 4.1). To assess the potential effects of cell protein digestion in the viral culture, a digested cell control (Treatment 2; Table 4.1) was prepared by predigesting with trypsin uninfected C6/36 cells, stopping the reaction with STI, and diluting with 1 volume of 14-day-old cells and 2 volumes of blood. The undigested control (Treatment 1; Table 2.1) was prepared with 1 volume of DENV-2 culture, 1 volume of uninfected C6/36 cell culture, and 2 volumes of blood. The infectious blood meal virus titer was $10^{9.2}$ $TCID_{50}$/mL for the undigested control and $10^{8.8}$ $TCID_{50}$/mL for both the *in vitro* trypsin digested samples.

TABLE 4.1

Treatments used to test for the effects of *in vitro* digestion with bovine trypsin*

1. **Undigested control**
   1 vol. Viral culture plus 1 vol. Cell culture plus 2 vol. blood
2. ***In vitro* trypsin pre-digested cell culture control**
   1 vol. Cell culture → 15 min Bovine Trypsin → 15 min STI → add 1 vol. Viral culture and 2 vol blood
3. ***In vitro* trypsin pre-digested viral culture**
   1 vol. Viral culture → 15 min Bovine Trypsin → 15 min STI → add 1 vol. Cell culture and 2 vol. blood

* STI = soybean trypsin inhibitor.

Mosquito dissection and RNA extraction. Mosquito mid-guts, thoraces, and ovaries were dissected in 25 μL of RNAlater (Sigma). Total RNA was extracted in duplicate using the RNAeasy kit (Qiagen, Valencia, CA) from pools of five midguts or five thoraces. Total RNA was eluted in 50 μL of RNAase-free water. Ovaries were fixed in 4% paraformaldehyde in PBS and examined by confocal microscopy.

Late trypsin qRT-PCR. Late trypsin mRNA was measured 24 hours after the blood meal by SYBR green qRT-PCR (Qiagen). Primers were F1 (5'-ACAGTACCAGTATTCG-GCAAA-3') and R1 (5'-GAGAACTTGGAATGG-GAACT-3'). The target fragment (147 bp) was TA cloned into a pCR2.1 (Invitrogen, Carlsbad, CA) and used to generate a standard curve with 10^1--10^8 copies/reaction. Reactions (20 μL) with 4 μL of total RNA were carried out in an Opticon-2 system (MJ Research, Reno, NV). cDNA was synthesized at 50°C for 30 minutes, reverse transcriptase was inactivated at 95°C for 15 minutes, PCR involved 44 cycles of 15 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. Fluorescence readings were taken at 72°C after each cycle. A final extension at 72°C for 5 minutes was completed before deriving a melting curve (70--95°C) to confirm the identity of the PCR product. qRT-PCR measurements were made in triplicate. Results were normalized with ribosomal protein S6 (RPS6) as an internal standard, also measured by qRT-PCR, and expressed in copies/midgut.

DENV-2 RNA qRT-PCR. DENV-2 (+) RNA was measured by qRT-PCR as above using primers For (5'-ACAAGTCGAACAACCTGGTCCAT-3') and Rev (5'-GCCGCACCATTGGTCTTCTC-3') directed to the NS5 gene (Laue et al. 1999). A standard curve was generated by analyzing 10^1 to 10^8 copies/reaction of plasmid

containing the 177 bp target fragment. Results were normalized with RPS6 and expressed in copies/midgut. DENV-2 (–) RNA was measured by adding the For primer, denaturing RNA at 80°C for 5 minutes before the cDNA synthesis, and the second primer (Rev) was added only after inactivation of the reverse transcriptase for 15 minutes at 95°C. Controls lacking both primers during the cDNA synthesis were done to confirm reverse transcriptase inactivation.

RPS6 qRT-PCR. RPS6 mRNA was measured by qRT-PCR as above using primers RPS6(5+) (5'-CGTCGTCA-GGAACGTATCCG3-') and RPS6(5–) (5'-TCTTG-GCAGCCTTAGCAGC-3'). A standard curve was generated analyzing 10^1 to 10^8 copies/reaction of plasmid containing the 118 bp fragment. Results were expressed in copies/midgut.

DENV-2 IFA. DENV-2 was detected by indirect immunofluoresence in head squashes (Bennett et al. 2002). A mouse-derived monoclonal antibody 3H5 (Henchal et al. 1985), directed against a flavivirus E protein epitope, was the primary antibody.

Late trypsin and DENV-2 Western blots. *Aedes aegypti* midguts infected with DENV-2 were dissected and proteins were extracted in pools of five midguts by homogenizing them with 50 μL of 1X PBS containing protease inhibitors. Supernatant was collected after centrifugation (12,000 rpm for10 minutes). To denature the proteins, supernatants were mixed with 1 volume of 2X SDS sample buffer and boiled for 5 minutes. The equivalent of one midgut/lane (20 μL) was run in a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. The protein was transferred to a polyvinylidine difluoride (PVDF) membrane using the Transblot system (Bio-Rad, Hercules, CA). Western blot for DENV-2 was done with mouse monoclonal antibody

1A6A-8 against E-glycoprotein (Roehrig et al. 1998), and late trypsin was detected with a specific mouse monoclonal antibody (Graf et al. 1988). First antibody was detected with an anti-mouse/phosphatase conjugate.

