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ABSTRACT

Title of Dissertation:	THE INFLUENCE OF MULTIPLE HOST CONTACTS ON THE ACQUISITION AND TRANSMISSION OF DENGUE-2 VIRUS BY AEDES AEGYPTI
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I tested assumptions of the hypothesis that multiple host contacts by Ae. aegypti contribute to dengue virus transmission.

The hypothesis assumes multiple meals that combine a viremic meal with a meal from an immune host will not interfere with mosquito infection. I administered enemas, which simulated a second meal from an immune host, to Ae. aegypti that had fed 24 hours earlier from a drop of dengue-2 virus infected blood. Infection rates of the mosquitoes receiving immune sera (85%) were significantly lower than the controls (92%). Lower infection rates reduce the potential for virus transmission. In vitro transmission rates of the mosquitoes that became infected were not altered by the immune sera.

The multiple contacts hypothesis also assumes Ae. aegypti is not depleted of infectious virus by probing and/or engorging on a host. Using an in vitro transmission assay, I tested Ae. aegypti infectivity and found that after 20 consecutive probes on a guinea pig, Ae. aegypti still transmitted virus as well as controls. Likewise, imbibing a

replete meal from a guinea pig did not alter infectivity. Once infective, Ae. aegypti is an efficient vector and can transmit virus each time it probes or imbibes blood from a host.

Dengue viruses might make it more difficult for Ae. aegypti to locate or imbibe blood and thus ensure that multiple contacts occur. I tested this prediction by comparing the blood feeding behavior of dengue-2 virus infected Ae. aegypti with uninfected mosquitoes and found no difference. Dengue-2 virus does not alter the feeding behavior of Ae. aegypti.

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ABSTRACT

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John L. Putnam, Doctor of Philosophy, 1993

Dissertation directed by: Dr. Thomas W. Scott, Professor, Department of Entomology

It has long been known that Ae. aegypti contact more than one host per ovarian cycle, but until recently the hypothesis that multiple host contacts contribute to dengue virus transmission had not been empirically evaluated. I tested assumptions of this hypothesis.

Multiple host contacts by Ae. aegypti could result in mosquitoes imbibing two blood meals--one from a viremic host and the other from an immune host. The hypothesis assumes such an event will not interfere with mosquito infection. I administered enemas, which simulated a second meal from an immune host, to Ae. aegypti that had fed 24 hours earlier from a drop of dengue-2 virus infected blood. Infection rates of the mosquitoes receiving immune sera (85%) were significantly lower than the controls (92%). Lower infection rates reduce the potential for virus transmission. In vitro transmission rates of the mosquitoes that became infected were not altered by the immune sera.

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Dengue viruses might alter the blood feeding behavior of Ae. aegypti by making it more difficult to locate or imbibe blood and thus ensure that multiple contacts occur. I tested this prediction by comparing the blood feeding behavior of dengue-2 virus infected Ae. aegypti with uninfected mosquitoes and found no difference. Dengue-2 virus does not alter the feeding behavior of Ae. aegypti.

THE INFLUENCE OF MULTIPLE HOST CONTACTS ON THE ACQUISITION AND TRANSMISSION OF DENGUE-2 VIRUS BY *AEDES AEGYPTI*

by

John L. Putnam

Dissertation submitted to the Faculty of the Graduate School
of The University of Maryland in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
1993

Advisory Committee:

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DEDICATION

To Laksamee and Kyle,

who rightfully complained the most.

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Chapter I

Introduction

Overview

This dissertation examines the hypothesis that multiple host contacts by Aedes aegypti (L.) contribute to dengue virus transmission. In this first chapter, I set the stage for my research project by defining multiple host contacts and presenting the reasons for proposing that multiple contacts contribute to dengue virus transmission. Also, I discuss the illnesses caused by dengue viruses and the transmission of dengue viruses. Finally, I introduce the experiments I conducted to test the multiple host contacts hypothesis.

Multiple Host Contacts by Mosquitoes

The traditional paradigm of mosquito blood feeding asserts that mosquitoes feed on blood only once per gonotrophic cycle and therefore contact only one host in a single gonotrophic cycle (Klowden, 1988). Laboratory studies of the physiological regulation of host-seeking behavior appear to support this contention. Following a replete blood meal, mosquitoes generally do not seek hosts while they digest the blood meal and develop eggs. This inhibition of host-seeking is governed by a two component system (Klowden, 1988). Initially, stretch receptors in the

blood-swollen abdomen inhibit further host-seeking. As the blood meal is digested, the ovaries become vitellogenic and induce the release of a host-seeking inhibitory factor from the fat body. This oocyte-induced inhibition suppresses host-seeking until the eggs are laid. These two mechanisms would "appear to limit host-seeking behavior to once each gonotrophic cycle" (Klowden, 1988) and thus restrict contacts to one host per gonotrophic cycle.

It is well documented, however, that mosquitoes, including Ae. aegypti, do not limit their host contacts to one per gonotrophic cycle (McClelland and Conway, 1971; Pant and Yasuno, 1973; Boreham and Lenahan, 1976; Klowden and Lea, 1979; Magnarelli, 1979; Mitchell et. al, 1979; Ritchie and Rowley, 1981; Klowden, 1988; Gubler, 1988). Mosquitoes that contact more than one host per gonotrophic cycle are described as engaging in multiple host contacts. I define a contact as the insertion of a mosquito's fascicle into the host's tissue. Initially, the mosquito thrusts the fascicle through the host's tissue (probes) in search of blood (Griffiths and Gordon, 1952). A defensive host may dislodge a mosquito during a probe (Service, 1971; Walker and Edman, 1985) or, if the mosquito cannot locate blood the mosquito may simply desist (Ribeiro et al, 1985). A multiple host contact may then occur if the mosquito continues its search for blood on another host. These interrupted probes may be epidemiologically important because if the mosquito is

already infected dengue viruses can be inoculated each time it probes a susceptible host (Gubler and Rosen, 1976).

Once blood is found the mosquito begins to engorge or imbibe the blood. A defensive host may interrupt a mosquito while the mosquito is imbibing blood and prevent the mosquito from imbibing a complete blood meal. If the partial blood meal is not large enough it will not initiate distention-inhibition or oocyte-induced inhibition of host-seeking (Klowden, 1988). Thus, a mosquito with a partial blood meal will continue to seek hosts and attempt to feed until it has imbibed enough blood to initiate inhibition.

For several reasons, even mosquitoes that feed to repletion may attempt to imbibe blood more than once before laying their eggs. Mosquitoes that are nutritionally stressed may require two complete blood meals to initiate oogenesis; one blood meal to prepare the follicles for vitellogenisis, a second to complete vitellogenisis (Briegel, 1990). Since oogenesis does not occur after the first blood meal, these mosquitoes do not experience oocyte-induced inhibition and therefore will attempt to refeed within a single ovarian cycle (Klowden, 1988). Finally, older mosquitoes tend to seek hosts even after a replete meal because they require more blood to induce distension-inhibition and have a delayed onset of oocyte-induced inhibition (Klowden, 1988). To summarize, multiple host contacts may occur when mosquitoes are interrupted while

probing or imbibing blood, or when not inhibited by the physiological mechanisms that govern host-seeking behavior.

Consequences of Multiple Host Contacts

Relative to the one-contact-per-gonotrophic-cycle scenario, contacting more than one host should increase dengue virus transmission. If mosquitoes contact hosts more frequently, transmission rates should increase because the mosquitoes are more likely to "acquire parasites from an infected host, as well as disseminate infections to uninfected hosts" (Klowden and Lea, 1979).

Many interacting factors regulate the transmission of arboviruses by mosquitoes. One way to examine the interaction of these factors is with a mathematical model that concisely relates the components of transmission and quantifies their effect on virus transmission. The model I will use for illustrative purposes in this research project is vectorial capacity. Vectorial capacity has long been used to describe the transmission of malaria parasites by mosquitoes (Dye, 1986; Garrett-Jones, 1964). Recently, it has been applied to arboviruses (Reisen, 1989; Reiter, 1988; Scott and Weaver, 1989). The vectorial capacity of a population of mosquitoes ($\underline{C}$) is the daily rate that potentially infective inoculations arise from an infective host and is defined by the following equation:

$$C = \frac{ma^2 p^n}{-\log_e p}$$

where m is the mosquito density (number of mosquitoes present/person/day), a is the proportion of mosquitoes that feed on humans each day, p is the daily survival rate of mosquitoes and n is the length of the extrinsic incubation period. The product ma is the number of mosquitoes that feed on a dengue infective human in a single day and p^n is the proportion of those mosquitoes that live through the extrinsic incubation period. Therefore, map^n is an estimate of the daily number of infective Ae. aegypti generated from a single human viremic with dengue. A proportion of those mosquitoes then feed on humans again, at rate a (hence a^2). These infective mosquitoes continue to feed on humans for a total number of days that is equal to their life expectancy, $1/-\log_e p$. (That is $1/-\log_e p$ estimates the average life expectancy when daily survival equals p.)

From the vectorial capacity equation it is clear that multiple host contacts can have a substantial effect on dengue virus dissemination. Multiple host contacts increase the biting rate, which increases a, the proportion of mosquitoes that feed on humans each day. Because a is squared, small increases in a result in relatively large non-linear increases in C. Multiple host contacts have the potential to increase both the number of mosquitoes that become infected and the number of infective inoculations.

The hypothesis that multiple contacts by Ae. aegypti contribute to dengue virus transmission is supported by at least three other observations. First, Ae. aegypti is very easily disturbed while attempting to feed and it feeds on humans during the day, when humans are most active (Gubler, 1988). This combination affords ample opportunity for the interruption of contacts, either by defensive or non-defensive host movements. Second, other researchers report that Ae. aegypti contacts several hosts within one gonotrophic cycle (MacDonald, 1956; Sheppard et. al, 1969; Gould et. al 1970; Yasuno and Pant, 1970; McClelland and Conway, 1971; Pant and Yasuno, 1973; Trpis and Hausermann, 1986; Klowden, 1988; Gubler, 1988). Most recently, Dr. Thomas Scott has documented the occurrence of frequent multiple feeding by Ae. aegypti in both Thailand (T.W. Scott, personal communication) and Puerto Rico (Scott et al., 1993). Finally, multiple host contacts provide a possible explanation for the clustering of dengue fever cases within a single house and with a similar date of onset of illness (Waterman et. al, 1985; Gubler, 1988).

Dengue Infections

The dengue viruses (dengue-1,2,3 and 4) are four serologically similar but distinct flaviviruses transmitted primarily by Ae. aegypti (Gubler, 1988). These viruses cause a wide range of human illness (Gubler, 1988). Some

infections are asymptomatic, others cause a mild febrile ailment, and some infections may cause death. Classic dengue fever is characterized by fever, severe headaches, joint pains, retroocular pains and a rash. The severity of the aches and pains associated with dengue fever led some to call this disease "breakbone fever" (Sabin, 1959). Typically, dengue fever lasts 3-7 days and though self limiting, most patients are prostrate throughout its course. Severe syndromes of dengue fever called dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), occur primarily in children under the age of 15. DHF/DSS is characterized by fever and vascular permeability that leads to internal hemorrhaging followed by shock. Untreated, DHF/DSS causes fatality rates of 40-50%, but if properly treated with intravenous fluids, fatality rates drop to less than 1% (Benenson, 1990).

