DISSERTATION

MIDGUT INFECTION BARRIERS (MIB) AND MIDGUT ESCAPE BARRIERS (MEB) THAT CONDITION DENGUE TYPE 2 VIRUS (DEN-2) SUSCEPTIBILITY IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, CO

Spring 2003

ProQuest Information and Learning Company
300 North Zeeb Road
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COLORADO STATE UNIVERSITY

February 21, 2003

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY KRISTINE E. BENNETT ENTITLED 'MIDGUT INFECTION BARRIERS (MIB) AND MIDGUT ESCAPE BARRIERS (MEB) THAT CONDITION DENGUE TYPE 2 VIRUS (DEN-2) SUSCEPTIBILITY IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)' BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MIDGUT INFECTION BARRIERS (MIB) AND MIDGUT ESCAPE BARRIERS (MEB) THAT CONDITION DENGUE TYPE 2 VIRUS (DEN-2) SUSCEPTIBILITY IN *AEDES AEGYPTI* (DIPTERA: CULICIDAE)

Vector competence of *Aedes aegypti* for DEN-2 is a quantitative trait conditioned by several loci throughout the genome, and as such can be highly variable. The objectives of this study were to: 1) detect variation in midgut infection (MIB) and midgut escape barriers (MEB) in natural populations of *Ae. aegypti*, 2) to use marker assisted selection (MAS) to create lines of *Ae. aegypti* with specific DEN-2 infection phenotypes and test these for susceptibility to other DEN serotypes and genotypes and 3) to use quantitative trait locus (QTL) mapping to identify areas of the genome associated with MEB.

Aedes aegypti collections from Mexico and the USA show extreme variation in MIB, MEB and vector competence (VC) for DEN-2. VC ranges from 24-83%, MIB rates range from 14-59% and MEB rates range from 4-43%. A loose correlation between MIB and MEB suggests the possibility of common genetic factors conditioning these phenotypes.

Ae. aegypti lines with 2 distinct DEN-2 infection phenotypes were created. The *D2S3* line is highly susceptible to DEN-2 while the *D2MEB* line exhibits a large MEB for DEN-2 infection. MAS was not used for selection of these lines. These lines have maintained a high level of fitness over several generations. The susceptibility

phenotypes for the *D2S3* and *D2MEB* lines were selected using DEN-2, and are not consistent for other DEN serotypes. This suggests that different genetic factors may quantitatively determine susceptibility to DEN infection for the different serotypes.

QTL mapping was performed using F_1 intercrosses and reciprocal crosses of the *D2S3* and *D2MEB* lines. Two families were used for mapping. In one, the sample size was increased by creating a recombinant inbred line, and the F_5 generation was used for QTL mapping. The increased sample size gave substantially more statistical power in detecting QTL. This crossing design was able to identify 3 QTL for MIB and 8 QTL for MEB.

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ACKNOWLEDGMENTS

I would first like to thank my committee members for taking time out of their busy schedules to serve as mentors for my journey through graduate school. I have learned much from each of them. Dr. Beaty has been a continuous source of motivation to achieve and a shrewd teacher in the art of biopolitics. Dr. Black (not a biopolitician) has been a source of great knowledge and an approachable role model, as well as a friend and advisor. I will always remember trips to Pingree. Dr. Barillas Mury has been an amazing source for new ideas and has taught me to try to look at a problem from different angles. She is additionally one of the most well balanced people I know, and this has been a source of hope that I can make science work in my life. Dr. Blair has been a roommate at conferences and a woman of great integrity and intelligence – a wonderful role model. Dr. Miller has been a source of reality – and has taught me to try to think of disease in terms of the situations surrounding it.

I owe a huge debt of gratitude to those who have helped me along this journey in the form of pupae picked, DNA extracted, PCR and gels run, and technical skills shared. I owe an even larger amount to those who have helped me in terms of support, understanding, and tolerance. In most cases, people belong to both of the above groups. Dr. Steve Higgs very patiently introduced me to cell culture and virology. Research associates Jennifer Holmes, Kim Samms, Misty Jenkins, Karen Fleming, and Don Flick were key in completing this work. A most amazing work study student, Ryan Jochim, has seen me through at least 3 years of my graduate work, and has been an infinite source of

assistance and friendship. Fellow graduate students on campus and at the Arthropod Borne and Infectious Disease Laboratories have been excellent sounding boards for ideas and complaints. Among the most memorable and close to my heart are Jenny Taylor (the only other student in my cohort insane enough to embark on the full PhD program), Elizabeth Hunsperger, Saul Lozano Fuentes, Jeff Root, Ruth Fulton and Kevin Myles. Several post doctoral fellows have been instrumental in encouraging me to complete my PhD. Dr. Norma Gorrochotegui Escalante has been a great friend and mentor. Dr. Brad Blitvich has shown me what hard work and dedication can achieve. Dr. Alvaro Molina Cruz has provided technical insight and impressed me with his quality of science.

The amazing support I have received from family and friends has been essential in my success at Colorado State University. In particular I must thank my husband, Robert Rulli, to whom this dissertation is dedicated, for always being there and being supportive and encouraging through my prelims and defense, as well as all the times in between. Finally, I have to acknowledge my dog Geli, who has been a most amazing companion and willing listener throughout.

DEDICATION

This dissertation is dedicated to my husband and best friend, Robert A. Rulli. Beyond love, support and patience, he has provided comic relief and a solid grounding in reality that has been a constant (and necessary) reminder that life's simple joys are often its best gifts.

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Introduction:

Female mosquitoes of most species must take a blood meal to obtain the proteins necessary for development of their eggs. The fact that females usually have multiple gonadotropic cycles, and that some species, such as *Aedes aegypti* and *Anopheles gambiae*, even feed several times within a single cycle, makes them ideal vectors of diseases caused by eukaryotic pathogens such as malaria and filarial parasites, and arboviruses. Arthropod borne diseases cause extensive morbidity and mortality annually (James 1992). Malaria alone causes illness in at least 300 million people each year, and the resultant estimated death toll is between 1 million (WHO 1998 www.rbm.who.int) and 2.7 million (Butler 1997) annually. Approximately 40% of the world's population lives in areas of malaria transmission.

Arthropod borne viruses (arboviruses) are among the most important vector borne diseases seen today. Mosquito borne viruses in particular have seen a global resurgence in the past 2 decades (Gubler 1996). Yellow fever (YF) is one such arboviral disease of importance. *Aedes aegypti* mosquitoes transmit this flavivirus. *Aedes aegypti* is so often associated with YF transmission that it is commonly known as the yellow fever mosquito. Although there is a highly effective and safe vaccine, epidemics of YF still occur in parts of Africa and South America. There have been many epidemics in the last 20 years in West Africa (Lepiniec *et al.* 1994, Monath 1994, Nasidi 1989, De Cock *et al.* 1988, Miller *et al.* 1989), and mortality rates have ranged from 25-50 percent.

West Nile virus (WNV) has recently seen a resurgence in Europe (Hubalek and Halouzka 1999) and was seen for the first time in North America in 1999 (Lanciotti *et al.* 1999, Nash *et al.* 2001). The outbreak began in New York, and has moved progressively westward and southward (Marfin *et al.* 2001, O'Leary *et al.* 2001). WNV is a flavivirus transmitted primarily by mosquitoes in the genus *Culex*. West Nile is not usually severe in healthy humans, and severe disease and mortality is most associated with infection in the elderly, the young and the immunocompromised. It also causes an important illness of horses and some bird species, but has a broad host range including mammals and reptiles (Hayes 1988, McLean *et al.* 2002). The emergence of WNV in the United States has led to unprecedented mortality in indigenous and captive birds (Kramer and Bernard 2001), and has caused morbidity and mortality in horses (McLean *et al.* 2002).