Results

Inhibition of midgut trypsin activity and blood digestion by STI. Late trypsin accounts for most of the endoproteolytic activity during blood digestion in the *Ae. aegypti* midgut, and its expression is regulated by the activity of early trypsin (Noriega and Wells 1999). Mosquitoes were membrane fed a DENV-2–infected blood meal with or without 2 mg/mL STI. The extent of midgut trypsin inhibition by STI was assessed by measuring late trypsin mRNA and protein levels after blood feeding. Minimal amounts of late trypsin mRNA were detected by qRT-PCR in midguts from STI-treated mosquitoes 24 hours after blood feeding. These were not significantly different from prefeeding levels and were 97% lower than in untreated individuals (Figure 4.1A). Late trypsin Western blot analysis on midguts was consistent with mRNA data. Midguts from STI-treated mosquitoes showed minimal amounts of late trypsin protein 24 hours after blood feeding compared with untreated, and no late trypsin protein was detected 48 hours after blood feeding (Figure 4.1B). Both observations indicate that midgut trypsin activity was substantially inhibited by STI.

The level of inhibition of blood digestion by STI was determined by assessing oogenesis because this correlates with the amount of blood digested by the mosquito (Klowden 1987). Whereas untreated mosquitoes presented normal egg development by 48 hours after blood feeding (Figure 4.1C), most STI-treated mosquitoes had no detectable egg development (Figure 4.1D). Consistent with these results, STI-treated

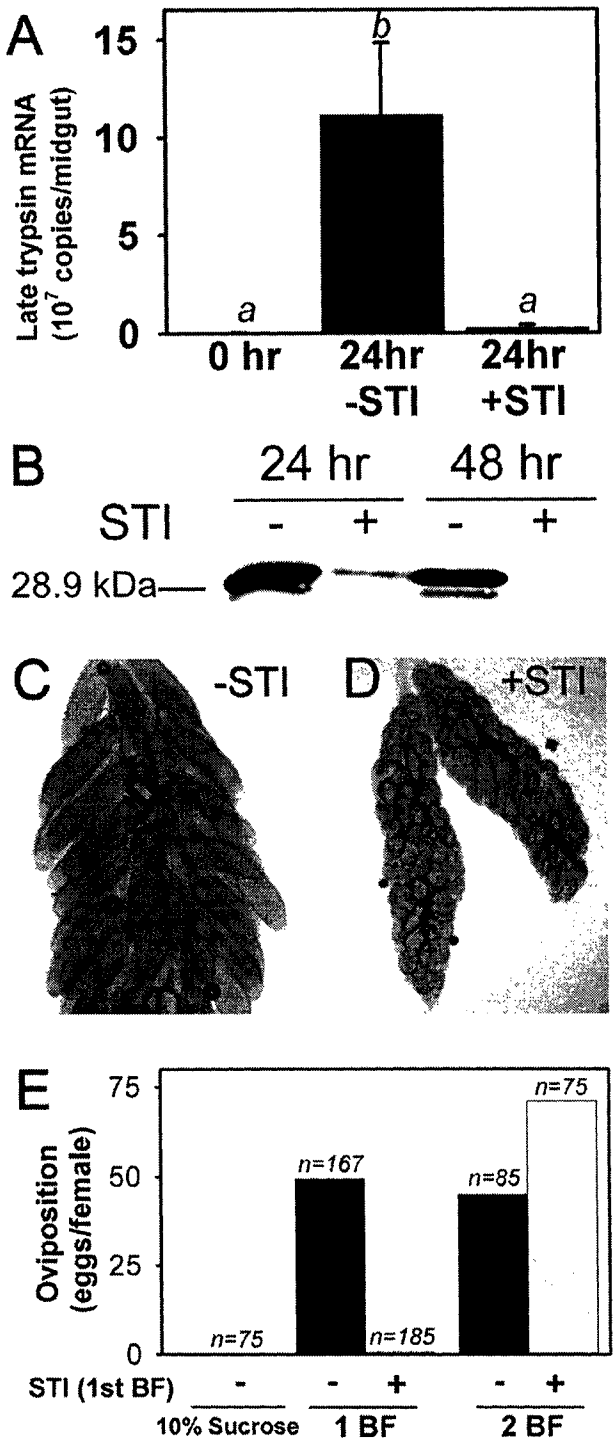

FIGURE 4.1. Inhibition of *Ae. aegypti* midgut trypsin activity by STI. Midgut late trypsin mRNA levels by qRT-PCR (**A**), midgut late trypsin protein by Western blot (**B**), ovary development (**C, D**), and oviposition (**E**) after a blood meal with (+) or without (–) STI. A second uninfected blood feeding (2BF) without STI was done 7 days after the infectious blood feeding. Error bars represent standard error; different letters are significantly different (ANOVA, $P < 0.05$). qRT-PCR data were normalized with ribosomal S6 mRNA levels. Ovary images were taken at the same magnification. The number of mosquitoes (n) is indicated in E.

mosquitoes produced only 1% of the eggs laid by untreated mosquitoes (Figure 4.1E). Mosquitoes treated with STI remained engorged for an average of 3 days after blood feeding and then excreted the blood meal. The almost complete inhibition of oogenesis by STI indicates that the inhibition of trypsin activity nearly completely inhibited blood digestion.

The reversibility of the inhibition of digestion by STI was tested by providing mosquitoes with a second, untreated blood meal 7 days after receiving the infectious blood meal with STI. Administration of a second blood meal resulted in higher levels of oviposition in the group that had received STI in their first meal (Figure 4.1E). This shows that the initial lack of oviposition was due to the nutritional effect of STI and that it had not damaged the ovaries. No significant differences in mortality 7 days postinfection was observed between STI treated (8.6% ± 9) or untreated mosquitoes (7.1% ± 5.8), and STI had no observable toxic effects.