Originally considered "an adult disease of the expatriate" in tropical Asia, dengue fever has now spread throughout the Pacific, Africa and the Americas (Gubler, 1988). It currently has a global distribution in the tropics. The incidence of dengue infection has also increased and now, worldwide, dengue infections cause more morbidity and mortality than any other arboviral disease (Defoliart et. al, 1986; Gubler, 1987; Gubler, 1988). In southeast Asia alone, hundreds of thousands of people contract dengue fever annually (Henchal and Putnak, 1990).

Worldwide, over 1.5 billion people are at risk of dengue infection (Halstead, 1980) and Lederberg et. al (1992) estimate more than 2 million cases of dengue fever occur each year. Concurrent with the increased incidence of dengue fever has been an increase in DHF/DSS and the frequency of epidemics (Gubler, 1988). Gubler (1988) points to two factors to explain the dramatic increase in incidence of dengue infections in recent years; failure to control mosquitoes and the increased spread of dengue viruses due to the rapid travel afforded by jet airplanes.

Dengue Virus Transmission Cycles

Humans, lower primates and Aedes mosquitoes, the only natural hosts for dengue viruses (Gubler, 1988), interact in three transmission cycles (Rudnick, 1965; Gubler, 1988). One cycle occurs in forests and involves transmission of dengue viruses between lower primates by forest dwelling Aedes mosquitoes. The second cycle is a rural or semirural cycle in which peridomestic Aedes mosquitoes transmit viruses to humans. The third cycle is an urban cycle involving virus transmission between humans by domesticated Aedes mosquitoes. Aedes albopictus and Ae. aegypti are considered the principle vectors of dengue viruses with Ae. aegypti being the most important epidemic vector (Gubler, 1988).

Dengue Virus Transmission Patterns

Where dengue viruses are endemic, transmission continues throughout the year, but usually there is an increase in transmission during the rainy season (Gubler, 1988). This pattern has been described for Thailand, Indonesia, the South Pacific, and the Americas (Halstead et. al, 1969; Gubler, 1988). It is not known what causes the number of human dengue infections to increase during the rainy season (Defoliart et. al, 1986; Gubler, 1988). Similarly, factors that cause increases in transmission of other arboviruses, which can lead to epidemics, have not been explained (Reiter, 1988).

Several attempts have been made to explain the association of increased dengue virus transmission with rainy seasons. Sheppard et al. (1969), working in Bangkok, Thailand, studied the movement, density and longevity of Ae. aegypti. They speculated that increases in Ae. aegypti population density and movement caused seasonally predictable increases of dengue virus transmission. After conducting mosquito surveys and mark-recapture studies, Sheppard et al. (1969) concluded that "the fluctuations in the amount of movement, the expectation of life and the population size (of mosquitoes) throughout the year are inadequate to account for changes in the incidence of dengue hemorrhagic fever." Since changes in adult female Ae. aegypti population size and life expectancy did not

correlate with DHF outbreaks, Yasuno and Pant (1970) examined the biting frequency of Ae. aegypti as an alternative explanation. They hypothesized that Ae. aegypti may feed more frequently during the outbreak period and thus initiate an outbreak. Their work with Ae. aegypti in Bangkok supported this hypothesis and they suggested that seasonal increases in biting rates may "explain the seasonal nature of DHF outbreaks" (Pant and Yasuno, 1973). Pant and Yasuno (1973) also suggested that seasonal increases in temperatures and the resulting reduction in the dengue virus's extrinsic incubation period in the mosquito may also explain the seasonality of dengue viruses. Watts et al. (1987) conducted a laboratory test of the temperature portion of Yasuno and Pant's hypothesis. Their results support the notion that seasonal changes in the incidence of dengue infections are consistent with temperature fluctuations decreasing the extrinsic incubation period and thereby increasing the likelihood of virus transmission by Ae. aegypti.

Gubler (1988) suggested that changes in the mosquito flight behavior might influence seasonal transmission of dengue viruses. He speculated that during the rainy season, when most dengue outbreaks occur, mosquitoes may remain indoors. This behavior might increase mosquito longevity and contact with humans and in that way increase the probability of transmission.

Testing the Hypothesis

The hypothesis that multiple host contacts contribute to dengue virus transmission contains several assumptions. Two assumptions of the hypothesis are the focus of this dissertation.

One assumption I address is that all Ae. aegypti infected with dengue virus will transmit virus each time they contact a susceptible host. It is plausible, however, that the salivary ducts of Ae. aegypti are depleted of virus following a probe or blood meal and that subsequent contacts do not result in virus transmission. This has been proposed for other mosquito-borne pathogens (Turell et. al. 1987; Rosenberg et. al, 1990). If Ae. aegypti is depleted of infectious dengue virus quickly while probing or engorging, the contribution of multiple host contacts to dengue virus transmission could be less than predicted by vectorial capacity calculations. In Chapter II I discuss experiments testing the assumption that Ae. aegypti remains infective regardless of how many times it contacts a host.

The second assumption I examine is that imbibing multiple blood meals will not affect the dengue virus infection process in Ae. aegypti If true, it follows that an increase in the number of host contacts, as a result of multiple feeding--imbibing blood more than once in a single ovarian cycle--could result in an increase in the number of infected mosquitoes. On the other hand, multiple feeding

results in the combining of blood meals in a mosquito's gut. If one of the meals is infected with dengue virus and the other is from a dengue virus immune host then antibodies in the blood from the immune host may bind to and neutralize viruses in the mosquito's gut. This may prevent the mosquito from becoming infected (Patrican and Bailey, 1989). Also, digestive enzymes released for earlier blood meals may inactivate viruses in subsequent meals (Gass, 1977). Thus, rather then increasing infection rates, multiple feeding in endemic areas, where immune hosts are plentiful, might decrease dengue infection rates in Ae. aegypti (Burkot, 1988). In Chapter III I discuss an experiment that tests the assumption that multiple blood meals do not interfere with the infection of Ae. aegypti with dengue viruses.

In Chapter IV I describe an experiment testing a prediction derived from the hypothesis that multiple host contacts contribute to dengue virus transmission. I predict that if multiple contacts benefit dengue viruses, then dengue viruses might influence the feeding behavior of Ae. aegypti so as to increase the frequency of multiple contacts. Similar modifications of host behavior by parasites are known to exist (Molyneux and Jefferies, 1986).

By examining details of the multiple host contacts hypothesis, my dissertation will contribute to a better understanding of the role Ae. aegypti in dengue virus transmission. Knowledge gained from my research may be

applicable to Ae. aegypti-borne diseases, such as yellow fever, as well as other mosquito-borne viruses or mosquito-borne pathogens.

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Chapter II

The Effect of Multiple Host Contacts on the Infectivity of Virus Infected Mosquitoes

Introduction

Several researchers have attempted to identify the factors important to the transmission of dengue viruses. I reviewed their efforts in Chapter I. One factor that may be an important contribution to dengue virus transmission is Ae. aegypti's tendency to engage in multiple host contacts. Traditional views hold that mosquitoes contact a host only once per gonotrophic cycle (Klowden, 1988). During that single visit the mosquito supposedly imbibes all the blood it needs to develop a clutch of eggs and returns to blood feed again only after laying those eggs (Klowden, 1988). Researchers have previously reported, however, that Ae. aegypti frequently contacts more than one host per gonotrophic cycle. (MacDonald, 1956; Sheppard et al., 1969; Gould et al., 1970; Yasuno and Pant, 1970; Yasuno and Tonn, 1970, McClelland and Conway, 1971; Pant and Yasuno, 1973; Trpis and Hausermann, 1986; Scott et al., 1993; Scott et al., in press). Until very recently (Scott et al., in press) no research has tested the long standing hypothesis that multiple contacts contribute to dengue virus transmission.

Relative to contacting only one host per gonotrophic cycle, multiple contacts could increase transmission rates by increasing the opportunity for a mosquito to become infected by engorging blood from a viremic person, or if already infected, to transmit virus to a susceptible person (Klowden and Lea, 1979; Scott et al., 1993). Thus, multiple host contacts may contribute substantially to the maintenance and spread of dengue viruses. Seasonal increases in multiple host contacts may accelerate transmission and start outbreaks (Rosenberg et al., 1990a; Scott et al., in press). Between outbreaks, multiple host contacts may help maintain dengue virus transmission.

One assumption of the hypothesis that multiple host contacts contribute to dengue virus transmission is that infectivity of Ae. aegypti is unaltered by probing and/or imbibing a blood meal. If Ae. aegypti infectivity is reduced following a host contact, subsequent contacts may not result in transmission of an infectious dose of dengue virus. This would diminish and perhaps negate the effect that multiple contacts might have on the spread of dengue virus through a human population by infected mosquitoes. Aedes aegypti salivate while probing (Griffiths and Gordon, 1952; Gubler and Rosen, 1976; Ribeiro et al., 1984; Ribeiro, 1987). It is possible that salivation during a probe purges the lumen of the salivary ducts/glands of dengue virus and that during subsequent host contacts virus is not

transmitted (Turell and Bailey, 1987; Rosenberg et al., 1990b). Similarly, imbibing blood may exhaust the supply of infectious virus in the lumen of the salivary ducts and glands because mosquitoes also salivate while engorging (Kashin, 1966). Thus, the act of contacting a host may reduce the infectivity of dengue virus infected Ae. aegypti (Burkot, 1988). The experiments discussed in this chapter test whether the infectivity of dengue-2 infected Ae. aegypti is reduced following probing or engorging.

Materials and Methods

Overview of experimental procedure. I infected Ae. aegypti either parenterally or orally with dengue-2 viruses. Following various extrinsic incubation periods the mosquitoes were allowed to probe or engorge to repletion on a guinea pig. At various times after probing or engorging I determined if the mosquitoes were infective with an in vitro transmission assay. I compared transmission rates of the mosquitoes that probed or engorged on the guinea pig to transmission rates of cohort mosquitoes that had neither probed or blood fed prior to their transmission attempt.

Mosquitoes. I conducted all the experiments with F_2 generation Ae. aegypti from San Juan, Puerto Rico. The Dengue Branch, San Juan Laboratories (SJL) in San Juan, Puerto Rico, provided me with eggs from mosquitoes that had been reared from field collected eggs. I reared the larvae

in an environmental chamber at 26° C. Larvae were reared at low densities to ensure homogeneity in the size and nutritional status of the emerging adults. Adults infected intrathoracically were held at 32° C. Those infected orally were held at 30° C. In both cases the relative humidity was 80% and the photoperiod was 12:12 (L:D). The mosquitoes were provided a 5% sucrose solution, except for the 24-hour period prior to an experiment. Sugar was removed at that time because mosquitoes are known to salivate while sugar feeding (Eliason, 1963) and this might deplete their salivary glands of virus.