Factors Involved in Increased Disease Incidence: It is not completely understood why these diseases are increasing, but several hypotheses have been put forth. Such factors as alterations in global climate (Patz *et al.*1995), disturbance of ecosystems (Epstein 1995), increased urbanization and poverty (Gubler 1996, Monath 1994), insecticide resistance (Brown and Brown 1974, Goldman *et al.* 1986, Mouches *et al.* 1987, Thomson *et al.* 1993, Hemingway *et al.* 2002, McCarroll and Hemingway 2002, Ranson *et al.* 2002a and b), and the collapse of public health infrastructure and mosquito abatement have all contributed to this resurgence. There are undoubtedly many other issues that factor into the rise in arboviral disease, and often given geographic areas have unique problems that must be overcome. These individual situations must be considered in any control strategy, and a failure to look at them has often been the downfall of control efforts. These factors, in addition to the facility with which humans and cargo

now travel globally, have contributed to the distribution and increased prevalence of viruses and their vectors.

The unfortunate truth is that these problems are not likely to go away any time soon. It would be difficult, and most likely impossible to control these factors, and in all probability, vector borne disease will remain a problem for the foreseeable future. Vector control is one means of disease control that seems to be fairly effective when well thought out and correctly implemented. Unfortunately, as mentioned above, the increasing prevalence of insecticide resistance, often to multiple groups of insecticides (Mouches *et al.* 1987, Thomson *et al.* 1993, Goldman *et al.* 1986, Brown and Brown 1974) makes traditional forms of vector control difficult in many regions of the world. Along with increasing vector resistance to insecticides, there is a growing opposition by the public for application of insecticides. There are both legitimate and illegitimate fears that insecticides may be harmful to the environment and to non-target organisms. Clearly, disease control will require new strategies for keeping the vector in check.

Vector Capacity/Vector Competence: One important consideration when looking at vector control is determination of the true transmission potential. Two main terms are used when talking about transmission of vector borne pathogens, and it is important to understand the difference between them. Vector competence is defined as the intrinsic permissiveness of a vector to infection, replication, and transmission of a pathogen (Hardy 1988). Vector competence does not determine whether or not transmission actually could occur. It simply defines whether or not a potential vector is capable of supporting, replicating, and delivering a pathogen. Vector capacity encompasses many additional factors, and actually calculates the likelihood of pathogen

transmission. It takes into account vector competence, but additionally considers vector abundance, host preference, longevity, and the extrinsic incubation period of the given pathogen in the given vector. A generally accepted formula for vectorial capacity is:

$$\text{Vectorial capacity} = mbca^2 p^n / -\text{Ln}(p)$$

where m is the number of female mosquitoes per person, b is the probability that an infectious mosquito transmits the pathogen while biting a susceptible host, c is the probability that a mosquito acquires an infection while biting an infected human, a is the number of bites per person per day, n is the duration of the extrinsic incubation period (EIP), and p is the survival rate of the mosquito. Thus, even if laboratory experiments demonstrate that a given vector is competent to transmit a pathogen, more information is necessary before it is possible to determine the likelihood of pathogen transmission. The majority of discussions in this dissertation will focus on vector competence, as a component of and a means by which to disrupt vector capacity.

A better understanding of the interactions between the virus and its vector will help scientists find more effective ways to control disease transmission. Recent advances in molecular genetic techniques have given researchers more tools with which to investigate disease vectors (Besansky and Collins 1992, Collins 1994, Crampton *et al.* 1994, Carlson *et al.* 1995, Beaty 2000, Black *et al.* 2000, 2001, Atkison and Michel 2002). Discovering the mechanisms that determine vector competence will provide insight into approaches to alter vector competence as well as providing more effective targets for disease control. Potential uses for this knowledge include the development of novel insecticides, repellents, attractants and vaccines, which could be beneficial in decreasing disease transmission.

Manipulation of the Mosquito Vector: Most attempts at eradication of disease vectors have failed. In most cases, eradication is not even a rational option, as it would require careful planning, global cooperation, extreme persistence and a bottomless budget. The new mindset is that control efforts should forgo attempts at eradication and instead concentrate on finding ways to control vectors in ways that minimize exposure to bites or decrease vector competence. One way in which this might be accomplished is to replace natural mosquito populations with altered mosquitoes that are incompetent vectors. If the transmission cycle can be broken, eradication may no longer be a necessary component of vector-borne disease control strategies.

Using Gene Expression to Interupt Vector Competence: One avenue for altering vector competence has come through the expression of exogenous genes or gene sequences in normally competent vectors. One successful means by which these sequences have been expressed is the double subgenomic virus expression system derived from the alphavirus Sindbis (dsSIN) (Xiong *et al.* 1989). A Sindbis expression system has been engineered to efficiently express green fluorescent protein in *Ae. aegypti* midguts after oral infection (Olson *et al.* 2000). Expression of heterologous viral sequences in sense or antisense orientation in the mosquito vector has been used to prevent replication and transmission of these viruses (Powers *et al.* 1996, Olson *et al.* 1996, Blair *et al.* 2000, Olson 2002). DsSIN expression of antisense sequences has also been successfully used to inhibit expression of enzymes (Johnson *et al.* 1999). Most recently, the dsSIN and DNA-based expression systems have been engineered to express inverted repeat RNA derived from the virus genome to induce degradation of viral RNA through a process known as RNA interference (RNAi) (Adelman *et al.* 2001, 2002, Olson

2002). The Sindbis virus expression system is transient, and because Sindbis virus naturally infects humans and other animals, it is not a practical solution for disease control. It has proven, however, to be a useful laboratory tool for gene characterization in mosquitoes, and in addition to its other uses, has allowed the study of gene function by allowing researchers to silence or overexpress mosquito genes. The dsSIN expression system has also shown that virus transmission and enzyme activity can be blocked in a whole mosquito.

Stable and Heritable Gene Expression: The next obvious step is to find ways of creating stable, heritable expression of genes of interest, or sequences in mosquitoes that will render them incapable of transmitting pathogens. Several strategies are being investigated to achieve this goal. Heterologous sequences have been expressed in vectors using naturally occurring endosymbionts (Beard *et al.* 1993), *Aedes* densonucleosis viruses (Family Parvoviridae) (Afanasiev *et al.* 1994), and pseudotyped retroviruses (Yee *et al.* 1994). In addition, transposable elements have been used to actually transform the vector (Jasinskiene *et al.* 1998, Coates *et al.* 1998, Pinkerton *et al.* 2000, Allen *et al.* 2001, Catteruccia *et al.* 2000).

Despite the facility with which *Drosophila* genomes are transformed using the *P*-element, transformation of mosquitoes has been difficult. The first stable transposable element mediated transformation of *Ae. aegypti* was achieved using the *Hermes* transposable element (Jasinskiene *et al.* 1998) and subsequently the *Mariner* transposable element (Coates *et al.* 1998). Green fluorescent protein (GFP) and other fluorescent proteins have been used as markers for easy recognition of transgenic individuals. This has been successful in *Ae. aegypti* (Pinkerton *et al.* 2000), *Cx. quinquefasciatus* (Allen *et*

al. 2001), and *An. stephensi* (Catteruccia *et al.* 2000). Transformation has now been accomplished through the use of transposable elements *Hermes* from the house fly, *Musca domestica* (Warren *et al.* 1994), *piggyBac* from the cabbage looper, *Trichoplusia ni* (Wang *et al.* 1989), *Minos* from *Drosophila hydei* (Franz *et al.* 1994), and *Mos*1 from *Drosophila mauritiana* (Jacobson *et al.* 1986) as gene vectors. Transformation rates range from 0.5-13%. Using transgenic mosquitoes for disease control in the field has been a very controversial topic, and would not be logistically simple (Crampton *et al.* 1990, Spielman 1994, Ashburner *et al.* 1998). The use of transgenic mosquitoes in the lab, whether or not a viable option for actual disease control, is certain to increase understanding of the factors that condition vector competence, and thus will be important in finding other solutions for disease control.