Effect of STI on DENV-2 midgut infection. STI-treated mosquito midguts developed a DENV-2 infection but at much lower titers and with slower replication of the virus when compared with midguts from untreated mosquitoes. The levels of DENV-2 (+) RNA from days 4 to 7 in midguts from STI-treated mosquitoes were 91--97% lower than in the untreated group (Figure 4.2A). In the case of viral protein, midguts from STI-treated mosquitoes did not have immunoblot detectable DENV-2 E protein up to day 14 after blood feeding, whereas in midguts from untreated mosquitoes it was detectable at day 4 (Figures 4.2B and 4.2E). The amount of DENV-2 (-) RNA was also measured to evaluate replication intermediates as indicators of active infections (Cleaves et al. 1981, Liu et al. 1997, Vaughan et al. 2002, Westaway et al. 2003). Midguts from STI-treated

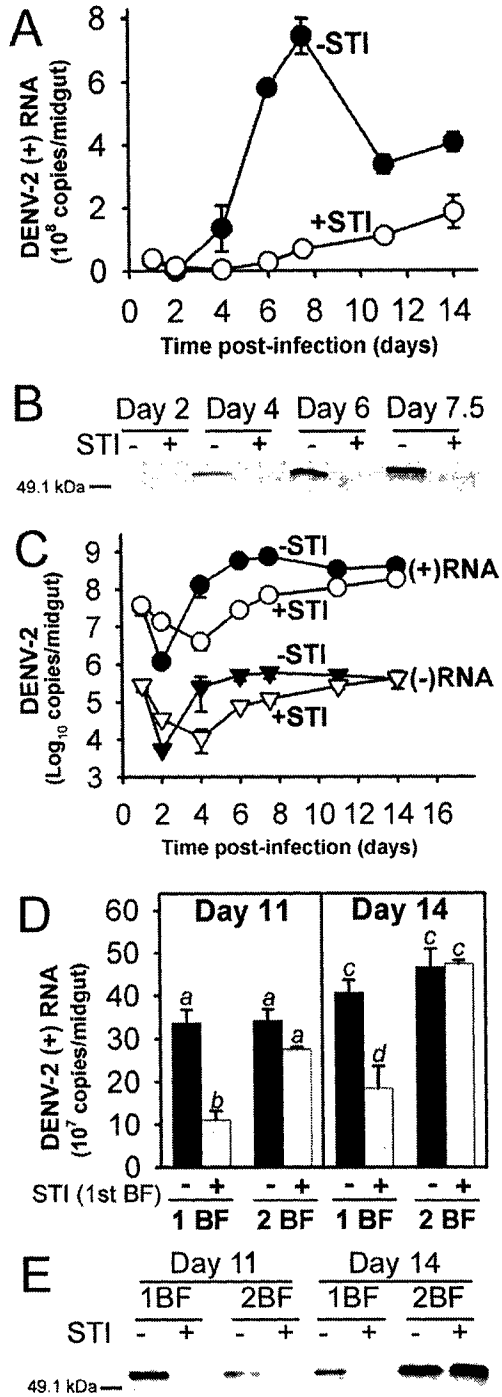

FIGURE 4.2. Effect of inhibiting *Ae. aegypti* midgut trypsins with STI, during digestion of a DENV-2–infected blood meal, on the viral infection of the midgut. Midgut DENV-2 (+)RNA by qRT-PCR (**A**, **C**, **D**), Western blot of midgut DENV-2 E protein (**B**, **E**), and DENV-2 (–)RNA by qRT-PCR (**C**) after a DENV-2–infected blood meal with (+) or without (–) STI. A second uninfected blood feeding (2BF) without STI was done 7 days after the infectious blood feeding. Error bars represent standard error; different letters within a day are significantly different (two-way ANOVA, $P < 0.05$). qRT-PCR data were normalized with ribosomal S6 mRNA levels.

mosquitoes also had lower levels of DENV-2 (-) RNA from days 4 to 11 when compared with untreated mosquitoes (Figure 4.2C). Although the amount of DENV-2 (-) RNA was 10^2--10^3 lower, the courses of infection as detected by DENV-2 (–) RNA and DENV-2 (+) RNA followed a similar pattern (Figure 4.2C).

We also tested the hypothesis that STI lowers DENV-2 infection because of lack of nutrients. Mosquitoes received a second uninfected blood meal with no STI 7 days after the infectious one. When given a second blood meal, the levels of DENV-2 (+) RNA measured in midguts of STI-treated mosquitoes were not significantly different from those of untreated ones (Figure 4.2D) at days 11 and 14. The second blood meal also recovered viral E protein expression by day 14, not by day 11, as detected by Western blot (Figure 4.2E). These results suggest that the lower DENV-2 midgut infection with STI is in part caused by the lack of nutrients, most probably amino acids, normally provided by blood digestion.