I used the Rexville strain of Ae. aegypti, also from the SJL, for cultivating dengue-2 viruses and as recipient mosquitoes during the assay of dengue virus by mosquito inoculation, which is described below. The Rexville strain of mosquitoes were reared as described above but at higher larval densities.

Virus. All experiments were conducted with a dengue-2 virus strain isolated in 1986 from a 5 month old infant who became ill and died in San Juan, Puerto Rico. This isolate has since undergone 2 passages in Toxorhynchites amboinensis and has a titer of $10^{6.6}$ 50% mosquito infectious doses (MID_{50})/ml. I held aliquots of the virus at -70° C and thawed them just prior to use.

Infection of Ae. aegypti with dengue-2 virus. I infected female Ae. aegypti one of two ways: 1) by

intrathoracic inoculation or 2) by allowing them to engorge from a drop of virus infected blood. Inoculations were conducted as described by Rosen and Gubler (1974). Briefly, I immobilized the mosquitoes by chilling and then inoculated them with 76 MID_{50} of dengue-2 virus contained in a total volume of 0.19 µl. This procedure consistently resulted in a 100% infection rate.

For oral infections I allowed Ae. aegypti mosquitoes to engorge from "hanging drops" of infected blood (Gubler and Rosen 1976; Miller et al., 1982). Blood meals were prepared as follows. I ground 20-25 dengue virus infected female Ae. aegypti in 0.5 ml of fetal calf sera with tissue grinders held in wet ice. The mosquitoes had been infected approximately 10 days earlier via intrathoracic inoculation and held at 30° C. I cleared the mosquito-fetal calf sera triturate by centrifuging it at 15,000 g's for 15 seconds in an Eppendorf microcentrifuge. The supernatant (virus suspension) was mixed with triple washed human red blood cells (HRBC) and a 50% sucrose solution (10 parts virus suspension with 9 parts washed HRBC with 1 part 50% sucrose solution). Human blood had earlier been drawn from volunteers, mixed with an anticoagulant (ethylene diamine tetra acetic acid) and stored in Alsever's solution at 5° C. Immediately after formulation, a 50 µl aliquot of the blood meal was removed for virus titration and stored at -70° C. The remainder of the blood meal was then warmed in a 37° C

water bath for 4 minutes and presented to the mosquitoes by suspending drops of the blood meal on the nylon netting covering the top of the mosquito cages. Mosquitoes were allowed 30 minutes to engorge, greater than 80% engorged within the first 15 minutes. Only mosquitoes imbibing to stage IV or greater on the Pilitt/Jones scale (Pilitt and Jones, 1972) were retained for experiments.

Virus Assay and Virus Titration. I used the mosquito inoculation technique (Rosen and Gubler, 1974) in combination with the direct fluorescent antibody technique (DFAT) (Kuberski and Rosen, 1977) to assay for dengue-2 viruses. Triturates or FCS from the capillary tubes used in the in vitro transmission assay were inoculated into 10 female Ae. aegypti Rexville strain. Following a 10 day incubation at 30° C, I tested at least 5 mosquitoes for the presence or absence of virus with the DFAT. SJL provided me with the fluorescein-labeled anti-dengue virus antibodies that I used in the DFAT. Virus was titrated by inoculating 10-fold dilutions into groups of Ae. aegypti Rexville strain mosquitoes, which were then held and assayed for virus as described above. At least five mosquitoes per dilution were assayed for virus. I used the Kärber method to calculate virus titres (Lennette and Schmidt, 1979).

Transmission model. To determine if a mosquito was infective after probing or engorging blood, I used a transmission model that incorporated two components: 1) a

guinea pig for the mosquitoes to probe or engorge from; and 2) Aitken's (1977) in vitro transmission assay to determine if the mosquitoes were infective.

To anesthetize the guinea pig, I inoculated between 0.05-0.15 ml of a Ketamine/Xylazine mixture into a rear thigh muscle with a 29 gauge needle. This anesthetized the guinea pig for approximately one hour. I shaved the guinea pig with electric animal shears to provide a clear view of the tissue. For probing experiments the mosquitoes probed the guinea pig's back. For blood feeding experiments the mosquitoes imbibed blood to repletion on the guinea pig's abdomen, where blood venules are more plentiful (K. Nepote, personal communication).

To assay a mosquito's infectiveness I anesthetized the mosquito with CO_2, removed the wings and legs and inserted the mosquito's mouthparts into a 50 µl capillary tube containing 4 µl of 10% fetal calf serum (FCS) and 10% sucrose in Dulbecco's phosphate buffered saline (Aitken, 1977). I allowed the mosquitoes to salivate into the FCS solution for 10 minutes. The FCS solution, containing the mosquito saliva, was then held on wet ice until I assayed it for dengue-2 virus by the mosquito inoculation technique described above (Rosen and Gubler, 1974; Kuberski and Rosen, 1977). The contents of the capillary tubes were inoculated into recipient mosquitoes within 2 hours. Presence of detectable dengue virus in the FCS solution indicated that

the mosquito that salivated into the solution was capable of transmitting virus, that is, the mosquito was infective. For each experiment I also assayed the saliva of one uninfected mosquito as a negative control.

Testing the effects of probing on infectivity. I infected cohort Ae. aegypti mosquitoes either orally or parenterally and held them 14 days at 30° C or 32° C, respectively. I randomly selected half of the cohort and induced them to probe an anesthetized guinea pig 5, 10 or 20 times in immediate succession. To monitor probing, individual mosquitoes were placed into small plastic cages fitted with a flat-glass viewing "window". I used a microscope to observe the mosquito as it searched the guinea pig's tissue for blood and I timed each probing bout with a stop watch. I started timing a probe when I observed the mouthparts penetrating into the tissue. I determined the fascicle had entered tissue by watching the mosquito's head after the proboscis had been positioned on the skin. When the head started to move towards the guinea pig's skin I started timing. Preliminary work with an electronic monitoring device similar to Kashin's (1966) indicated this was a reliable cue for indicating that the fascicle had entered the guinea pig's tissue. I stopped timing when the mosquito fully withdrew its mouthparts from the tissue, when the mosquito began imbibing blood, or when 45 seconds of continuous probing had elapsed. Cessation of probing and

initiation of blood engorging was obvious; the mosquitoes discontinued all movement and "froze" *in situ*. I forcibly stopped mosquitoes from engorging or from probing more then 45 seconds by gently lifting the cage holding the mosquito. The cage was then straightaway placed back on the guinea pig so the next probing bout could start.

Immediately after the prescribed probing regiment ended, I assayed the mosquito's infectiveness with the *in vitro* transmission assay described above. I also assayed the control mosquitoes--mosquitoes that had not probed--to determine their capability to transmit virus. Immediately after the transmission assay I froze both the control and experimental mosquitoes at -70° C. Later I determined the infection status of the mosquitoes with the DFAT (Kuberski and Rosen, 1977).

To evaluate the effect of probing on *Ae. aegypti* infectiveness, I compared the transmission rate of dengue-2 infected mosquitoes that had just completed a probing regiment to the transmission rate of dengue-2 infected mosquitoes that had not probed.

Testing the effect of imbibing a blood meal. I infected a cohort of *Ae. aegypti* mosquitoes per os with dengue-2 virus and held them 7, 10 or 14 days at 30° C. I allowed half of the cohort to engorge to repletion on an anesthetized guinea pig and then held the mosquitoes at 30° C for 6 or 48 hours. Following this interval I assayed the

mosquito's infectiveness with the *in vitro* assay described above. Controls were mosquitoes that had not imbibed a blood meal. Immediately after the transmission assay I froze the mosquitoes at -70° C. The infection status of every mosquito was confirmed by the DFAT (Kuberski and Rosen, 1977). For my first two experiments (7 and 14 day incubation periods), I retested the mosquitoes negative for infection by head squash. The bodies of the decapitated mosquitoes, held at -70° C, were homogenized individually in a tissue grinder and the triturates were assayed for virus by the mosquito inoculation technique described earlier. I never found any mosquitoes that were negative by head squash to be positive by body-assay so I discontinued the assay of the mosquito bodies.

To evaluate if imbibing a replete meal affected *Ae. aegypti* infectiveness, I compared the transmission rate of mosquitoes that just completed imbibing a replete blood meal to that of mosquitoes that had not imbibed a previous blood meal.

Statistical analysis. I used a paired t-test to test for statistical significance at $\alpha = 0.05$ (Sokal and Rohlf, 1987).

Results

Probing 5, 10 and even 20 times consecutively did not significantly alter the infectivity of parenterally infected

Ae. aegypti ($P > 0.05$, df = 2 or 3) (Table 1). After 5 probes, 100% of the mosquitoes transmitted virus (n=15). Following 10 probes, the mean transmission rate ± SE was 87 ± 7.4% (n=15), and it was 94 ± 4.4% after 20 consecutive probes (n=15). However, orally infected Ae. aegypti, that probed 20 consecutive times, transmitted dengue viruses at a significantly higher rate than did mosquitoes that had not probed prior to the transmission assay ($P < 0.05$, df=2) (Table 2). Mosquitoes that had probed transmitted 100% of the time (n=14) versus a mean transmission rate of 64 ± 9.8% by the controls (n=15).

The infectivity of orally infected Ae. aegypti was unaffected by blood feeding (Table 3). Mosquitoes that blood fed on a guinea pig 7 days after imbibing an infectious blood meal were assayed for infectivity 6 and 48 hours later (n=15). At 6 hours none of the mosquitoes assayed were infective (n=15). Forty-eight hours after engorging blood from a guinea pig, the mean transmission rate of dengue-2 viruses was 32 ± 11.1% (n=17), which was not significantly different from the 28 ± 11.1% rate (n=17) recorded for the controls ($P > 0.05$, df=3). Other Ae. aegypti were held for a 10 day incubation period and then allowed to engorge on a guinea pig. Six hours later an average of 30 ± 6.1% (n=20) transmitted dengue-2 viruses, compared to a 28 ± 6.1% (n=24) mean rate of transmission by the control mosquitoes. Again, these results were not

Table 1. *In vitro* transmission rates of dengue-2 virus by parenterally infected *Aedes aegypti* after having probed a guinea pig 5, 10, or 20 times consecutively compared with mosquitoes that had not probed before the transmission attempt.

	% Transmission (transmission detected/total infected mosquitoes assayed)					
	Experiment 1		Experiment 2		Experiment 3	
Replicate	Following 5 probes	Control[a]	Following 10 probes	Control	Following 20 probes	Control
1	100 (5/5)	100 (5/5)	80 (4/5)	100 (5/5)	100 (3/3)	100 (4/4)
2	100 (5/5)	100 (5/5)	80 (4/5)	100 (5/5)	100 (4/4)	75 (3/4)
3	100 (5/5)	80 (4/5)	100 (5/5)	60 (3/5)	100 (4/4)	100 (1/1)
4	-	-	-	-	75 (3/4)	100 (4/4)
Mean	100 (15/15)	93 (14/15)	87 (13/15)	87 (13/15)	94 (14/15)	94 (12/13)
SE	0	6.6	7.4	7.4	4.4	4.4
Mean probe duration (sec)	33.7	-	28.4	-	28.4	-

[a] - Cohort mosquitoes that had not probed prior to the *in vitro* transmission assay.
Within each experiment no treatments were significantly different (paired t-test, $P > 0.05$).