Dengue Viruses

Dengue Disease Manifestations: Although not pleasant, infection with a dengue virus generally causes a self-limiting, febrile illness (Clarke 2002, Guzman and Kouri 2002). A typical case of dengue fever (DF) begins abruptly 2-7 days after an infectious bite. The initial symptoms include high fever, headache, retroorbital pain, lumbosacral aching pain, conjunctival congestion and facial flushing. The fever may last for 6-7 days, or it may be biphasic. Myalgia or bone pain, anorexia, nausea, vomiting, weakness and prostration follow the initial symptoms. Some individuals, especially children, may experience respiratory symptoms. A transient rash may appear on the first or second day. A secondary rash, which starts on the trunk and spreads to the face and limbs, typically follows this on days 3-5. Convalescence can be prolonged and may include general

weakness, depression, bradycardia, and ventricular extrasystoles (Monath and Heinz 1996).

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are more severe forms of dengue virus infection. In most cases, the disease initially presents as DF, but after 2-5 days rapidly progresses to include prostration, restlessness, irritability, shock with cold clammy extremities, diaphoresis, rapid respiration, rapid pulse, and hypotension. Spontaneous hemorrhages can occur, both internally and externally. DHF is distinguished from DF by the presence of fever, thrombocytopenia and hemoconcentration. Hypotension or profound shock characterizes DSS (Monath and Heinz 1996).

Treatment for DF and DHF/DSS is supportive only, and includes bed rest, antipyretics and analgesics. When there are signs of dehydration, treatment that includes careful monitoring of electrolytes and fluids by hydration therapy and maintenance of the circulating fluid volume is extremely important. This type of supportive therapy has succeeded in decreasing the case fatality rate from nearly 40% to less than 5%.

Perhaps the most alarming factor in the recent dengue pandemic has been the rise in the number of individuals progressing to DHF and DSS. Reasons for this increase may include antibody dependent immune enhancement (Halstead 1982, Halstead 1988) and/or increased virus virulence (Rico-Hesse *et al.* 1997, Holmes and Burch, 2000).

Dengue Epidemiology: Although there are many arboviruses that have increased in incidence in the past few decades, none have become as important as the dengue viruses. Dengue is currently considered (along with several other important illnesses) to be an emerging disease (Gubler 1996). In the case of dengue, the term "emerging" refers

less to the fact that it is a novel virus than to the fact that there has been an increase in the number and severity of cases globally. Indeed, it is sometimes considered to be the most important vector-borne viral disease of human beings (Gubler 98, Monath 94). Two and a half billion people (2/5 of the global population) are at risk for dengue infection. There are currently approximately 50-100 million cases of classical dengue fever (DF) annually. More worrisome are the estimated 500,000 cases per year of DHF/DSS (Monath 1994, Gubler and Clark 1995, Isturiz *et al.* 2000, Sharma *et al.* 2000, Kantachuvessiri 2002, Halstead *et al.* 2002). In 2001, the Americas reported more than double the number of DHS/DSS cases as reported in the same area in 1995 (WHO Fact Sheet No 117 Revised April 2002). Between 1981 and 1995, there had already been a four-fold increase in the incidence of DF as compared to the total incidence in the preceding 30 years (Gubler 1998). The number of dengue cases is clearly increasing globally (Dove 1998, Gratz 1999, Chareonsook *et al.* 1999, Aviles *et al.* 1999, and Yamada *et al.* 1999, Clarke 2002), which justifies its classification as an emerging disease. The prognosis is not good, as the number of cases of dengue fever and DHF is projected to increase with increased urbanization (Cho Min 2000, Condon *et al.* 2000, Isturiz *et al.* 2000, Nogueira *et al.* 2000, Barbarosa da Silva *et al.* 2002, Endy *et al.* 2002, Flisser *et al.* 2002, Gubler 2002, Guzman 2002, Kantachuvessiri 2002).

Dengue is currently endemic in over 100 countries in the Americas, Africa, the Eastern Mediterranean, Southeast Asia, and the Western Pacific. Epidemics now occur regularly in Southeast Asia, India, the western Pacific, and much of Mexico and Central and South America (Clarke, 2002). DHF has increased, and many new countries now report epidemics of this life threatening disease. Prior to 1970 only 9 countries reported

DHF epidemics. As of 1995, that number had increased more than 4 fold (WHO Fact Sheet No 117 Revised April 2002).

Until recently, dengue has been considered a disease of the tropics and/or of developing countries. This is however; changing rapidly, as dengue is diagnosed more and more commonly in locations like northern Mexico and the southern United States (Pena *et al.* 1999). The disparity between the number and severity of cases seen in the southern United States and northern Mexico still leads to the conclusion that socioeconomic factors may play an important role in the epidemiology of dengue. However, epidemics have also been seen in Australia (Hanna *et al.* 1998), Pakistan (Paul *et al.* 1998), and India (Clarke 2002). The emergence of West Nile Virus in the United States demonstrates that vector borne disease is not just a problem of the tropics and developing countries. Given the right circumstances, dengue may become a serious health threat in the United States, particularly in impoverished areas.

Global travel has contributed to the increased distribution of both DEN virus and *Aedes aegypti*. In addition to its toll on human health in the form of morbidity and mortality, DF/DHF/DSS has been an economic burden not easily tolerated by the communities hardest hit. Enormous sums of money have been spent on vector control, medical care, and hospitalization costs. Much more has been lost in the form of decreased productivity and through the disruption of tourism (vonAllmen *et al.* 1979, Gubler 1988, Meltzer *et al.* 1998).

DEN viruses naturally have sylvatic, rural/suburban, and urban transmission cycles (Gubler 1988). Sylvatic cycles have been documented in Southeast Asia between susceptible monkey species and mosquito vectors of the *Ae. (Finlaya) niveus* complex

(Rudnick 1965, 1978). Similarly, a sylvatic cycle in West Africa has been documented involving *Ae. (Diceromyia) furcifer, Ae. (Diceromyia) taylori,* and the *Ae. (Stegomyia) africanus* complex (Cordellier *et al.* 1983, Cornet *et al.* 1984, Fagbami *et al.* 1977). Although a sylvatic cycle has not yet been discovered in South and Central America, it is presumed to exist (Rosen 1958). The importance of these sylvatic cycles as reservoirs for viruses to emerge in human urban and suburban epidemics is not clear. Even in areas (e.g. South America) where no sylvatic cycle has been discovered, virus remains endemic. Additionally, virus isolates from sylvan cycles in West Africa have been shown to be genetically distinct from isolates obtained in urban cases. This suggests that the two cycles may be unrelated (Kerschner *et al.* 1986).