DENV-2 dissemination. The effect of inhibiting midgut trypsin activity on DENV-2 dissemination from the midgut was studied by measuring DENV-2 (–) RNA in the thorax, which is direct evidence of DENV replication in a tissue and decreases the possibility of measuring (+) RNA from contaminating viral particles released by the midgut during dissection. No significant viral dissemination was detected before day 7. Mosquitoes that received STI in the infectious blood meal showed lower amounts of DENV-2 (–) RNA in the thorax by days 11 and 14 postinfection than untreated mosquitoes (Figure 4.3A). Similar results were obtained by immunofluorescent assay (IFA) on heads. Only 16% of untreated mosquitoes were negative for DENV-2 E protein in the head by day 14 as compared with 35% of STI-treated ones (Figure 4.3B). STI

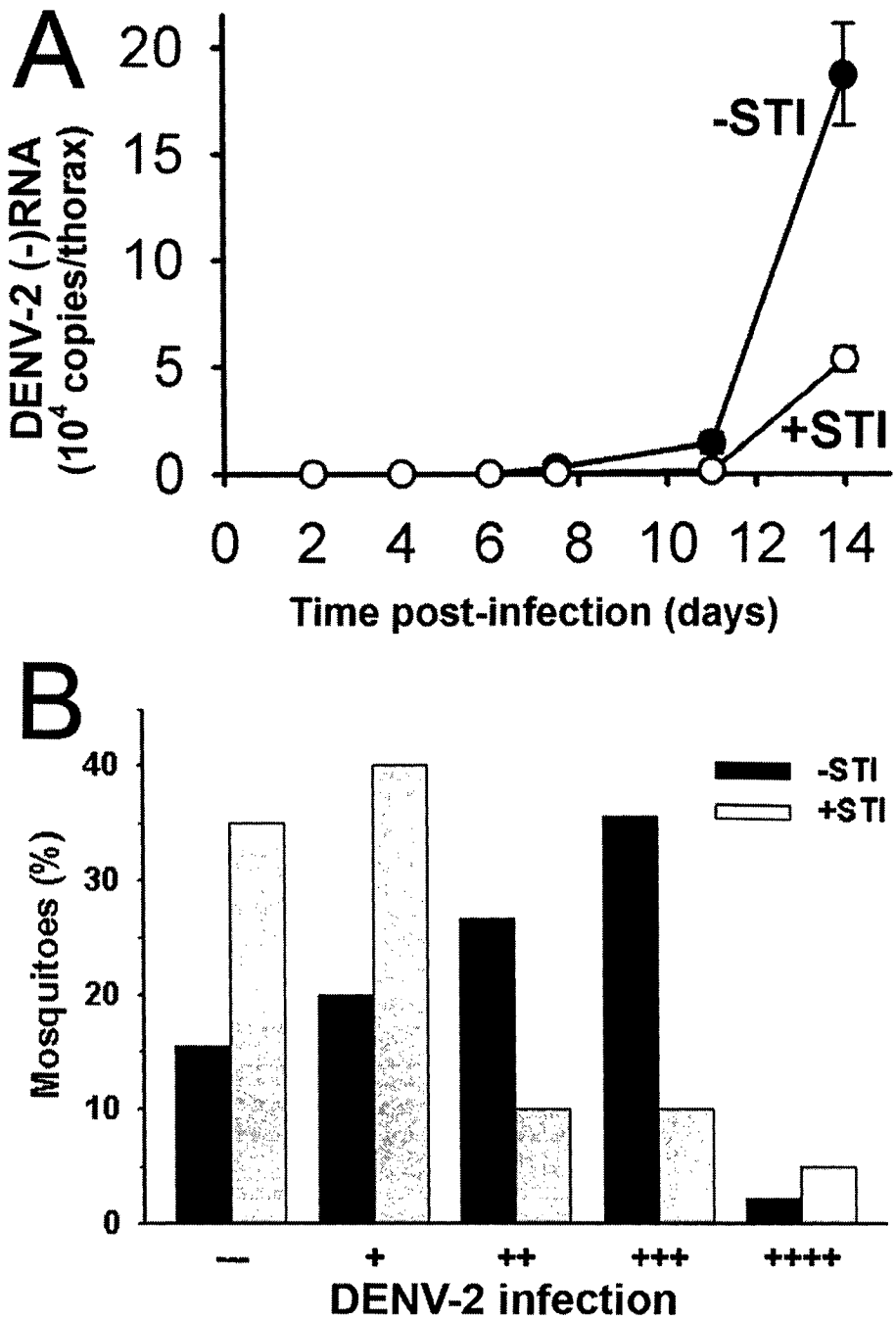

FIGURE 4.3. Effect of inhibiting *Ae. aegypti* midgut trypsins with STI, during digestion of a DENV-2–infected blood meal, on the dissemination of the virus from the midgut. DENV-2 (–) RNA by qRT-PCR in thorax (**A**), IFA of DENV-2 E protein in mosquito heads by day 14 postinfection (**B**) with a DENV-2–infected blood meal with (+) or without (–) STI (-STI $N = 45$; +STI $N = 20$). qRT-PCR data were normalized with ribosomal S6 mRNA levels. Head infection levels were uninfected (–) and infected in four increasing levels (+, ++, +++, ++++). Error bars represent standard error. Frequency distributions for head infections with and without STI were significantly different (χ^2, $P < 0.05$).

treatment also led to a lower infection titer in the head (Figure 4.3B). These results indicate that midgut trypsin activity also affects the rate of dissemination from the midgut and could therefore constitute a midgut escape barrier.