Table 2. *In vitro* transmission rates of dengue-2 virus by orally infected *Aedes aegypti* after having probed a guinea pig 20 times consecutively compared with mosquitoes that did not probe prior to the transmission attempt.

Replicate	% Transmission (transmission detected/total infected mosquitoes assayed)	
	Following 20 probes	No probes prior to transmission
1	100 (4/4)	50 (2/4)
2	100 (3/3)	60 (3/5)
3	100 (6/6)	83 (5/6)
Mean	100[a] (13/13)	64 (10/15)
SE	0	9.8
Mean probe duration	32 sec	-

[a] - Significantly greater than mosquitoes that did not probe (paired t-test, $P < 0.05$).

Table 3. *In vitro* transmission rates of dengue-2 virus by orally infected *Aedes aegypti* after having engorged to repletion on a guinea pig compared to mosquitoes that did not engorge prior to transmission attempt.

	% Transmission (transmissions detected/total infected mosquitoes assayed)							
	7 day extrinsic incubation				10 day extrinsic incubation		14 day extrinsic incubation	
Rep	6 hrs post replete	Control	48 hrs post replete	Control	6 hrs post replete	Control	48 hrs post replete	Control
1	0 (0/2)	0 (0/1)	0 (0/3)	0 (0/1)	50 (3/6)	33 (2/6)	67 (2/3)	50 (2/4)
2	0 (0/2)	0 (0/3)	60 (3/5)	33 (1/3)	33 (1/3)	40 (4/10)	86 (6/7)	83 (5/6)
3	0 (0/4)	0 (0/3)	67 (2/3)	0 (0/5)	20 (1/5)	40 (2/5)	83 (5/6)	100 (3/3)
4	-	-	0 (0/6)	50 (4/8)	17 (1/6)	0 (0/3)	33 (1/3)	100 (6/6)
Mean	0 ~~(0/8)~~	0 ~~(0/7)~~	32	28	30	28	67	83
Total	0/8	0/7	5/32	5/17	6/20	8/24	14/19	16/19
SE	0	0	11.1	11.1	6.1	6.1	8.5	8.5

Within each extrinsic incubation period no treatments were significantly different (paired t-test, $P > 0.05$)

significantly different ($P > 0.05$, $df = 3$). Finally, mosquitoes that engorged to repletion on the guinea pig 14 days after their infectious blood meal and were assayed for infectivity 48 hours later, transmitted dengue-2 viruses at the same rate as the controls (mean transmission rates of 67 ± 8.5% (n= 19) and 83 ± 8.5% (n=19) respectively, $P > 0.05$, $df = 3$).

The hanging drops used to infect the mosquitoes contained 1 $x10^{7.2}$ to 1 x $10^{8.6}$ MID_{50}/ml of dengue-2 virus.

Discussion

The rationale for testing infectivity following probing or engorging is that the saliva expressed during these activities may flush the lumen of the salivary glands and its ducts of dengue-2 virus. This might leave an Ae. aegypti mosquito functionally uninfective (Turell and Bailey, 1987; Rosenberg et al., 1990a). If that were the case, multiple host contacts might not increase transmission rates, as has so long been assumed. Such was not the case.

My initial experiments were with Ae. aegypti, parenterally infected with dengue-2 virus. These mosquitoes were capable of transmitting virus after having probed a guinea pig 5, 10 or 20 consecutive times (Table 1). Probing 20 times at an average of 24.8 seconds/probe results in an average total probe duration of approximately 9.5 minutes. Aedes aegypti mosquitoes commonly take only 2 to 3 minutes

to obtain a blood meal and only a portion of that time is spent probing (Christophers, 1960). Therefore, 20 probes is a strenuous test of Ae. aegypti's ability to remain infective.

These data suggests that once infective Ae. aegypti remains so regardless of probing history. That is, probing does not cause Ae. aegypti to run out of dengue-2 virus.

One concern with these initial experiments was that the unnatural route of infection (intrathoracic inoculation) might cause an abnormal infection. The high rate of transmission (mean rates of 87% to 94% by the controls) supported this suspicion (Gubler and Rosen, 1976). If the mosquitoes had heavy infections they might not have run out of virus after nearly 10 minutes of probing simply because there was more virus present than in naturally infected mosquitoes.

Accordingly, I repeated the experiment with Ae. aegypti that became infected orally after engorging from a drop of infectious blood. The mean transmission rate of the controls dropped to 64%, but the mosquitoes that probed 20 times immediately prior to the transmission assay transmitted 100% of the time. This was a significantly higher rate of transmission than the controls. This peculiarity has been reported before. Hurlbut (1966) found that a second sample of saliva from Culex pipiens pipiens had a higher concentration of St. Louis encephalitis virus

than a sample taken an hour earlier. Hurlbut (1966) reasoned that the first fraction of saliva contained "old secretions" with inactive virions. He speculated that salivation stimulated "increased shedding of virus" from the tissues of the salivary gland and that these virions were more infectious.

Whatever the mechanism the end result is the same; probing and the consequent salivation does not decrease Ae. aegypti's infectivity, rather infectivity remains the same or greater than that of mosquitoes that do not probe. That being the case, dengue viruses that increase the amount of time Ae. aegypti spends probing might have a selective advantage, because mosquitoes forced to probe longer are more likely to transmit virus. Experiments designed to test this prediction are addressed in Chapter IV.

To my knowledge, no one has ever quantified probing rates of a natural Ae. aegypti population or evaluated the impact of multiple probing on dengue-2 transmission in an endemic setting. Probing behavior does not easily lend itself to field study; once humans are aware that mosquitoes are present, the humans become defensive and thereby affect the "natural" probing rates. However, at least two points support the premise that multiple probing by Ae. aegypti occurs frequently. First, my experience with Ae. aegypti indicates it is easily disturbed when it is probing and flys away at even the slightest movements by the host. In

Village 6, Hua Sam Rong, Thailand, I watched Ae. aegypti mosquitoes repeatedly fly off my ankles when I wiggled my toes. I also saw Ae. aegypti fly away from dozing humans when the people shifted in their sleep or twitched at bothersome houseflies. Second, Ae. aegypti is a diurnal mosquito, as such, it seeks blood meals when humans are most active. I suspect interruption of host contacts is likely when a mosquito that appears to have its blood feeding easily interrupted attempts to blood feed on an active host. Such interruptions could include interruption of probing.

Conversely, there is reason to believe that Ae. aegypti does not probe frequently within a single ovarian cycle. Ae. aegypti is also cited as a mosquito that can probe and engorge unnoticed by its human host (Christophers, 1960; Gubler, 1988). I learned in Hua Sam Rong, Thailand, that catching Ae. aegypti in the act of probing or imbibing a blood meal requires constant vigilance. On several occasions I discovered partially replete Ae. aegypti engorging blood from my ankles only because I was regularly searching my ankles for mosquitoes. The mosquitoes had alighted, probed and started to engorge without me becoming aware of their presence. Thus, one could argue that Ae. aegypti are frequently unnoticed and probes are rarely interrupted.

Several reports support the assertion that multiple probing is epidemiologically important. Both Gubler (1988)

and Waterman et al. (1985) report finding clusters of dengue fever cases within a single dwelling and with a similar date of onset of illness. The simplest explanation for such an event is that one infective Ae. aegypti probed or partially engorged on each of the residents and infected them all. More recently, a foreign family of four visiting Bangkok, Thailand, all contracted dengue within a few days of each other (B. Innis, personal communication). The family had been staying together at the time they were infected. A probable scenario for this observation is that a single mosquito probed or blood fed on several people before desisting. Given the experimental data presented above, it is now credible that a single mosquito could transmit virus to four people by probing four or more consecutive times on different people.

The other component of multiple host contacts that I examined was the effect of blood feeding on Ae. aegypti infectivity. I started my studies with mosquitoes that had incubated virus for 7 days. When I checked the infectivity of the mosquitoes 6 hours after engorging to repletion, neither the experimental or the control mosquitoes transmitted virus; the mosquitoes were not yet infective (Table 3).

Next, again using mosquitoes that had incubated virus for 7 days, I waited 48 hours between a replete meal and the assay of infectivity. Some of the mosquitoes were now

infective, but imbibing a blood meal did not alter the transmission rate (Table 3). Similarly, the transmission rate of mosquitoes incubated longer (14 days) and held for a 48 hour interval between replete meal and transmission assay, was unaffected (Table 3).

The most stringent test of whether imbibing a blood meal reduces transmission efficiency involved mosquitoes that I allowed to engorge on day 10 of the incubation period and then assayed for infectivity 6 hours later. This combination forced lightly infected mosquitoes (average transmission rate of only 28% for the controls) to try to transmit only 6 hours after a blood meal. This simulates an Ae. aegypti engorging during the morning and engorging again in the afternoon. I recorded an average transmission rate of 30% for the mosquitoes that had imbibed blood 6 hours earlier and this rate was not significantly different from the transmission rate of the controls (28%).

Many permutations of incubation periods, time intervals and blood meal size are possible, but testing lightly infected mosquitoes, 6 hours after a replete meal was most pertinent to the field studies being conducted in conjunction with these laboratory experiments. Lightly infected mosquitoes would seem to be the ones most likely to run out of virus; a replete meal offers the most time for the mosquitoes to expectorate their supply of virions; a 6 hour interval is the limit of the histological technique for

determining the time interval between multiple meals (Scott et al., 1993). No loss of infectivity under these circumstances is consistent with the assertion that imbibing blood does not reduce the infectivity of ensuing host contacts.

Other than monkeys, no animal model is available for dengue virus investigations. Using monkeys was not possible for my study so I developed the guinea pig-capillary tube model described earlier. Initially, I tried to develop a model using suckling mice. To test the mice as model hosts, I inoculated some 1-day-old suckling mice, intramuscularlly, with New Guinea C strain dengue-2 virus and let infected mosquitoes blood-feed on other suckling mice. One week later, 19 of the inoculated mice and 10 of the mice bitten by mosquitoes were tested for dengue virus infection by assaying their brains for virus antigen with the mosquito inoculation technique (Kuberski and Rosen, 1977). Only two of the inoculated mice were infected. I did not detect virus in any of the mice that had been bitten by infected mosquitoes. I held eleven of the inoculated mice for 4 weeks and then I assayed them for seroconversion with a hemagglutination test (Clark and Cassals, 1958) and an enzyme immunosorbent assay (Ksiazek, personal communication). None of these mice seroconverted. Following these results, I abandoned suckling mice as a way

to assay mosquito transmission of dengue virus and developed the guinea pig-capillary tube model.