Peridomestic species of *Aedes* are primarily responsible for rural/suburban transmission. These include: *Ae. (Stegomyia) albopictus* in Southeast Asia and possibly South America, *Ae. (Stegomyia) luteocephalus* in Africa, *Ae. (Stegomyia) polynesiensis* in the Pacific, and potentially *Ae. (Gymnometopa) mediovittatus* in the Caribbean (Gubler 1988, Gubler *et al.* 1985). *Aedes albopictus* and *Aedes polynesiensis* have served as vectors in dengue epidemics (Fan *et al.* 1989, Gould *et al.* 1968, Metselaar *et al.* 1980), but the primary vector in urban transmission is *Ae. aegypti.*

Dengue Viruses: Dengue viruses belong to the genus *Flavivirus* in the family Flaviviridae. This family is made up of single stranded RNA viruses of positive polarity (Monath 1996, Rice 1996, Heinz *et al.* 2000, Burke and Monath 2001). Although the mode of transmission has been understood since the early 1900's (Kruif 1926, Graham 1903), the etiology of dengue fever was not determined until the mid 1940s (Sabin 1952).

Dengue viruses have a genome length of approximately 11,000 bases. The genome codes for three structural and seven nonstructural proteins. The entire genome is translated as a single polyprotein that is cleaved cotranslationally and posttranslationally by host and viral proteases into the functional viral proteins. Nonstructural proteins are coded for at the 3' end of the genome and include: NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. Structural proteins are coded for at the 5' end of the genome and include: capsid protein (C), premembrane/membrane protein (prM/M), and envelope protein (E). The dengue virus single stranded RNA genome is associated with a core protein in an icosahedral nucleocapsid. A virus-encoded membrane protein surrounds the nucleocapsid and is embedded in the envelope. The mature virion obtains a lipid envelope from host cell membranes but adds its own virus encoded envelope glycoprotein (Henschal and Putnak 1990, Monath and Heinz 1996, Burke and Monath 2001).

Dengue Viruses, Genetic Diversity: There are four distinct dengue virus serotypes that comprise the dengue group of viruses, which have all been associated with both DF and DHF (Wisseman and Sweet 1961, Monath and Heinz 1996, Burke and Monath 2001). Among the 4 serotypes, there is substantial genetic diversity. Indeed, the distinct serotypes are not necessarily more similar to one another in nucleotide and amino acid sequence than they are to other members of the genus *Flavivirus* (Kuno, 1998).

Each of the four dengue serotypes can be further subdivided into genotypes. It is generally accepted that DEN-1 has at least 3 genotypes, consisting of viruses from Thailand, the Philippines, and the Caribbean (Chu *et al.* 1989). DEN-2 is made up of 5 genotypes (Rico-Hesse 1990). Although there is some discrepancy as to the number designation of each genotype, the following are generally accepted. Genotype I is

designated Puerto Rico and includes strains from the Americas, India, and the South Pacific. Genotype II is designated Jamaica, and includes strains from the western Caribbean. (Trent *et al.* 1983, Walker *et al.* 1988, Trent *et al.* 1990). Genotype III includes viruses from Burma and Thailand, and Genotype IV includes viruses from the Philippines. Genotype V groups viruses originate from the Seychelles Islands and Sri Lanka (Trent *et al.* 1983, Monath *et al.* 1986, Lewis *et al.* 1993). DEN-3 is made up of 4 genotypes (Lanciotti *et al.* 1994). Genotype I consists of viruses from Indonesia, Malaysia, the Philippines and the South Pacific Islands. Genotype II consists of viruses from Thailand. Genotype III is made up of viruses from Sri Lanka, India, Africa, and Samoa. Genotype IV is made up of viruses from Puerto Rico. DEN-4 has only 2 genotypes. Genotype I includes viruses from the Philippines, Thailand, and Sri Lanka, while Genotype II consists of viruses from Indonesia, Tahiti, the Caribbean Islands (Puerto Rico, Dominica) and Central and South America (Lanciotti *et al.* 1997). These genotypes have been shown to diverge as much as 12% in nucleotide sequence in the envelope gene, which determines most of the antigenic characteristics of the virus (Lewis *et al.* 1993, Westaway and Blok 1997). Structural differences in the virion have been observed as well, and have been correlated with disease severity (Leitmeyer *et al.* 1999). A high mutation rate contributes to the great genetic diversity and rapid evolution in dengue viruses. Dengue, along with many other RNA viruses is sometimes referred to as a quasispecies. Quasispecies are closely related but nonidentical viral genomes within a single organism (Holland 1982, Domingo and Holland 1994, Domingo *et al.* 1998, Domingo *et al.* 2001, Domingo 2002). Using a plant model, the results of Schneider and Roossinck (2001) suggest that different hosts may actually accelerate or decelerate the

rate of viral evolution by respectively permitting or preventing high levels of diversity in the viral population. This is certainly relevant to dengue, as it must infect both vertebrate and invertebrate hosts.

A high mutation rate is characteristic of all RNA viruses, because they lack the proofreading enzymes that DNA-based systems use to maintain high fidelity during genome replication. (Drake 1993). For the dengue viruses, synonymous base substitution occurs at a significantly higher rate than non synonymous substitution, especially in some genome regions such as the E gene. This suggests that the virus has strong functional constraints on certain areas of its genome, and that purifying selection occurs (Zanotto *et al.* 1996).

Another potential mechanism for enhancing diversity is viral recombination. This could occur via a copy choice mechanism, which requires that the RNA polymerase switch template strands midway through replication. This can occur between the genomes of 2 different viruses that have infected the same cell and has the potential to form a hybrid molecule (Worobey *et al.* 1999, Worobey and Holmes 1999). It is not known how commonly this occurs, but simultaneous infection of human hosts with different genotypes and even different serotypes of dengue virus has been observed (Gubler 1995, Lorono-Pino *et al.* 1999). Whether or not recombination occurs in the mosquito vector has not been determined. Although there is not evidence of recombination between serotypes, there is evidence of widespread recombination among genotypes within serogroups (Worobey *et al.* 1999, Woroby and Holmes 1999).

Because intra-serotype recombinants are so easily identified when phylogenetic analysis is applied using more than one portion of the genome, it has been hypothesized

that recombination may not be a frequent event. If high rates of recombination were occurring, one would expect virus sequences to be much more homogeneous, and for recombinants to be less easily identified (Holmes and Burch 2000). Recombinational events obviously depend on the coinfection of a single mosquito with more than one virus genotype, which may also be a relatively rare occurrence. If this is true, recombination will be a less important factor in virus evolution than mutation (Holmes and Burch 2000). Recombination, in addition to high mutation rates, may partially explain differences in virus virulence, which is one of the factors hypothesized to contribute to an increase in the number of DHF cases (Rothman 1997).

There are other factors that contribute to increased genetic diversity, such as migration or gene flow. Because the different serotypes and genotypes of dengue can now be transported over long distances by human hosts and mosquitoes, they have a potentially broad geographical distribution (Rico Hesse 1990). Distribution of the different serotypes and genotypes over large geographical distances may also contribute to an increased incidence of recombination among serotypes and genotypes through simultaneous infection of individual mosquitoes.

Antibody Dependent Immune Enhancement: Infection with one serotype confers life-long immunity to exposure to homologous, but not heterologous serotypes. DHF is seen most commonly in patients suffering a secondary infection with a dengue serotype heterologous to the primary infection, suggesting a potential for antibody dependent immune enhancement (ADE) (Halstead 1982, 1988). In the ADE model, antibody binds to, but is unable to completely neutralize dengue virions. These virus-antibody complexes bind to Fc-receptor bearing peripheral blood monocytes and

macrophage-like cells, which are infected by the virus more efficiently than they would be had the virus not been bound by non-neutralizing antibody. ADE becomes particularly important for vaccine development; the vaccine must necessarily be able to induce protection against all 4 serotypes. A vaccine targeted at a specific serotype might actually serve to increase the incidence of DHF/DSS by protecting against the target serotype, but allowing other serotypes easier access to target cells and giving them a replicative advantage that could lead to more severe disease.