***In vitro* digestion with bovine trypsin.** Mosquitoes were fed a meal with a 50% volume of sheep blood, 25% uninfected cell culture, and 25% DENV-2–infected culture. Three different treatments were compared (see Table 4.1). The infectious blood meal virus titer was $10^{9.2}$ $TCID_{50}$/mL for the undigested control and $10^{8.8}$ $TCID_{50}$/mL for both the *in vitro* trypsin-digested samples. At day 4 postinfection, trypsin pre-digestion of the viral culture rescued the level of DENV-2 (+) RNA in the midgut to levels even higher than in mosquitoes fed a meal without STI or one in which uninfected cell culture was predigested (Figure 4.4A). This result indicates that direct contact of trypsin with the virus is necessary to enhance the infectivity. This enhancement was confirmed by Western blot analysis, where in spite of the presence of STI in the meal, DENV-2 E protein was clearly detected (Figure 4.4B). Interestingly, the observed enhancement was transient, as by day 6 postinfection there was no difference in DENV-2 infectivity between midguts with the meals in which the uninfected or the DENV-2 infected culture were predigested with trypsin (Figure 4.4A), and the E protein could not be detected in either sample (Figure 4.4B). Mosquitoes that fed on DENV-2 culture or trypsin-treated uninfected cell culture that were then inhibited with STI had large reductions in oviposition rates (96% and 95.5%, respectively) as compared with the untreated control (84.75 eggs/female, $N = 94$).

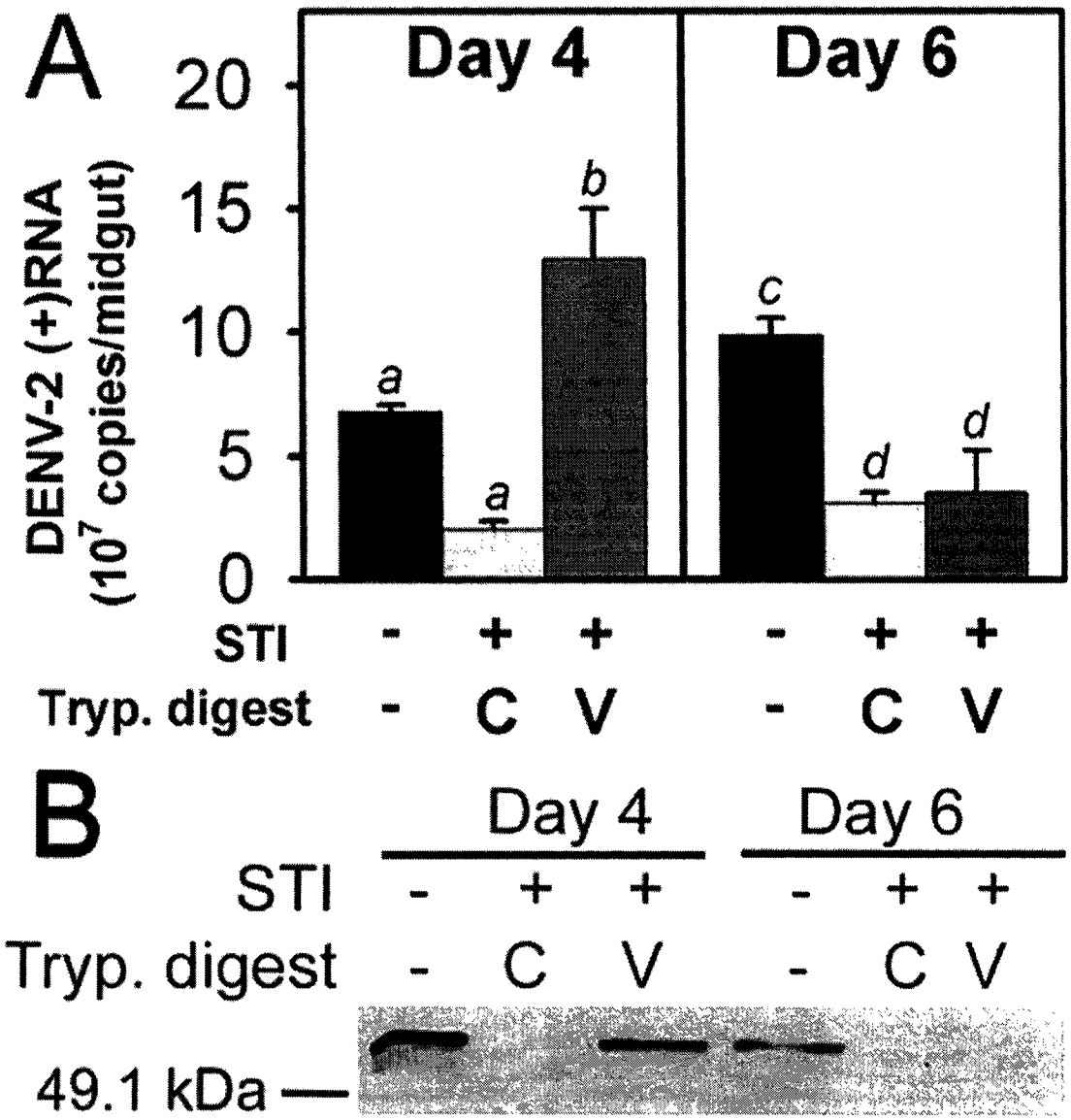

FIGURE 4.4. Effect of bovine trypsin *in vitro* digestion of DENV-2 culture on infection of *Ae. aegypti* midguts. DENV-2 (+) RNA by qRT-PCR (**A**) and DENV-2 E protein Western blot (**B**) were detected in midguts. *In vitro* digestion with bovine trypsin was done on DENV-2 culture (V) or cell culture control (C) and was stopped with STI. Error bars represent standard error; different letters within a day are significantly different (two-way ANOVA, $P < 0.05$). qRT-PCR data were normalized with ribosomal S6 mRNA levels.