An optimal dengue transmission model would accurately assess dengue transmission rates, but it is not known how much virus a mosquito must inoculate to cause a human infection. Kraiselburd et al. (1985), working with Rhesus monkeys, found that 9.5 MID_{50} of dengue-2 virus infected 50% of the monkeys. The minimum amount of dengue-2 virus my transmission model detected was 21 MID_{50} ([total quantity of fluid in capillary tube]/[amount inoculated into each assay mosquito] = 4μl/0.19μl). Assuming monkeys and humans are equally sensitive, my transmission model underestimates the true transmission rate. That is, some of the assays I declare "virus free" may in fact contain enough virus to infect a human. The mosquito inoculation technique, however, is considered the most sensitive dengue virus isolation technique (Rosen and Gubler, 1974) and therefore, is the best assay.

Multiple host contacts have the potential to substantially increase transmission rates of dengue viruses by Ae. aegypti (Boreham and Garrett-Jones, 1973). The research presented in this chapter supports the assumption that all contacts by infective Ae. aegypti are indeed infective and that Ae. aegypti remain infective regardless of how frequently the mosquitoes contact hosts. There is no evidence that probing or engorging reduces the infectivity

of an Ae. aegypti infected with dengue-2 virus. These findings are consistent with the hypothesis that multiple host contacts by Ae. aegypti are an important factor in the amplification and transmission of dengue virus.

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Chapter III

Infection and Infectivity of Aedes aegypti with Dengue-2 Virus: The Effect of Mixing Viremic Blood Meals with Blood Meals from Immune Hosts

Introduction

Infection with dengue viruses induces life long immunity in humans. In areas where dengue viruses are prevalent, dengue fever is usually a childhood disease and most of the adult human population is immune to one or more dengue serotypes (Gubler, 1988). Aedes aegypti that imbibe blood from humans in such an environment are likely to imbibe a blood meal from a human immune to dengue viruses. Multiple feeding by Ae. aegypti--mosquitoes that imbibe more than one blood meal per ovarian cycle (Scott et al., 1993)--may therefore imbibe at least one immune meal. If a mosquito combines a virus immune blood meal with a blood meal from a viremic host, the neutralizing antibodies in the immune blood meal may interfere with the infection process and the mosquito may not become infected (Patrican and Bailey, 1989). Thus, rather than increasing transmission, as is so often predicted (Boreham and Garrett-Jones, 1973; Boreham and Lenahan, 1976; Magnarelli, 1979; Conway and McBride, 1991; Scott et al., in press), multiple feeding may be reducing transmission by reducing infection rates in mosquitoes.

To my knowledge, Patrican and Bailey (1989) are the only ones who have investigated this notion with arboviruses. Their studies with Rift Valley fever virus indicated that infection, dissemination and transmission were unaffected when viremic and immune blood meals were combined during an interrupted feeding. For the study described here, my objective was to determine if ingesting an immune blood meal 24 hours after imbibing a viremic meal interfered with virus infection and transmission. I investigated a 24 hour separation of blood meals because mosquitoes collected in San Juan, Puerto Rico indicated that the majority (67%) of Ae. aegypti that took multiple meals were imbibing blood at 24 hour intervals (Scott et al., 1993).

Materials and Methods

Overview of experimental procedure. Aedes aegypti were permitted to imbibe a dengue-2 viremic blood meal, held 24 hrs at 30° C and then, to duplicate the ingestion of a second meal, given an enema containing dengue-2 virus immune sera or non-immune sera. After incubating the mosquitoes for 14 more days at 30° C, I determined the infection and infectivity rates of the mosquitoes. Infectivity was measured with an in vitro transmission assay. I compared the infection rate and transmission rate of the mosquitoes that had received an enema of immune sera to the infection

and transmission rate of the mosquitoes that had been given an enema of non-immune sera.

Mosquitoes. All experiments were conducted with the F_2 generation of wild Ae. aegypti collected in San Juan, Puerto Rico. Rearing procedures are described in Chapter II.

I used Ae. aegypti Rexville strain mosquitoes for cultivating dengue-2 viruses and as recipient mosquitoes in the dengue virus assay by mosquito inoculation (see Chapter II).

Virus. All experiments were conducted with a dengue-2 virus strain isolated in 1986 from a 5 month old infant from San Juan, Puerto Rico. Passage history, titre and holding procedures are presented in Chapter II.

Virus Assay and Titration. I used the mosquito inoculation technique (Rosen and Gubler, 1974) described in Chapter II to assay material for dengue-2 viruses. Individual mosquitoes were checked for infection with the DFAT (Kuberski and Rosen, 1977). Virus was titrated in 10-fold dilutions as described in Chapter II.

Sera. The Dengue Branch, San Juan Laboratories, San Juan, Puerto Rico provided me with dengue-2 virus immune sera. The sera was drawn from a patient approximately 2 months after a primary infection of dengue-2 virus. The patient had never been infected with dengue viruses prior to the dengue-2 infection. The immune sera had a hemagglutination inhibition (HAI) titre of 1:80 (Clark and

Cassals, 1958). To test the sera's dengue-2 neutralizing capabilities I homogenized 10 dengue-2 infected mosquitoes in 0.25 ml of the immune sera, prepared a blood meal with the virus-immune sera suspension (see Chapter II) and allowed Ae. aegypti to imbibe the blood meals from hanging drops (Gubler and Rosen, 1976; Miller et al., 1982). An aliquot of the blood meal was stored at -70° C for virus titration. I held the engorged mosquitoes for 10 days at 30° C and then tested them for the presence or absence of virus using the DFAT to determine if the immune sera had neutralized the dengue-2 virus. For comparison, I replicated the aforementioned procedure using a virus suspension made from 20 dengue-2 infected mosquitoes homogenized in 0.5 ml of fetal calf sera.

The non-immune human sera used for the experiments was drawn from a person who had never resided in a dengue virus endemic area. The sera was negative for dengue virus antibodies in the HAI test (Clark and Cassals, 1958).

Infection of Ae. aegypti with dengue-2 virus. The first meal, or infecting meal, was presented to the mosquitoes in the form of a "hanging drop" of dengue-2 virus infected blood (Gubler and Rosen, 1976; Miller et al., 1982). Details of blood meal preparation are presented in Chapter II.

Delivery of enemas. Twenty four hours after the mosquitoes ingested the infecting blood meal I administered

a second "meal" as an enema (Briegel and Lea, 1975). Enemas permitted frugal use of the dengue-2 immune sera, ensured consistency in the size of the second meal, and solved the difficulties I had enticing mosquitoes to imbibe a second blood meal from a hanging drop (typically, less than 5% of the mosquitoes would imbibe blood from a second drop.)

The enemas consisted of human sera mixed 1:1 with triple washed human red blood cells (HRBC). See Chapter II for details on HRBC preparation. The human sera was either dengue-2 immune sera or sera with no dengue-2 neutralizing antibodies (non-immune sera). I held aliquots of the sera at -70° C until just prior to an experiment, then the sera was thawed and mixed with HRBC. Two µl of the sera-HRBC mixture were picked up with a micropipette. Using a 30 ml syringe, I drew the sera-HRBC mixture from the micropipette into a glass capillary tube that had been heated and pulled into a needle. The tip of the needle was immediately dipped in saline to prevent blood or sera from drying in the tip and plugging it (Klowden, 1981). I then inserted the needle into the anus of a mosquito that had been immobilized by chilling. The blood-sera mixture was then pushed into the mosquito's midgut with air pressure generated by the 30 ml syringe connected to the needle. Based on visual estimates of the degree of repletion, two µl was approximately the same amount of blood my Ae. aegypti ingested from a hanging drop.

Evaluation of enemas for administering viremic blood meals. Viremic enemas would permit me to examine blood feeding scenarios in which the second meal of a double-meal was viremic. As explained earlier, I could not use hanging drops to evaluate such a scenario because the mosquitoes would not imbibe twice, in close succession, from hanging drops.

Attempts by other researchers to use enemas to deliver an infected blood meal into a mosquito's midgut sometimes resulted in unusually high infection rates (M. Klowden, personal communication). This was interpreted as evidence that the enemas had breached the gut barrier and were essentially inoculated directly into the hemocoel. To evaluate if I could use enemas to deliver dengue-2 infected blood to the midgut of Ae. aegypti, I prepared dengue-2 virus infected blood meals as described in Chapter II. I administered some of blood to mosquitoes as enemas (as described above) and fed the remainder to mosquitoes via hanging drops (Chapter II). I incubated the mosquitoes for 10 days at 30° C and then determined the infection rate with the DFAT.

Transmission Assay. To assess the ability of the mosquitoes to transmit dengue virus, I used Aitken's (1977) in vitro transmission assay as described in Chapter II, but with the following modifications. I used 30% FCS in Dulbeco's PBS to collect the mosquito saliva rather than a

5% solution. The capillary tubes containing the mosquito saliva were stored at -70° C and assayed for virus at a later date. By freezing the capillary tubes, rather than assaying them on the same day as the experiment, I was able to increase the number of mosquitoes I could test per experiment. I assayed only the tubes corresponding to mosquitoes that were positive by the DFAT.

Statistical analysis. I used a paired t-test to test for statistical significance at $\alpha = 0.05$.

Results

Evaluation of dengue-2 neutralizing immune sera. Dengue-2 virus infected blood meals made with the immune sera infected significantly less mosquitoes per os than the blood meals prepared with non-immune sera ($0.001 < P < 0.01$, $df = 2$). The results are summarized in Table 4. A mean ± SE of 1 ± 1.0% of the mosquitoes became infected after imbibing dengue-2 virus infected blood meals mixed with immune sera. Conversely, 45 ± 8.1% of the mosquitoes became infected after imbibing infected blood meals mixed with a non-immune sera (fetal calf serum).

There was no significant difference in the virus titres of the blood meals prepared with immune sera and non-immune sera ($P > 0.05$, $df = 2$). The 3 blood meals prepared with immune sera had a mean ± SE virus titre of $1 \times 10^{7.9} \pm 1 \times 10^{0.1}$ MID_{50}/ml (Table 4). The blood meals prepared with non-

Table 4. Infection rates of *Aedes aegypti* that imbibed dengue-2 virus infected blood meals prepared either with human dengue-2 virus immune sera or non-immune sera (fetal calf serum) and the virus titre of each blood meal as determined by the mosquito inoculation technique.

	Mixed with Immune Sera		Mixed with Non-Immune Sera	
Replicate	% Infected (+/total)	MID_{50}/ml[a]	% Infected (+/total)	MID_{50}/ml[a]
1	0 (0/28)	$10^{7.8}$	45 (15/33)	$10^{7.9}$
2	0 (0/28)	$10^{7.8}$	59 (27/46)	$10^{8.2}$
3	3 (1/30)	$10^{8.2}$	31 (14/45)	$10^{8.4}$
Mean (total)	1[b] (1/86)	$10^{7.9c}$	45 (56/124)	$10^{8.2}$
SE	1.0	$10^{8.1}$	8.1	$10^{8.1}$

[a] - Mosquito infectious dose 50/ml

[b] - Significantly less than mean infection rate of blood meals prepared with non-immune sera (paired t-test; $P < 0.01$).

[c] - Not significantly different than virus titre of blood meals prepared with non-immune sera (paired t-test; $P > 0.05$).

immune sera averaged 1 x $10^{8.2}$ ± 1 x $10^{0.1}$ MID_{50}/ml.