Virus Virulence: Not all DHF can be associated with secondary infections (Gubler and Clark 1995), and differences in virus virulence have been suggested as another determinant of dengue disease outcome. The first occurrence of large numbers of DHF cases in the Americas coincided with the introduction of a new strain of DEN-2 that was similar to virus circulating in Southeast Asia (Rico-Hesse 1990). Similar results were seen when DEN-3 was introduced into the Americas (Gubler and Clark 1995, Gubler 2002, Guzman 2002). It is difficult, however, to distinguish effects from the introduction of new viruses from ADE or other factors that contribute to disease severity.

The rapid mutation rates seen in RNA viruses may allow for the evolution of more virulent strains that can lead to more severe disease outcome (Rico-Hesse *et al.* 1997, Bergstrom *et al.* 1999, Holmes and Burch 2000). Viruses that become more efficient at invading host cells, or which gain the ability to replicate to higher titers may be responsible for some DHF/DSS cases. The arthropod vector may also select viruses that can replicate to high titer. In the quasispecies model, viruses are in a constant state of competition. Those that are able to replicate to high titers are more likely to be ingested by an arthropod, and thus more likely to be amplified and transmitted. The mosquito

vector can also serve as a bottleneck for the virus because only a small number of virions are ingested and eventually infect the mosquito midgut. In at least one study of DEN-3 viruses, bottlenecks are hypothesized to be the most important factor in the emergence of new virus strains in Thailand (Wittke et al. 2002). Bottlenecks usually reduce the fitness of an organism. In the case of viruses, Muller's ratchet, or the continual decrease in mean fitness of the clonal lineage by stochastic loss of the least mutated class (Haigh 1978, Bergstrom *et al.* 1999), has been proposed to decrease virus virulence due to the accumulation of deleterious mutations. This may explain the loss of pathogen virulence in some laboratory systems. More recently, the quasispecies model has been tested, and results suggest that, while the accumulation of deleterious mutations leads to a decrease in fitness, the rapid mutation rate also allows evolution of virus strains with increased fitness (Bergstrom *et al.* 1999). The bottlenecks experienced in the mosquito vector may actually help certain virulent strains become more prevalent.

Aedes aegypti

***Aedes (Stegomyia) aegypti* (Linnaeus)**: Walter Reed and the US Army Yellow Fever Commission were the first to demonstrate that the yellow fever virus was transmitted by mosquitoes (McCarthy 2001). *Aedes aegypti* was proven to be the yellow fever virus vector when James Carroll in 1900 (testing a hypothesis of Carlos Finlay), infected himself and other volunteers with YF through the bite of an infected mosquito (de Kruif 1926). Dengue fever was subsequently proven to be biologically transmitted by *Ae. aegypti* (Graham 1903). This set the stage for further research on vector borne arboviruses.

The dramatic increase in the number of dengue cases is due in part to the resurgence of *Ae. aegypti*. Eradication campaigns in the 1950's and 1960's nearly eradicated *Ae. aegypti* from many countries in the Americas. However, when these campaigns were stopped in the 1970's, the mosquito was able to return to its pre-campaign prevalence, and its current distribution exceeds previous boundaries.

Aedes aegypti is a day feeding, synanthropic, container-breeding mosquito that has adapted well to cohabitation with humans. The species has a global distribution in tropical and subtropical areas between the 40°N and 40°S latitudes. It can be subdivided into at least 2 subspecies. *Ae. aegypti formosus*, the "black" subspecies, occurs in sub-Saharan Africa in sylvatic habitats and has been shown to be quite resistant to infection with dengue. It is not usually considered to be a significant vector in urban epidemics. However, it was found to be the primary vector in at least one YF virus epidemic in Nigeria (Miller *et al.* 1989). *Ae. aegypti formosus* preferentially oviposits in treeholes, which keeps it from infesting urban areas, and thus from being a real pest to humans. *Ae. aegypti aegypti*, the "light" subspecies, is primarily an urban mosquito, is found globally, has been shown to be highly susceptible to infection with dengue, and is the vector most associated with dengue epidemics. *Ae. aegypti aegypti*, preferentially oviposits in artificial containers such as tires and garbage, which keeps it in close proximity to humans. In fact, it feeds almost exclusively on human blood (Tabachnick 1991, Scott *et al.* 1993, 2000a). This adaptation to feeding on human blood may benefit *Ae. aegypti aegypti* by providing increased energy reserves and a fitness advantage (Harrington *et al.* 2001, Costero *et al.* 1998). Selection may actually favor females that feed on human blood without feeding on sugar (Scott *et al.* 1997, Costero *et al.* 1999, Harrington *et al.*

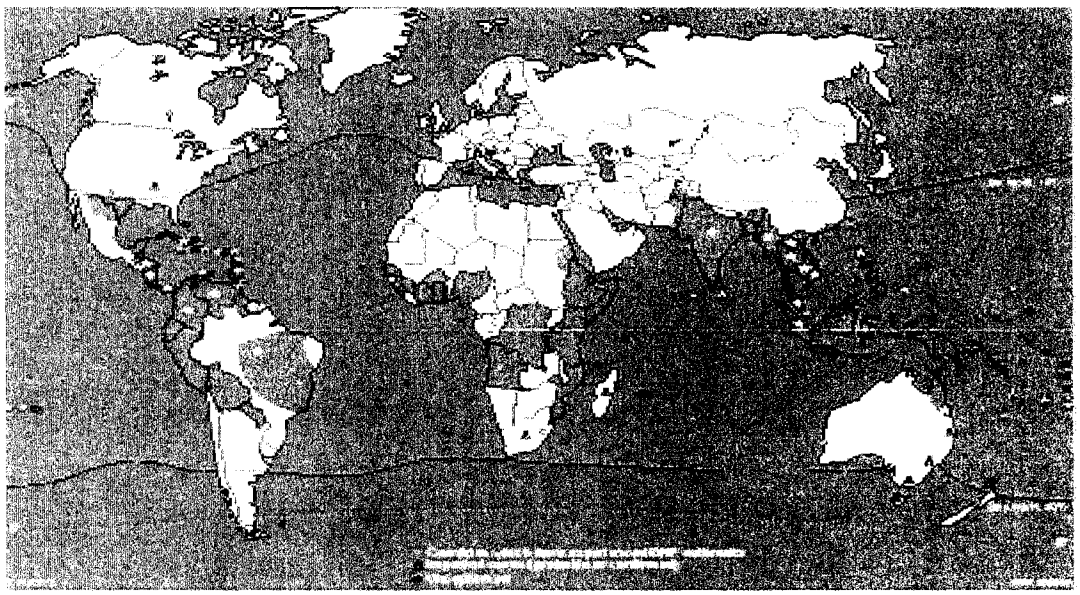
2001). This close association with humans is probably what allowed the mosquito to disperse globally from its believed origin in Africa (Christophers 1960).

Increases in human population size, poverty, urbanization, and global travel have helped *Ae. aegypti aegypti* thrive and spread. These factors, particularly more efficient global travel, have also allowed the 4 dengue serotypes to spread and have led to epidemics in which all 4 serotypes may be co-circulating. The ADE theory would explain the dramatic increase in the numbers of DHF/DSS cases annually, as individuals suffer secondary dengue infections with heterologous serotypes. This could also lead to increased virus virulence as viruses spread, mutate and potentially recombine.