Discussion

The use of qRT-PCR to determine DENV-2 RNA levels in specific mosquito tissues allowed a fast and detailed analysis of the time course of DENV-2 infection. After the administration of a regular infectious blood meal, not containing STI, there was a decrease in midgut DENV-2 titer on the second day postinfection (Figure 4.2C). This decrease corresponds to the eclipse phase due to elimination of the virus present in the blood meal (Hardy 1988, Woodring et al. 1996), and it was followed by a steady increase in DENV-2 RNA up to 7 days postinfection (Figure 4.2A). This time course of midgut infection was similar to patterns of infections of *Culex tarsalis* (Kramer et al. 1981) and *Culex pipiens* midgut (Lamotte 1960) with Western equine encephalitis virus and Japanese B encephalitis virus, respectively. At day 11, however, a decline in DENV-2 RNA levels was observed (Figure 4.2A), which could be due in part to a general reduction in midgut metabolic activity. Alternatively, one cannot dismiss the possibility that the midgut could be actively eliminating the virus. Administration of a second noninfectious meal did not significantly increase DENV-2 RNA levels at days 11 and 14 (Figure 4.2D), indicating that midgut viral RNA levels reached at this time are not influenced by nutrient availability. In contrast, the second meal apparently resulted in a pronounced increase in E protein expression at day 14 (Figure 4.2E), indicating that the availability of dietary amino acids could be limiting protein translation. By the time midgut DENV-2 RNA levels begin to decline (day 11), the virus can be detected in the thorax and increases dramatically in this tissue by day 14 postinfection (Figure 4.3A). At day 14, the virus has also disseminated to the head, as 84% of the females were positive for DENV-2 by immunofluorescence (Figure 4.3B).

Inhibition of midgut trypsin activity during an infectious blood feeding led to a significantly lower titer of DENV-2 in the midgut and to a lower rate of DENV-2 dissemination (Figures 4.2 and 4.3). Midgut trypsin activity appears to be required for DENV-2 to achieve optimal levels of infection, replication, and dissemination in the midgut. Inhibition appears to be due in part to a nutritional effect, as a second uninfected blood meal recovered DENV-2 mRNA titers of STI-treated mosquitoes (at days 11 and 14) and protein E translation (at day 14) back to control levels (Figure 4.2D).

The *Ae. aegypti* midgut undergoes major changes in morphology and gene expression after blood feeding. These changes include dispersion of mitochondria and rearrangement of the rough endoplasmic reticulum, suggesting large-scale metabolic shifts in the blood-fed midgut (Bertram and Bird 1961, Rudin and Hecker 1979). Blood feeding also triggers changes in expression of at least 333 midgut genes, including upregulation of genes involved in protein and amino acid metabolism, peritrophic matrix-formation, and iron metabolism (Sanders et al. 2003). Our results suggest that the metabolic changes in the midgut elicited by blood meal digestion are required for optimal DENV-2 replication and dissemination. In STI-treated mosquitoes, midgut DENV-2 mRNA titers increased slowly over time (Figure 4.2C), but E protein could not be detected up to 14 days postfeeding, indicating that the nutritional effect was more dramatic at the level of protein translation. The inhibition of blood digestion presumably prevented the establishment of an adequate mid-gut amino acid supply to support the synthesis of detectable amounts of DENV-2 E protein in the midgut. This nutritional deficit did not affect other tissues to the same extent as the midgut, as IFA detected E protein in the heads of 65% of STI-treated mosquitoes (although at lower levels than the

control group) by day 14 (Figure 4.3B). The lack of detection of DENV-2 E protein by Western blot in the midgut of STI-treated mosquitoes up to day 14 suggests lack of sensitivity of this method. DENV-2 E protein must have been produced in the midgut in order for the virus to disseminate.

The increase in DENV-2 replication after a second blood meal ingestion could have epidemiologic implications. Ingestion of multiple uninfected blood feedings may boost a previous incipient infection. This seems plausible, as *Ae. aegypti* tends to take small sequential blood-feeds on humans (Harrington et al. 2001). Our results show that even very low levels of virus can remain "dormant" in the midgut for a week (and probably longer) and then be reactivated by ingestion of uninfected blood, generating high infection titers.

The fact that the level of trypsin activity affects DENV-2 midgut infection and dissemination suggests that natural variations in the rate of induction, or absolute expression levels of midgut trypsins, could result in midgut infection and dissemination barriers. Natural variation in midgut trypsin activity could be genetically or environmentally determined. Consistent with this hypothesis, quantitative trait loci (QTL) analysis of a genetic cross between mosquitoes susceptible and refractory to dengue infection revealed that the disseminated infection rate correlated positively and additively with the number of susceptible alleles present for both early trypsin (Chr. II) and late trypsin (Chr. III) genes (Bosio et al. 2000). It is also known that environmental temperature affects both the rate of enzyme synthesis and activity in *Ae. aegypti* (Briegel and Lea 1975, Gooding 1996). Blood digestion in *Ae. aegypti* at 32°C is twice as fast compared with 22°C (Briegel and Lea 1975).

In vitro digestion of the viral culture (in C6/36 cells) prior to the addition of STI to the blood meal increased the amount of DENV-2 mRNA and E protein in the *Ae. aegypti* midgut by day 4 (Figures 4.4A and 4.4B) but not by day 6. This suggests that tryptic digestion of viral surface proteins enhanced the interaction of DENV-2 with the midgut cells during the first days postinfection but was unable to support viral replication. It is unlikely that the transient increase in infectivity associated with trypsin predigestion of the viral culture was due to a nutritional effect, as this phenomenon was not observed in the control group in which the uninfected cell culture was predigested instead of the DENV-2–infected culture. Direct contact of the protease with the virus was required to enhance its association with the midgut. Furthermore, *in vitro* pre-digestion of the meal (infected or uninfected) had little nutritional value, as only minimal egg production was observed in all STI-treated samples.