Evaluation of enemas for administering viremic blood meals. The infection rate of mosquitoes administered viremic blood via enema was not significantly different than the infection rate of mosquitoes that imbibed the viremic blood ($0.2 < P < 0.4$, df = 3) (Table 5). The mosquitoes receiving the viremic enema had a mean ± SE infection rate of 80 ± 7.0%. The mosquitoes that imbibed the viremic blood had an infection rate of 61 ± 7.0.

Multiple meals experiment. The infection rate of mosquitoes administered a blood meal enema mixed with dengue-2 virus immune sera was significantly less than the infection rate of mosquitoes receiving an enema with non-immune sera ($P = 0.05$, df = 3) (Table 6). The mosquitoes receiving the immune sera had a mean ± SE infection rate of 85 ± 0.9%. The mosquitoes receiving the non-immune sera had an average infection rate of 92 ± 0.9% The infecting blood meals had dengue-2 virus titres ranging from 1 x $10^{8.0}$ to 1 x $10^{8.9}$ MID_{50}/ml with a mean of 1 x $10^{8.3}$ ± 1 x $10^{0.1}$ MID_{50}/ml.

The dengue-2 virus transmission rate of the mosquitoes given an enema containing dengue-2 virus immune sera was not significantly different from the transmission rate of the mosquitoes administered enemas with non-immune sera ($P = 0.3$, df = 3), (Table 6). The mosquitoes receiving the immune sera had a mean ± SE transmission rate of 51 ± 5.4%,

Table 5. Dengue-2 virus infection rates of Aedes aegypti that either imbibed virus infected blood from a hanging drop or had it delivered into the midgut by enema.

	% Infected (+/total)	
Replicate	Blood meal imbibed from a hanging drop	Blood meal administered by enema
1	64 (9/14)	100 (12/12)
2	78 (7/9)	56 (14/25)
3	65 (20/31)	64 (9/14)
4	39 (9/23)	100 (18/18)
Mean (total)	61[a] (45/77)	80 (53/69)
SE	7.0	7.0

[a] - Not significantly different from mean infection rate of mosquitoes administered enemas (paired t-test; $0.2 < P < 0.4$).

Table 6. Dengue-2 virus infection and transmission rates of Aedes aegypti that imbibed virus infected blood meals and 24 hrs later received a 2 µl enema of either human dengue-2 immune sera or non-immune sera mixed 1:1 with human red blood cells.

	% Infected (+/total)		% Transmitting[a] (+/total)	
Replicate	Immune sera	Non-immune sera	Immune sera	Non-immune sera
1	89 (8/9)	92 (11/12)	50 (4/8)	9 (1/11)
2	87 (13/15)	91 (10/11)	38 (5/13)	50 (5/10)
3	83 (10/12)	92 (12/13)	60 (6/10)	42 (5/12)
4	82 (9/11)	94 (16/17)	56 (5/9)	50 (8/16)
Mean	85[b] (40/47)	92 (49/53)	51[c] (20/40)	38 (19/49)
SE	0.9	0.9	5.4	5.4

[a] - Only infected mosquitoes were assayed for infectivity.
[b] - Significantly less than the infection rate of mosquitoes that received enemas of non-immune sera (paired t-test; P = 0.05).
[c] - Not significantly different from the transmission rate of mosquitoes that received enemas of non-immune sera (paired t-test; P = 0.3).

while 38 ± 5.4% of the mosquitoes receiving non-immune sera transmitted dengue-2 virus.

Discussion

Evaluation of dengue-2 neutralizing immune sera. Rosen et al. (1989), citing unpublished data, reported that dengue virus-antibody complexes are dissociated when inoculated into mosquitoes and therefore neutralized virus is not easily detectable by the mosquito inoculation technique. Their article is the only report I am aware of that addresses this phenomenon. In their report, Rosen et al. (1989) described using two mosquito species for viral assays (Aedes albopictus and Toxorhynchites amboinensis), but did not specify which mosquito species possesses the dissociation characteristic.

I used Ae. aegypti and found it could be used to detect neutralized virus. I base this claim on the conflicting results of the per os infection rates and the titres of the blood meals prepared with immune sera (Table 4). The virus in the blood meals prepared with immune sera was clearly neutralized, because it infected only 1% of the mosquitoes that imbibed it. This was significantly lower than the per os infection rate of the controls (45%) and substantially lower than the 30-90% infection rates I typically got with blood meals prepared with non-immune sera (J. Putnam, unpublished data). When I used the mosquito inoculation

technique to titrate the virus content of these neutralized blood meals, however, I found they had the same titre as the blood meals prepared with non-immune sera (Table 4). That is, unlike the per os infection rate, there was no indication of neutralization. Based on the per os infection rates, the virus titre of the blood meals prepared with immune sera ($1 \times 10^{7.9}$ MID_{50}/ml) should have been substantially lower than the titres of the blood meals prepared with non-immune sera ($1 \times 10^{8.2}$ MID_{50}/ml). As in Rosen et al.'s (1989) report, the virus-antibody complexes in the blood meals prepared with immune sera were infective when inoculated into a mosquito, but were not infective when imbibed and exposed to gut epithelial cells.

This differential infectivity may be a result of virus-antibody dissociation following inoculation into mosquitoes, as proposed by Rosen et al. (1989), and merits further investigation. If dissociation of virus-antibody complexes does occur within the mosquito's hemocoel, determining the mode of action might provide a way to improve virus isolation and assay techniques. Alternatively, the differential infectivity may occur due to biochemical changes associated with the trauma of inoculation or because cells in the midgut are different from the cells within the hemocoel.

Multiple meals experiment. Receiving an enema of immune sera 24 hrs after ingesting infected blood reduced

the infection rate of Ae. aegypti but did not alter the transmission rate of the infected mosquitoes. Infection rates dropped from 92% to 85% (Table 6). Other studies have not noted a similar effect. Davis (1931), studying Stegomyia mosquitoes that had imbibed a partial meal on a monkey immune to yellow fever virus and then completed engorging on a monkey infected with yellow fever virus, found no effect on infection or infectivity. The experiment that reversed the order of the meals was inconclusive. Likewise, no effect on infection and transmission was noted for Aedes fowleri, Aedes mcintoshi and Culex pipiens that blood fed partially on hamsters viremic with Rift Valley fever virus and then completed engorging on immune hamsters (Patrican and Bailey, 1989).

In both of the studies I cited above there was no interval between the viremic and immune meals, i.e. the mosquitoes imbibed blood from the second host immediately after blood feeding on the first host. In my experiments the infectious meal and the enema were separated by 24 hrs.

The 7% reduction in infection rates that I found is certainly not definitive. I used hanging drops to infect mosquitoes and enemas to simulate a second meal, neither of which are "normal" blood feeding or infection processes for mosquitoes. These artificial methods have some inherent deficiencies. Hanging drops are known to be less infective than natural blood meals (Turell, 1988; Weaver and Scott,

1993). More infectious meals from a viremic host might be less susceptible to neutralization than the artificial ones I used. Perhaps this explains why my results differ from Davis (1931) and Patrican and Bailey (1989). Moreover, delivery of the second meal by enema may result in a unique spatial positioning of the first and second meals with unpredictable effects on virus infection of the mosquito gut.

I used my system of hanging drops and enemas because it was the best one available to me. I needed a system that placed two blood meals--one infected the other immune--into the gut of a mosquito. It would have been best to permit mosquitoes to imbibe blood from infected and immune animals, but neither monkeys nor humans were available to me. The next best alternative would have been blood feeding mosquitoes on two hanging drops, one infected with virus the other containing immune sera. I did not use such a system for two reasons: 1) I could not consistently entice the mosquitoes to imbibe a second meal from a hanging drop, and 2) my supply of dengue immune sera was limited and using the sera to make blood meals for hanging drops would have quickly depleted my stock of. By using enemas to simulate the second meal, I was able to use the sera sparingly and I could also control the size of the immune meal, which eliminated meal size as a confounding factor.

The epidemiological significance of multiple feeding that includes a meal from an immune host can be evaluated using the vectorial capacity equation (Chapter I). However, an additional component, c, must be added to the equation. The component c is the proportion of blood meals imbibed by susceptible mosquitoes on viremic humans that result in infection (Nedleman, 1985; Sattenspiel, 1990). The vectorial capacity equation then becomes

$$C = \frac{cma^2 p^n}{-\log_e p}$$

From my experiment, mosquitoes that imbibe an immune meal 24 hours after an infectious dengue-2 virus meal, have a c equal to 0.852 (Table 6). If a non-immune meal is imbibed as the second meal, c equals 0.922 (Table 6). If we assume the following:

m = 9 (T. Scott, personal communication)

a = 0.66 (i.e. every mosquito engorges twice within a 3 day gonotrophic cycle)

p = 0.85 (T. Scott, personal communication)

n = 8 (Watts et al., 1987)

the vectorial capacity (C) of a mosquito population that imbibes an immune meal 24 hours after an infectious meal is 5.6 inoculations per day. c for a mosquito population not imbibing an immune meal is 6.1 inoculations. A reduction in c from 6.1 to 5.6 results in approximately one less inoculation every two days.

The immune meal reduced $\underline{c}$ by reducing infection rates, but once the mosquitoes became infected the immune sera did not affect the mosquitoes's ability to transmit (Table 6). Apparently, once initiated, the infection process proceeded unhindered by the immune sera. Though immunoglobulins are known to pass into the hemocoel of a number of mosquitoes (Hollingdale and de Rossario, 1989), Vaughn and Azad (1988) reported that only minute quantities pass into the hemolymph of Ae. aegypti.

The reduction in the infection rate that I detected in the mosquitoes receiving immune sera needs confirmation. The experiments should be repeated using monkeys for the infectious and immune meals. Additionally, a third treatment should be added consisting of mosquitoes that do not imbibe a second meal. This would permit one to evaluate the effect of imbibing multiple meals (infected and uninfected) versus taking a single infected meal.

Notably absent from this study is an experiment reversing the order of the viremic and immune blood meals (i.e. an immune meal followed by an infected meal). I had difficulty getting second blood meals, that were viremic, into the mosquitoes. An evaluation of viremic enemas (Table 5) convinced me that enemas were not an option. Though mean infection rates induced by viremic enemas were not significantly different from infection rates of mosquitoes that imbibed the viremic blood, two of the four trials with

viremic enemas infected 100% of the mosquitoes (Table 5). In my research, only intrathoracic inoculations resulted in 100% infection rates. I may have abraded the hind gut while administering the enemas and inoculated virus directly into the mosquito. Therefore, I considered administering virus via enema as an unreliable method to simulate oral infection of mosquitoes.

Similarly, using hanging drops for the second meal proved inadequate. By holding the mosquitoes in a low humidity environment, I was able to entice some mosquitoes to engorge from a hanging drop 24 hours after receiving an enema of immune sera. However, when I dissected these mosquitoes I found that the viremic blood meal had been diverted to the ventral diverticulum. I dissected three mosquitoes every six hours for 24 hours and found that the blood was slowly moved into the midgut over the 24 hour period. Normally, blood is directed straight into the midgut (Clements, 1963). When the infectious blood meal is delayed in the diverticulum it permits additional digestion of the immune meal and decreases the likelihood that the immune meal will interfere with infection. When the first meal was from the hanging drop, the blood was also directed to the diverticulum, but the blood was moved to the midgut more quickly than when imbibed as a second meal, usually within 4 hours.