In theory, anywhere that *Ae. aegypti aegypti* occurs, there is potential for dengue transmission (Figure 1.1). In practice, however, this is not the case. Despite the fact that dengue transmission rarely if ever occurs without the presence of *Ae. aegypti aegypti*, the opposite is not true, and *Ae. aegypti aegypti* has a much wider distribution than the dengue viruses. The fact that *Ae. aegypti* has a range of susceptibilities to infection with flaviviruses that is, at least in part, genetically determined may account for some of the discrepancy between mosquito and virus distribution (Aitken *et al.* 1977, Tabachnick *et al.* 1985, Rosen *et al.* 1985, Lorenz *et al.* 1984, Vazeille *et al.* 2001).

Population Genetics of *Aedes aegypti*: Along with obvious phenotypic differences, *Ae. aegypti* populations differ genetically as shown by biochemical and molecular genetic markers and in its vector competence for dengue viruses (Gubler *et al.* 1979, Wallis *et al.* 1983, Rosen *et al.* 1985, Tardieux *et al.*, 1990, Vazeille-Falcoz *et al.* 1999, Vazeille *et al.* 2001). Genetic studies of allozymes in *Ae. aegypti* populations from around the world identified 8 genetic groups (Tabachnick 1991, Tabachnick and Powell

A.



B.

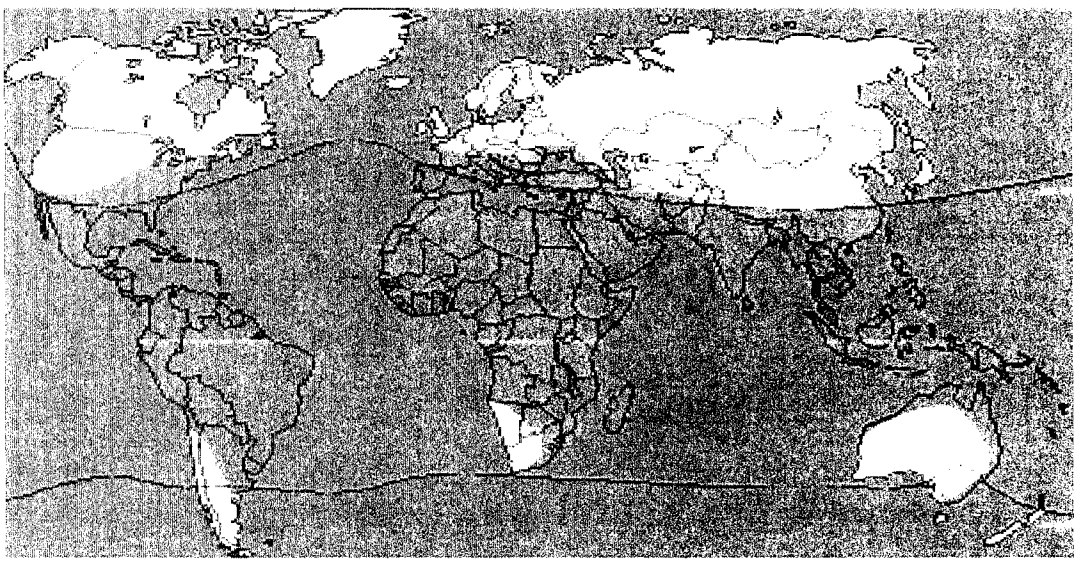


Figure 1.1: Global distribution of dengue circulation (A) and *Aedes aegypti* (B) taken from the website of the World Health Organization (WHO). The distribution of dengue completely overlaps the distribution of *Ae. aegypti*. The broader distribution of *Ae. aegypti* suggests the potential for further spread of dengue epidemics.

1979). These include 2 sylvan groups (*Ae. aegypti formosus*) in West and East Africa and 6 domestic groups (*Ae. aegypti aegypti*) in East Africa, Southeast USA, Southwest USA-Mexico, Central-South America, Caribbean, and Southeast Asia-Pacific.

Interestingly, the genetic variation found among these various groups of *Ae. aegypti* is relatively small when compared to that found in other insect populations (Tabachnick and Powell 1978 and 1979, Tabachnick *et al.* 1979, Wallis *et al.* 1983 and 1984). This finding suggests that the division into sylvatic and domestic subspecies occurred only recently in evolutionary history. The subspecies may have separated when the Sahara began to dry about 4,000 years ago (McIntosh and McIntosh 1981). Populations of *Ae. aegypti* would have been stranded in North Africa and adapted to living in water storage containers of humans (Tabachnick 1991). Additionally, the low level of genetic diversity within the subspecies suggests that the different populations have only recently spread and differentiated. Humans have been implicated as the primary factor in facilitating this spread and diversification via commerce and exploration (Black *et al.* 2002).

While the majority of studies have primarily focused on determining genetic differences among *Ae. aegypti* from a wide geographic distribution, variation on a smaller geographic scale within *Ae. aegypti* subpopulations may help determine why certain areas experience DF or DHF outbreaks at a higher frequency and with greater severity. Recent studies have investigated gene flow among *Ae. aegypti* populations at a more regional level (Apostle *et al.* 1996, Vazeille *et al.* 2001, Gorrochotegui-Escalante *et al.* 2000, Gorrochotegui-Escalante *et al.* 2002). Techniques that more sensitively detect variation among individual mosquitoes, such as single strand conformation polymorphism (SSCP),

PCR, cloning, and the use of more polymorphic markers, such as RAPDs and cDNAs, have allowed researchers to more accurately detect differences among mosquito collections. This has allowed for more accurate descriptions of gene flow and diversification in mosquito populations.

A study in Puerto Rico (Apostle *et al.* 1996) using RAPD-PCR markers examined gene flow in 16 mosquito collections from 6 cities. This study was able to find more than two times the heterozygosity (0.345) detected in previous studies using allozymes. Studies on mosquitoes collected from Mexico (Gorrochotegui-Escalante *et al.* 2000, Gorrochotegui-Escalante *et al.* 2002) have suggested that gene flow among *Ae. aegypti* populations varies among regions depending on the amount of human commerce and barriers to natural mosquito migration. This study demonstrated three major *Ae. aegypti* groups in Mexico corresponding to the geographic locations of Northeastern Mexico, the Pacific coast and the Yucatan. Northeastern collections were not strongly isolated by distance and showed moderate gene flow and low genetic diversity. Collections from the Yucatan had wide variation in genetic diversity, low levels of gene flow and were isolated by distance. Collections along the Pacific coast showed high genetic diversity and a large amount of gene flow. These studies suggested that populations of mosquitoes across Mexico can be expected to remain genetically homogeneous within distances of 150 km, assuming there is no local selection or natural barriers to migration. These studies also suggest that genes released into a given population should spread rapidly into populations within a 150 km radius.

It is clear that differences in gene flow among *Ae. aegypti* populations exist. These differences are based on such factors as natural geographic barriers, human

commerce, climatic differences and differences in mosquito behavior. The success of genetically based mosquito control strategies depends on understanding gene flow in the target populations. This information is necessary to determine how far a gene introduced into the population will spread. Gene flow information can also be useful for determining an effective use of pesticides in areas where insecticide resistance is a concern, as it can help predict the spread of insecticide resistance genes.

Physiological Genetics of Vector Competence: Vector competence of *Ae. aegypti* for arboviruses is dependent on the mosquito's susceptibility to midgut infection following an infectious blood meal and its subsequent permissiveness to virus dissemination in various organ systems including, ultimately, the salivary glands for transmission to a subsequent host. Variation in midgut infection (MI) and disseminated infection (DI) are due primarily to a midgut infection barrier (MIB) and a midgut escape barrier (MEB) (Figure 1.2).