Enhancement of arbovirus infectivity in the insect midgut by proteolytic processing has previously been shown for La Crosse virus (Bunyavirus) in *Oc. triseriatus* (Ludwig et al. 1989, Ludwig et al. 1991) or for Bluetongue virus (Orbivirus) in *Culicoides* biting midges (Mertens et al. 1996, Xu et al. 1997). In those cases, proteolytic processing of viral glycoprotein increased binding to midgut cells incubated *in vitro*. A similar mechanism could underlie the effect of trypsin on DENV-2 in *Ae. aegypti* we have observed *in vivo*. It has been shown previously that cleavage of prM protein into pr and M proteins is associated with an increase of infectivity of dengue viruses in Vero cells (Randolph et al. 1990) and of tick-borne encephalitis virus (Flavivirus) in C6/36 (Guirakhoo et al. 1991), porcine (Heinz et al. 1994, Stadler et al. 1997), and in BHK-21 cells (Elshuber et al. 2003). Cleavage of prM could be involved in the enhanced

infectivity of DENV-2 we observed, as trypsin has been shown to be capable of processing prM protein in tick-borne encephalitis virus (Elshuber et al. 2003). Further experiments are underway to elucidate the subcellular localization of DENV-2 during this period of enhanced interaction and the mechanism(s), such as modification of specific viral surface proteins, which could mediate this phenomenon.

In summary, we have shown that midgut trypsin activity affects DENV-2 infection and dissemination in the *Ae. aegypti* midgut. Midgut trypsin activity facilitates DENV infection in *Ae. aegypti* through a nutritional effect and probably also by direct proteolytic processing of the viral surface.

CHAPTER 5

CONCLUSIONS

The studies reported herein were centered on the quantitative analysis of DENV-2 RNA replication and dissemination in *Ae. aegypti*. The information obtained has contributed significantly to our understanding of the replication rate of DENV-2, JAM1409 in the midgut, head, and legs of select *Ae. aegypti* strains. This work has also made significant methodological contributions to the field of arbovirology, by extending the use of a still nascent technology, quantitative, real-time RT-PCR, and developing new protocols that may serve as a foundation for further study of vector-virus interactions and vector competence.

Two important discussions arise from these studies. The first involves a cost benefit analysis regarding the value of the quantitative study of an arbovirus in a vector. The other, by extension of the first, concerns questions of the impact of higher or lower virion concentrations on the vector competence of a mosquito. In other words, are quantitative analyses of the interaction between DENV-2 and *Ae. aegypti* worth the cost and how biologically and epidemiologically relevant are variations in DENV-2 RNA concentration in the vector?

The value of the immunofluorescent assay (IFA), commonly employed for detection of DENV antigen in fixed mosquito tissues, is without question. Much has been learned about vector competence using this convenient, cost effective, qualitative assay to characterize infection rates of a vector species with a given arbovirus. However,

as typically applied, the IFA provides only a qualitative scale of infection which is highly subjective in nature. It can not be used to study the quantitative differences in virus replication rate within the pool of infected samples--quantitative differences that may play a critical role in infection and escape barriers in various tissues. We are now in a period in which arbovirologists are focused on how host and viral factors interact to determine the fate of the virus in the vector. These factors almost certainly vary on a continuous scale and the ability to measure differences within and among individuals as well as their fluctuation over time is essential. Thus, the ability to accurately measure virus levels in a statistically robust fashion is vital. This may be accomplished by measuring infectious units of virus through the use of the plaque assay or by quantifying viral RNA using real-time RT-PCR. As with the IFA, each of these methods has its uses and advantages. They are not exclusive but complimentary and all three tools (as well as a Western blot) have been used in these studies.

Certainly employing a quantitative assay is expensive. Is it relevant?: Yes. It is clear that viral dose is a critical determinant of midgut infection (Chamberlain et al. 1954, Gubler et al. 1979, Miller et al. 1982, Houk and Hardy 1989, Bennett et al. 2002). There exists a threshold virus level below which midgut infection does not occur. Although the mechanism of cell invasion by DENV has yet to be demonstrated, it is intuitive to suggest that requisite events such as virus-receptor contact or proteolytic cleavage of viral proteins are a function of virus concentrations. Similarly, productive infection of the midgut epithelium requires that a sufficient number of virions avoid cellular antiviral defenses. Again these have yet to be adequately characterized and we are forced to hypothesize about the likely involvement of apoptosis or RNA interference in limiting

virus replication in the mosquito midgut or in the degradation of viral RNA already transcribed. The erratic changes in midgut DENV-2 RNA levels observed during the dissemination phase (6—10 dpi) support the possible involvement of host or viral factors that are causing significant changes in viral RNA levels. Here too, we can envision a threshold of infection above which the vector's defenses are overwhelmed and the virus triumphs. Thus virus concentration both in the blood meal and in the newly infected midgut epithelial cells is relevant in that it is likely a central concept to the midgut infection and escape barriers.

Relying solely on qualitative estimates of disseminated infection rates to assess vector competence may result in over estimation of actual DENV transmission potential. Knox and colleagues (2003) recently demonstrated that rates of DENV-2 and DENV-4 dissemination were not associated with transmission rates in *Ae. aegypti* while Gubler and Rosen (1976a) showed that 74% of *Ae. albopictus* with disseminated infections transmitted DENV-2. Similarly, only a portion of *Ae. aegypti* with YFV or DENV-2 antigen in the head successfully transmitted virus to suckling mice (Miller et al. 1982, Miller et al. 1989, Miller and Mitchell 1991). There is evidence of a correlation between the extent of salivary gland infection and likelihood of DENV transmission, indicating that the quantity of virus in the salivary glands may impact transmission (Gubler and Rosen 1976a).