The research in this chapter does not support the assumption that dengue virus infection in Ae. aegypti is unaffected by multiple feeding. An enema of immune sera, administered 24 hours after a viremic blood meal was ingested, halted the infection of some mosquitoes. This finding implies that multiple feeding in areas where immune hosts are present may reduce infection rates of Ae. aegypti. This is not consistent with the hypothesis that multiple host contacts contribute to dengue virus transmission. As stated earlier, these experiments should be repeated, using a system less artificial than the system I used.

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Chapter IV

Blood Feeding Behavior of Dengue-2 Virus Infected *Aedes aegypti*

Introduction

Many parasites manipulate their host's behavior to enhance their own reproductive success (Moore, 1984) and parasites transmitted by arthropods are no exception (Molyneux and Jefferies, 1986). One classic example is *Yersinia pestis*, the plague bacteria, which blocks the proventriculus of the rat flea (Bacot and Martin, 1914). This forces the infected flea to regurgitate into the host, resulting in an effective inoculation of the plague pathogen. Likewise, mosquito blood feeding behavior is sometimes altered by parasites to enhance transmission. Rossignol et al. (1984) found that *Aedes aegypti* infected with an avian malaria (*Plasmodium gallinaceum*) took longer to locate blood in guinea pigs. The parasite reduced the mosquito's production of apyrase. Normally, *Ae. aegypti* releases apyrase into a host while probing for blood. This inhibits platelet aggregation, the primary mechanism that mammals use to plug punctures in venules. The consequent increased bleeding from the punctured venules facilitates the mosquito's search for blood in the host's tissue. *Aedes aegypti* infected with *P. gallinaceum* have a reduced antiplatelet activity. Punctures made by these infected

mosquitoes are plugged quickly and malaria infected mosquitoes must probe the tissue longer to locate blood. This extended probing time enhances parasite transmission (Molyneux and Jefferies, 1986; Rossignol et al., 1986; Ribeiro, 1989).

In Chapter II, I showed that Ae. aegypti infected with the dengue-2 virus continued to transmit virus throughout the time they probed a guinea pig. There was no indication that probing diminished the mosquito's capability to transmit virus; Ae. aegypti continued to transmit dengue-2 virus even after probing 20 times consecutively. If dengue-2 virus increased Ae. aegypti's duration of probing and/or engorging, and thereby forced infected mosquitoes to contact more hosts than they normally would, the virus could take advantage of Ae. aegypti's transmission efficiency and increase its reproductive success. Moreover, as Molyneux and Jefferies (1986) state, there is a need to quantify the effects of parasites on vector feeding behavior "to provide a firmer basis for quantitative mathematical studies of transmission". Accordingly, to determine if dengue-2 virus modifies Ae. aegypti's blood feeding behavior, I compared the probing, engorgement, and the feeding duration of infected and uninfected mosquitoes. Feeding duration is the time required for a mosquito to locate blood and engorge to repletion, i.e. probing duration + engorgement duration.

Materials and Methods

Overview of experimental procedure. I infected Ae. aegypti via intrathoracic inoculation with dengue-2 virus. Infected and uninfected (control) mosquitoes were allowed to blood feed on a guinea pig and I recorded the duration of time required to locate blood and the time required to engorge to repletion (engorgement period). I compared the probing duration, the engorgement period, and the total time required to feed (probing duration + engorgement duration) of infected and uninfected mosquitoes to determine if feeding behavior had been altered by the infection with dengue virus.

Mosquitoes. I used the Rexville strain of Ae. aegypti for all my experiments. The larvae and adults were reared as described in Chapter II. After infection with dengue-2 virus, the mosquitoes were held at 32° C, 80% humidity with a 12:12 (L:D) photoperiod. The adult mosquitoes were provided a 5% sucrose solution, which was removed 24-48 hours prior to an experiment to encourage blood feeding.

Virus. I used the New Guinea C strain of dengue-2 virus supplied by the Walter Reed Army Institute of Research. This virus has been passed many times but details of the passage history are unknown. The virus was in a suspension of suckling mouse brain (SMB) and had a titer of

1 x $10^{8.8}$ MID_{50}/ml. Aliquots of the virus were stored at -70° C and thawed just prior to use.

Infection of Ae. aegypti with dengue-2 virus. I infected female Ae. aegypti by intrathoracic inoculation (Rosen and Gubler, 1974). Each mosquito received 0.19 µl of a 1 x 10^{-1} dilution of the virus-SMB suspension containing ca. 10^4 MID_{50} of dengue-2 virus. I used a 95% phosphate buffered saline-5% fetal calf solution as diluent. Control mosquitoes were inoculated with a 1 x 10^{-1} dilution of normal SMB. Following the inoculation, the mosquitoes were held at 32° C for 14 days.

All the mosquitoes I inoculated became infected. Considering the amount of virus I injected, the temperature during incubation and the extended length of the incubation period, these mosquitoes probably had heavy infections. The intensity of the fluorescence I observed during the viral assay indicated the mosquitoes were heavily infected. I hoped heavy infections would accentuate any effect the virus had on feeding behavior.

Virus assay. I checked each mosquito for infection with the DFAT (Kuberski and Rosen, 1977) as described in Chapter II.

Monitoring Probing and Engorging. I followed the procedures of Rossignol et al. (1984) to monitor mosquito blood feeding. A guinea pig was anesthetized with an intramuscular injection of a Ketamine and Xylazine mixture

as described in Chapter II. I allowed individual mosquitoes, held in small plastic cages fixed with a flat-glass viewing "window", to blood feed on the guinea pig's shaved abdomen. Each mosquito was observed through a binocular microscope and the duration of the probe and the time required to feed to repletion were recorded.

I started timing a probe when the fascicle of the mosquito entered the skin and stopped when the mosquito located blood (see Chapter II for details). If a mosquito withdrew its fascicle before finding blood, I stopped timing and restarted when it renewed probing. Mosquitoes were monitored for a maximum of 300 seconds of probing time. Those taking more than 300 seconds to locate blood were not included in the analysis. Greater than 90% of the mosquitoes located blood within the 300 seconds.

I started timing an engorgement period when the mosquito located blood and stopped timing when the mosquito withdrew its fascicle from the tissue. On some occasions (9 of 34 mosquitoes) the mosquitoes were unable to feed to repletion during the first engorgement period. These mosquitoes either searched for blood at the same site or withdrew their fascicle and probed a new site. For these mosquitoes I timed each engorgement period and summed them to obtain a total time required to engorge to repletion.

I was concerned that blood-finding and engorging efficiency might be related to the level of anesthesia in

the guinea pig, which wanes as the guinea pig metabolizes the drugs. To control for this potentially confounding effect, I alternately monitored control and infected mosquitoes throughout the experiment. Thus, equal numbers of control and infected mosquitoes probed and engorged on the guinea pig at each level of anesthesia.

Statistical analysis. I used a paired t-test to analyze the data. Control and infected mosquitoes were paired such that mosquitoes feeding during similar levels of anesthesia were matched. I considered $P \leq 0.05$ as indicating statistically significant differences.

Results

I compared the probing time of 36 pairs of mosquitoes (infected:control) and the engorging and feeding times of 34 pairs. The results are summarized in Table 7.

I did not find a significant difference in the amount of time required to locate blood ($0.5 < P < 0.9$). Dengue-2 virus infected mosquitoes took 6 to 289 seconds to locate blood with a mean time ± SE of 105 ± 9.5 seconds. Uninfected mosquitoes took 7 to 299 seconds with a mean ± SE of 102 ± 9.5 seconds to find blood in the guinea pig.

There also was no significant difference in the engorgement duration of dengue-2 virus infected and uninfected mosquitoes ($0.5 < P < 0.9$). The infected

Table 7. The mean duration of time that Aedes aegypti, parenterally infected with dengue-2 virus, spent probing, engorging and feeding (probing + engorging) compared to uninfected mosquitoes.

	Mean time ± SE (seconds)		
	Infected	Uninfected	n
Probing duration[a]	105 ± 9.5	102 ± 9.5	36
Engorgement duration[a]	168 ± 16.2	158 ± 24.7	34
Feeding duration[a]	297 ± 24.0	275 ± 24.0	34

[a] - Not significantly different from uninfected mosquitoes (paired t-test, $P > 0.05$).

mosquitoes took 39 to 375 seconds to engorge to repletion with a mean time ± SE of 168 ± 16.2 seconds. Uninfected mosquitoes required 47 to 911 seconds to engorge and averaged 159 ± 24.7 seconds.

The feeding time (probing + engorging time) of the infected mosquitoes was not significantly different from the feeding time of the uninfected mosquitoes ($0.05 < P < 0.9$). The infected mosquitoes took 78 to 759 seconds to complete a feed and averaged 298 ± 24.1 seconds. Uninfected mosquitoes took 75 to 1055 seconds with a mean time of 275.3 ± 24.1 seconds.

Discussion

By increasing the time a vector uses to obtain a blood meal, a vector-borne parasite may be enhancing the potential for its transmission (Molyneux and Jefferies, 1986; Rossignol et al., 1986). Increased feeding time forces the vector to salivate into a host for a longer period of time. This may increase the likelihood of delivering parasites to a receptive site or simply increase the size of the inoculum (Molyneux and Jefferies, 1986; Rossignol et al., 1986). Also, by forcing extended contacts the parasite may increase the number of hosts a vector contacts per engorgement; vectors may desist and move to another host or may be detected and behaviorally repelled by the host (Molyneux and Jefferies, 1986; Rossignol et al., 1986). Increasing the

number of hosts contacted might be particularly advantageous to arboviruses, which induce life long immunity, because it increases the likelihood of the vector contacting a susceptible host. Ribeiro et al.'s (1985) Monte Carlo simulation of Ae. aegypti probing behavior supports their contention that arthropod-borne "parasites may manipulate their hosts to enhance transmission". Using the model to test potential parasite manipulations, Ribeiro et al. (1985) predicted parasites would increase the vector's duration of probing. Several other researchers have reported evidence to support this prediction, which I review below.

Rossignol et al. (1984) found that an avian malaria, Plasmodium gallinaceum, increased blood location times of Ae. aegypti. Likewise, trypanosomes reduce the blood-finding efficiency of both the kissing bug, Rhodnius prolixus (Anez and East,1984) and the Tse tse Fly, Glossina morsitans morsitans (Jeni et al., 1980). Jefferies and Molyneux (1983) reported that mites (Ornithonyssus bacoti) infected with a filarial worm (Litomosoides carnii) probed longer than uninfected controls. Leishmania spp. appear to increase probing duration and the number of probes of phlebotimine flies (Molyneux and Jefferies, 1986), but a definitive study is lacking. Finally, Grimstad et al. (1980) found that Aedes triseriatus infected with La Crosse virus had an extended probing duration.