There is surprisingly little known about how dengue virions infect the mosquito midgut. Thus far, no receptors have been identified. Putative midgut receptors have been suggested for other arboviruses, such as the laminin receptor for alphaviruses (Ludwig *et al.* 1996). Research with other arboviruses, such as bunyaviruses and orbiviruses, suggests the need for proteolytic processing of virus proteins involved with host cell receptor attachment in the midgut for efficient vector infection (Ludwig *et al.* 1991, Ludwig *et al.* 1989, Mertens *et al.* 1996, Xu *et al.* 1997). This may also be true for dengue viruses, but has not yet been shown.

Despite the lack of detailed understanding of the mechanism of dengue infection of midgut epithelial cells, it is presumed that there must be some sort of receptor

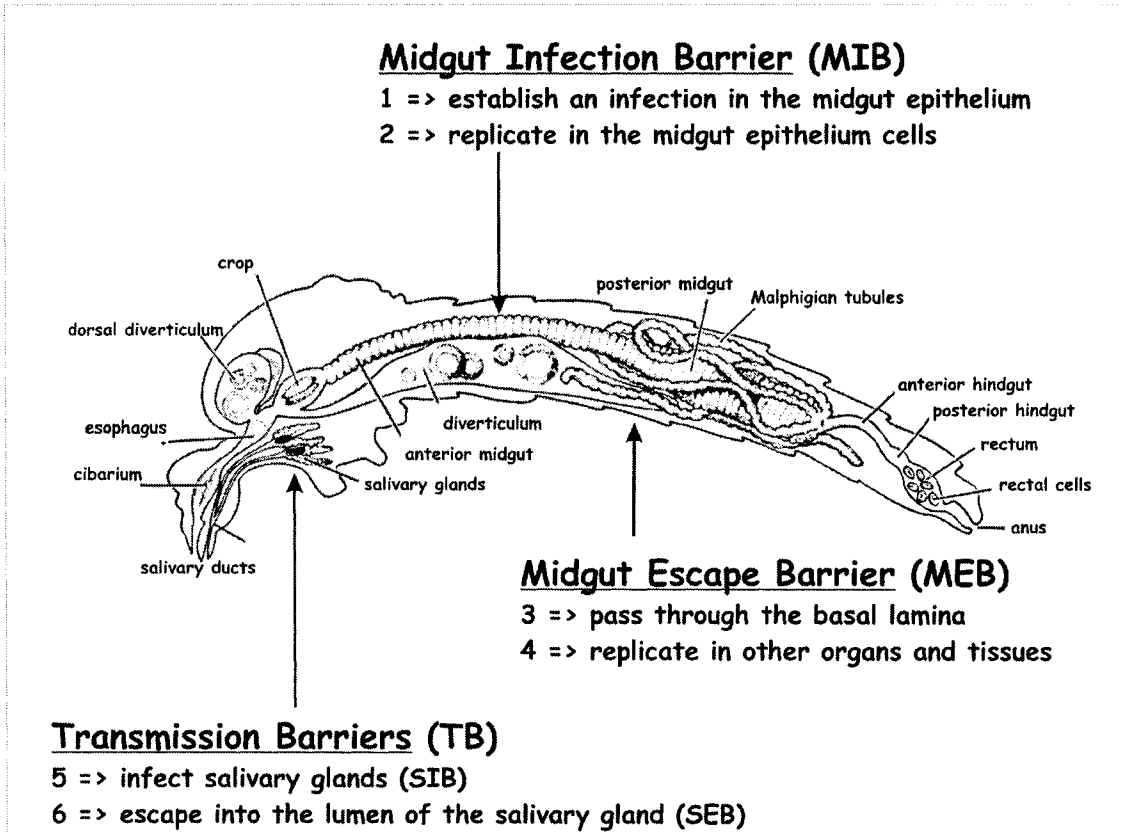


Figure 1.2: Physiologic Barriers to pathogen transmission. In order for transmission of a virus to occur, the virus must be able to overcome the midgut infection barrier (MIB) by infecting and replicating in the midgut epithelial cells. Virions must subsequently be able to overcome the midgut escape barrier (MEB) by passing through the basal lamina and disseminating to other tissues and organs, specifically the salivary glands.

mediated entry. After the virion gains access to the host cell, its genome must be translated and subsequently transcribed for replication and production of mature virions. If the virus is stopped at any of these steps (receptor binding, uncoating, translation, genome replication) the mosquito is said to have a MIB.

Once the virus has established a productive infection in the midgut, it must be able to disseminate from the midgut into the hemocoele to infect secondary target organs, including the salivary glands, if virus is to be transmitted to subsequent hosts. If the virus is unable to disseminate from the midgut, or if it disseminates but is unable to infect secondary organs, the mosquito is said to have a MEB.

When a mosquito ingests an extremely high titer of virus, an existing MIB can be overcome (Woodring *et al.* 1996). Why this is true, however, is not completely understood. It is hypothesized that some virus may bypass the midgut to infect secondary tissues and organs without replicating in the epithelial cells. This phenomenon is known as a "leaky midgut." In nature, horizontal infection of a mosquito typically occurs when it feeds on an infected host. Virus titers naturally vary widely among individuals, and this can potentially influence whether or not a mosquito will become infected with and be able to transmit the virus.

MIB are associated with attachment, penetration, uncoating, translation and transcription of the virus in the mosquito midgut. Studies on vector competence of *Ae. aegypti* have consistently shown that the MIB has a major effect on the potential for flavivirus transmission (Schoepp *et al.* 1990, Bosio *et al.* 1998, Gubler *et al.* 1979, Tabachnick *et al.* 1985). MIB is also an important factor in other systems, such as Western equine encephalitis (WEE) virus in *Culex pipiens* (Houk *et al.* 1986).

MEB can be associated with a number of factors. These include, but are not limited to inefficient assembly or maturation of virus in midgut cells, the failure of virions to leave midgut cells and pass through the basal lamina, and the inability of virus to infect secondary organs such as the salivary glands. MEB is presumably important in determining dengue transmission potential in *Ae. aegypti*, but, prior to work done for this dissertation, the involvement of MEB was not as well understood as that of MIB. MEB has been shown to be partially responsible for the resistance of *Ae. aegypti* mosquitoes to yellow fever virus (Miller and Mitchell, 1991). The fact that MEB has not been well characterized in many systems is partially due to the difficulty in distinguishing between effects of midgut infection and midgut escape. It is also due to a lack of basic knowledge of the mechanisms of viral invasion of midgut cells, and subsequent viral dissemination. MEB has, however, been shown to be important in other mosquito-virus systems. MEB is a major determinant of vector potential in the California group viruses (Bunyaviridae), where the middle sized RNA segment (encoding surface glycoproteins) seems to be critical in determining whether virus can successfully disseminate from the midgut. This is true even when large amounts of viral antigen can be found accumulating in the midgut (Miller 1982, Beaty and Bishop 1988). MEB has also been shown to be partially responsible for the inability of *Culex tarsalis* to transmit WEE virus (Kramer *et al.* 1981).

If virus does manage to infect, replicate in, and escape the midgut, it is released into the hemolymph where it disseminates to other tissues and organs. Virus must productively infect the salivary glands before it is subsequently transmitted during mosquito blood feeding. Salivary gland barriers have been shown in several systems (Whitfield *et al.* 1973, Whitfield *et al.* 1971, Takahashi and Suzuki 1979, Sriurairatna and

Bhamarapravati 1977, Takahashi 1980, Grimstad *et al.* 1985, Jupp 1985, Kramer *et al.* 1981, Beaty and Bishop 1988, Paulson *et al.* 1989). A salivary gland infection barrier (SIB) partially explains refractoriness of *Culex tarsalis* to WEE virus (Kramer *et al.* 1981). Salivary gland escape barriers (SEB) have been demonstrated in several systems including: *Ae. triseriatus* for infection with snow shoe hare (SSH) virus (Grimstad *et al.* 1985), *Culex tritaeniorhynchus* for JE virus (Takahashi 1980), *Ae. hendersoni* for California group viruses (Beaty and Bishop 1988), and *Culex theileri* for Sindbis virus (Jupp 1985). Thus far, SIB or SEB have not been described in *Ae. aegypti* transmission of flaviviruses.