It is well known that the dose of DENV in the blood meal impacts the infection rate of *Ae. aegypti* and *Ae. albopictus* fed an artificial blood meal (Gubler and Rosen 1976b, Bennett et al. 2002). Perhaps a similar threshold model can be applied to DENV transmission. Blood feeding *Ae. aegypti* are known to secrete varying quantities of saliva

(Devine et al. 1965) and it is possible that DENV transmission as well as disease outcome may be a function of viral load in the saliva and the dose of virus inoculated during probing (Gubler and Rosen 1976a). Recent successful attempts to quantify LACV and WNV in saliva provide promise that similar studies with DENV will provide answers to these questions regarding the relationship between the amount of disseminated virus and actual transmission (Vanlandingham et al. 2004, Colton et al. 2005).

The fact that midgut levels of DENV-2 were lower in the *D2MEB* strain supports the hypothesis that virus concentration in the midgut is associated with a strong MEB. Based on the relatively low levels of DENV-2 RNA in the midgut of *D2MEB* mosquitoes, we can formulate a hypothetical MEB model in which an unknown host factor is limiting viral replication or affecting the degradation of viral RNA in the midgut epithelium. This may involve an antiapoptotic pathway which is active in strains lacking a strong MEB but disrupted in *D2MEB* mosquitoes. Infected midgut cells may be dying, which lowers the overall infection of the midgut. Both DENV-2 and JEV have recently been shown to block caspase-dependent apoptotic cell death during early stages of infection in a mouse neuronal cell line (Lee et al. 2005). The antiapoptotic pathway appears to involve the activation of a lipid kinase, phosphatidylinositol 3-kinase (PI3-K) and its downstream target, Akt. Although the authors did not address the potential of a parallel mechanism in the mosquito, the fact that the PI3-K/Akt pathway is conserved in *Ae. aegypti* (Riehle and Brown 1999) highlights the possibility that a similar viral induced antiapoptotic mechanism may be at play in *Ae. aegypti*. Recent evidence also points to a potential antiapoptotic mechanism at work in the midgut of WNV infected *Culex pipiens quinquefasciatus* (Girard et al. 2005). Observations that salivary gland

cells were more susceptible to WNV replication and virus induced CPE led to the hypothesis that a midgut specific mechanism may be modulating virus replication.

With this knowledge base, it is safe to say that quantitative analyses of viral replication and dissemination are critical to enhance our understanding of vector competence in general, and midgut barriers in particular. However, caution is warranted in placing all one's eggs in a single basket. This work helped demonstrate the value of high throughput viral RNA quantitative assays while recognizing the fact that the biological unit of concern is an infectious virus. While many questions remain to be answered regarding the dynamic relationship between arboviral RNA levels and infectivity, our correlations between DENV-2 RNA copy number and number of PFU provide a contextual framework that facilitates rough translation between the two types of data. DENV-2 PFU were consistently lower than RNA copy number by 2--3 $\log_{10}$. The variability of the ratio of DENV-2 RNA copies to PFU is not surprising given the variability in defective, immature, or inactivated virus particles. The preponderance and stability of viral RNA relative to infectious virus can be advantageous particularly in cases of low virus concentrations or dormant infection as observed in STI treated and overwintering mosquitoes (Farajollahi et al. 2005). In such cases, reduced metabolic rates appear to be disrupting the production of infectious viral particles and/or structural proteins. This would explain the difficulty in isolating virus from overwintering mosquitoes without first breaking diapause by holding samples in an insectary.

The relationship between vector metabolism and virus replication is complex. Our findings indicate that with productive infections in mosquitoes lacking a MEB, overall midgut DENV-2 RNA levels appear to be only weakly dependent on bloodmeal

associated metabolic changes in the midgut. Viral RNA levels were not affected by a second bloodmeal (either at day 7 or 14) and were only correlated with total RNA concentration at days 0, 12, and 13. This applies to mosquitoes with a typical virus load in the midgut and not to individuals treated with STI. In these mosquitoes, the dormant virus clearly responded to a second bloodmeal by increasing both the rate of viral RNA transcription and E glycoprotein translation. Interestingly, control mosquitoes that showed no increase in DENV-2 RNA levels following the second bloodmeal, did increase translation of E protein. So although bloodmeal linked nutrient availability did not limit viral RNA synthesis, it did impact translation of viral protein. The inability of a viral RNA assay to detect changes in viral protein translation is a clear limitation when analyzing the vector-virus interaction. This should be a critical consideration in future studies using real-time RT-PCR to build expression profiles of genes which code for putative vector competence factors. The likelihood that viral and vector determinants of infection and dissemination rates may be translationally controlled necessitates evaluation of these candidate factors at both the RNA and protein level.

It is important to stress that these analyses have been limited to the use of a single serotype of DENV-2. Our focus was on the interaction of a single virus with *Ae. aegypti* mosquitoes from various strains under specific experimental treatments. Additionally we were interested in exploring the potential applications of real-time RT-PCR and DENV-2 quantification to assess the interaction of DENV-2 in *Ae. aegypti*. Even within this limited context we have observed significant variation in infection levels between individual mosquitoes. This underscores the complexity of the relationship between host and virus. In an effort to simplify this intricate system, it will be important to continue

using genetically homogenous *Ae. aegypti* strains with well characterized DENV-2 viruses, in the hopes of identifying specific effector molecules that condition the barriers to infection and dissemination.

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