I, conversely, found no evidence that dengue-2 viruses impaired the blood feeding efficiency of Ae. aegypti (Table 7). Similarly, Paulson et al. (1992) reported finding no effect of La crosse virus on the probing duration of Aedes hendersoni, and Li et al. (1992) found Anopheles stephensi unaffected by Plasmodium berghei infection.

As discussed earlier, increasing the duration of a feed could increase transmission rates by inducing multiple host contacts (Molyneux and Jefferies, 1986; Rossignol et al., 1986). The behavior of Ae. aegypti, however, is such that it is already prone to contacting multiple hosts within a single ovarian cycle. Aedes aegypti will interrupt feeding and fly away following even slight movements by humans (Christophers, 1960; Gubler, 1988; see Chapter II). Assuming Ae. aegypti will switch hosts between contacts, the tendency of Ae. aegypti to be easily interrupted when feeding should cause Ae. aegypti to contact several hosts to obtain one meal. Scott et al's (1993) recent finding that 67% of the Ae. aegypti in San Juan, Puerto Rico and ca. 50% in Hua Sam Rong, Thailand (T. Scott, personal communication) are feeding twice per gonotrophic cycle is indicative of this species proclivity for multiple feeding. With its principle vector already contacting several hosts per engorgement, the dengue-2 virus probably cannot improve its fitness by increasing the probing duration of its vector.

I propose that prolonging the feeding duration of Ae. aegypti offers no advantages to dengue-2 viruses because Ae. aegypti is already prone to contacting multiple hosts. The fitness of Plasmodium spp., however, may be increased by longer probing periods because the likelihood and magnitude of infection is related to the size of the inoculum (Burkot, 1988; Rosenberg et al., 1990). This difference may explain why P. gallinaceum induces a longer probing duration in Ae. aegypti and dengue-2 virus does not. More studies are needed to investigate the role of viral infection on mosquito blood-feeding behavior. Molyneux and Jefferies (1986) pointed out that all the studies on the feeding behavior of pathogen-infected vectors use various combinations of colony mosquitoes, in-vitro transmission models, laboratory versus true vertebrate hosts, artificial vector-parasite systems and artificial routes of infection. Iteractions investigated by these types of studies may be artifacts of laboratory procedures rather than evolutionarily relevant parasite modifications. Ponnudurai et al.'s (1991) finding that sporozoite ejection from mosquitoes is random and not correlated to probing duration challenges a principle rationale for extended probing duration--that longer probing times or more probes increase the number of malaria parasites injected. This should give researchers working in the field of vector-parasite interactions reason to reexamine the assumptions of the

hypothesis that extended probes benefit arthropod-borne parasites. My experiments indicate that the probing, engorging and feeding duration of Ae. aegypti are unaltered by dengue-2 virus.

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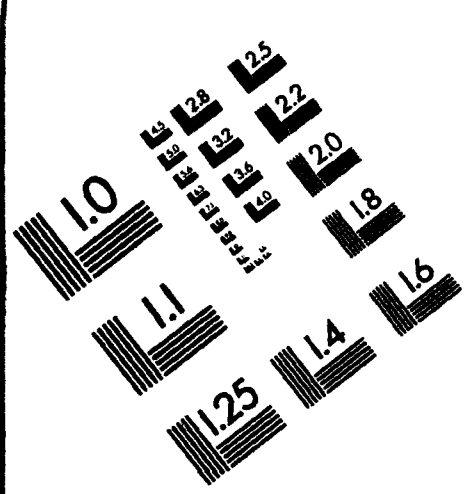

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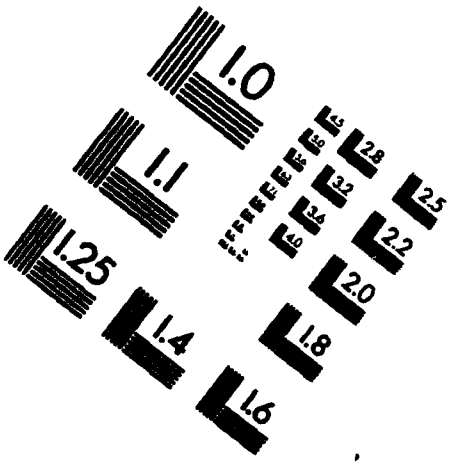

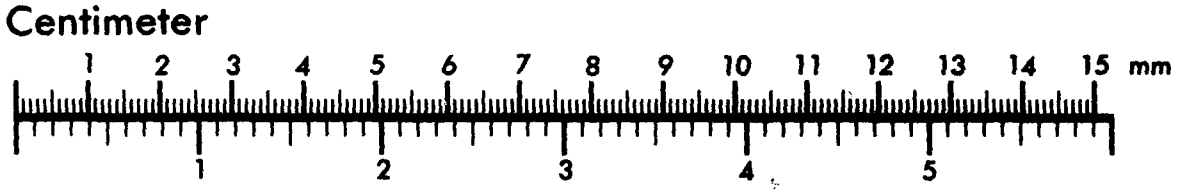

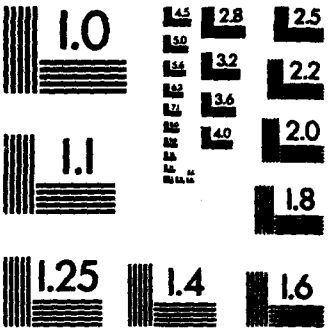

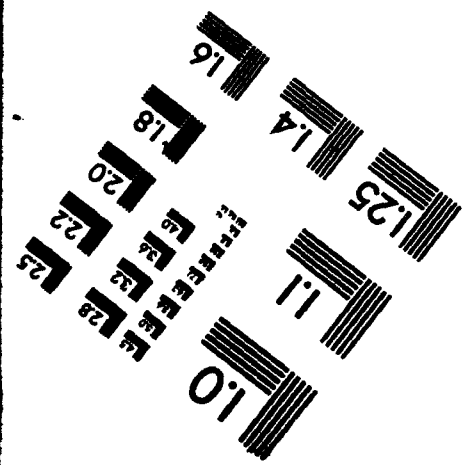

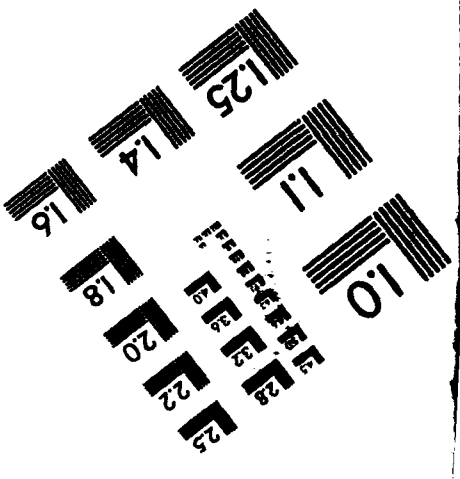

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Chapter V

Conclusion

Overview of Results

The primary purpose of my dissertation was to test assumptions of the hypothesis that multiple host contacts by mosquito vectors contribute to the transmission of dengue viruses. My results indicated that multiple host contacts might not increase the infection rate of Ae. aegypti as much as expected (Chapter III). Once infected though, Ae. aegypti are efficient vectors and continue to transmit virus even after probing 20 hosts in succession (Chapter II). This efficiency of Ae. aegypti to transmit dengue virus lends credence to the suggestion that spatial and temporal clusters of dengue fever cases might be the result of a single infected mosquito. At least two such occurrences are cited in the literature (Waterman et al, 1985; Gubler, 1988). These incidents provide examples of how multiple host contacts might be contributing to the transmission and epidemiology of dengue viruses.

I tested the prediction that dengue viruses might extend the probing and/or engorging duration of mosquitoes to increase the rate of multiple host contacts (Chapter IV). I reasoned that if multiple host contacts do contribute to transmission then there should be a selective advantage for dengue viruses that ensure that infected mosquitoes contact

multiple hosts. I found, however, that the feeding behavior of dengue virus infected mosquitoes is unaffected by the virus.

A criticism of my research could be my use of artificial infections, enemas, and in vitro transmission. It would have been better to have used natural hosts to infect the mosquitoes, provide dengue virus immune blood meals, and evaluate infectivity. Monkeys and humans are the only vertebrates susceptible to dengue virus infection, however, and they were not available to me. Accordingly, I developed model systems and tried to follow Platt's (1963) dictate to "study the simplest system you think has the properties you are interested in". To date, my efforts are the only ones to address the issues I studied with regard to dengue virus transmission. As such, they provide the best available data on the effect of multiple host contacts on dengue virus transmission.

My dissertation raises the point that multiple host contacts do not necessarily contribute to dengue virus transmission. The hypothesis that multiple host contacts do contribute to transmission is inherently attractive because the connection between multiple host contacts and increased biting rates seems intuitively obvious. Less obvious are the other consequences of multiple host contacts, such as the combining of immune and infectious blood meals. Burkot (1988), in fact, argued that multiple feeding due to

interrupted feeds may actually reduce transmission of *Plasmodium*.

Suggestions for Future Research

Confirmation of my findings is a logical next step in evaluating the multiple host contacts hypothesis. Evaluating the effect of immune blood meals on the infection process should be repeated with a less artificial model than I used. Likewise, using an *in vivo* transmission system to evaluate the effect of multiple probes and feedings on mosquito infectivity would be an improvement over my *in vitro* methods. Without such work the problems of interpreting *in vitro* transmission rates will remain.

Only field work in dengue virus endemic areas can provide the data to determine how important multiple host contacts are to dengue virus transmission. A long term study associating the frequency of multiple feeding and dengue fever's periodicity is still lacking. A link between dengue fever outbreaks and multiple feeding rates would substantially strengthen the assertion that multiple contacts are an important component of dengue virus transmission.

Also lacking is a field investigation on the frequency of probing behavior by *Ae. aegypti*. As explained in Chapter II, multiple probeing could add to the biting rate of *Ae. aegypti*, but currently we do not know how often *Ae. aegypti*

probes hosts in nature prior to obtaining a blood meal. Though research is currently being directed at multiple feeding, to my knowledge, no one is evaluating probing behavior under natural conditions.

The effect of multiple probing and multiple feeding could be greatly mitigated if mosquitoes returned to the same host to multiple probe or multiple feed. Experiments are needed to determine if Ae. aegypti are taking their multiple blood meals from the same individual.

Finally, Edman et al.'s (1993) recent and surprising finding that female Ae. aegypti in Thailand rarely feed on sugar offers an explanation for why Ae. aegypti imbibe multiple blood meals--the blood is used to meet energetic needs as well as for egg production. This hypothesis implies that the nutritional status of Ae. aegypti may influence biting rates because nutritionally stressed mosquitos may bite more frequently to obtain nutrients for survival. Consequently, the transmission of dengue viruses may be related to mosquito nutritional status.

Ultimately, each of the entomological parameters that affect dengue transmission will have to be united in a model that weighs the contribution of each component and its interaction with the other parameters. Such a model might permit predictions of impending outbreaks and generate hypotheses aimed at increasing our understanding of arboviral diseases. Details described in this dissertation

on the multiple host contacts hypothesis are a contribution to that effort.

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