Environmental Factors Contribute to Arbovirus Transmission: Biological barriers to infection can give a reasonable idea of vector competence, but there are many factors other than mosquito physiology that influence whether or not virus will actually be transmitted. These include such things as virus titer, environmental conditions, and larval and adult nutrition. When all these factors are taken into account, the ability to predict transmission potential becomes extremely complicated. These extrinsic, environmental factors are an integral part of determining whether or not a given vector will transmit a given virus at a given time (Hardy 1988).

Many studies have attempted to quantify the role environmental variation plays in vector competence. This is not an easy task, but an essential one in trying to make sense of the the intricacies of vector competence. Even in lab studies where experiments are carefully controlled for environmental variation, it is sometimes difficult to be sure that small variations in larval nutrition, temperature during development, temperature during the extrinsic incubation period, and virus dose, passage history or preparation method

have not affected the results. Bosio (1998) discovered that three different DEN-2 virus preparation protocols gave three different susceptibility results in *Ae. aegypti*.

The extrinsic incubation period (EIP) is the amount of time it takes a vector to be able to transmit a virus after it has ingested an infected blood meal. The length of the EIP is dependent on many things including the vector, virus and several environmental factors. The length of the EIP is generally inversely proportional to the temperature the vector is exposed to once it has ingested an infected meal. In early studies using *Haemagogus capricornii*, the EIP for yellow fever virus went from 28 days when insects were incubated at 25°C to just 12 days when they were incubated at 30°C (Bates and Roca-Garcia 1946). Studies have found similar results in other systems including: *Ae. triseriatus* with EEE virus (Chamberlain and Sudia 1955), *Cx. tritaeniorhynchus* with JE virus (Takahashi 1976), *Cx. tarsalis* with WEE virus (Kramer 1983), *Cx. pipiens* and *Ae. taeniorhynchus* with Rift Valley fever (RVF) virus (Turell *et al.* 1985), *Cx. pipiens quinquefasciatus* with St. Louis encephalitis virus (Hurlbut 1973), and *Ae. aegypti* with yellow fever virus (Davis 1932). The EIP for DEN-2 in *Ae. aegypti* went from 12 days at 30°C to 7 days at 32-35°C (Watts *et al.* 1987). When temperatures fluctuate over the course of the EIP, as one would expect in nature, the EIP approximates the EIP of mosquitoes kept at a constant temperature equal to the average exposure temperature over the course of the experiment (Bates and Roca-Garcia 1946, Chamberlain and Sudia 1955).

Changing the temperature to affect the EIP linearly only works within a given temperature range, which can vary among vectors and viruses (Hardy 1988). Incubation of mosquitoes below a given temperature does not allow virus replication (Hurlbut 1973),

while mosquitoes incubated at too high a temperature also show a decrease in virus titer (Kramer *et al.* 1983).

Larval nutrition also affects vector competence. Nutritionally deprived larvae become small adults. Smaller females (nutritionally deprived as larvae) of *Ae. triseriatus* were shown to transmit LAC virus more efficiently than larger females (well fed larvae) (Grimstad and Haramis 1984). While smaller mosquitoes were found to be more susceptible to infection, it was subsequently revealed that they also have a lower survivorship, making them less likely to survive through the EIP than their larger counterparts (Paulson and Hawley 1991). Thus, results of Grimstad and Haramis (1984) are valid but are likely to be inconsequential to virus transmission. *Culex tritaeniorhynchus* that were nutritionally deprived as larvae exhibited higher titers of Japanese encephalitis virus than their well-fed counterparts (Takahashi 1976). Smaller *Culex tritaeniorhynchus* females were more susceptible to West Nile virus (Baqar *et al.* 1980). One hypothesis is that increased susceptibility might be due to smaller females ingesting larger blood meals to compensate for their nutritional deprivation. It remains be determined, however, if body size is directly responsible for differences in vector competence, or if other factors such as physiological stress due to nutritional deprivation or overcrowding are more important. Interpreting these results is further complicated by studies that contradict these findings. Sumanochitrapon *et al.* (1998) found that larger *Ae. aegypti* females were more susceptible to infection with DEN-2.

Extrapolating these findings concerning vector competence to the field is extremely complicated. Although the majority of studies show that small females are more susceptible to infection with virus, many studies have also shown that larger body

size translates to longer life span. This is the case with *Ae. punctor* (Packer and Corbet 1989), *Ae. aegypti* (Briegel 1990), and *Ae. triseriatis* (Paulson and Hawley 1991), in the laboratory, and for *An. arabiensis* (Ameneshewa and Service 1996) in field populations. When calculating vectorial capacity, daily survivorship has been shown to be more important than susceptibility of the vector population (MacDonald 1973), and thus, any increase in susceptibility to infection from smaller size may be outweighed by an increase in longevity from larger body size.

Another factor that can influence whether mosquitoes become infected or not, is the dose of virus the mosquito ingests in an infectious bloodmeal. Low doses of virus may not be able to overcome the midgut infection threshold, or may increase the amount of time it takes for a productive infection to replicate to sufficient titer for dissemination. In *Haemagogus capricornii*, the EIP was 13 days when a "moderate" dose of virus was ingested, but just 10 days when a higher titer of virus was ingested (Bates and Roca-Garcia 1946). Three days is a large proportion of the vector life span, and can make a significant difference in transmission.

Certainly, there are other ways in which the environment can affect how easily a vector becomes infected with a virus. Adult age (Baqar *et al.* 1980) and photoperiod (Cates and Huang 1969) have both been shown to have an effect on susceptibility. The natural flora and fauna of an arthropod's midgut have also been shown to have an effect in *Anopheles spp* for infection with *Plasmodium falciparum* (Pumpuni *et al.* 1996) and in *Glossina* I for infection with *Trypanosoma spp* (Maudlin and Ellis 1985).

Vector competence for flaviviruses in *Ae. aegypti*: Variation in the ability of *Ae. aegypti* to become infected with and transmit flaviviruses has been well documented.

Many studies have shown that the *Ae. aegypti* range of susceptibility to infection with flaviviruses is, at least in part, genetically determined (Aitken *et al.* 1977, Tabachnick *et al.* 1985, Rosen *et al.* 1985, Vazeille *et al.* 2001, Black *et al.* 2002). There is considerable geographic variation in oral infection rates with yellow fever virus in populations of *Ae. aegypti* from around the world (Tabachnick *et al.* 1985). This susceptibility corresponded with the 8 genetic groupings found using allozyme analysis (Tabachnick and Powell 1979). *Aedes aegypti formosus* populations were least susceptible to infection (7-34% disseminated infection). *Ae. aegypti aegypti* populations from the Caribbean (34-53% DI) and east Africa (29-57% DI) were much more susceptible (Tabachnick *et al.* 1985).

Variation in the ability to transmit a given virus is not unique to *Ae. aegypti* and yellow fever virus. *Aedes albopictus* exhibits considerable polymorphism in its susceptibility to dengue viruses (Boromisa *et al.* 1987, Bosio *et al.* 1992, Gubler and Rosen 1976) and chikungunya (CHIK) virus (Tesh *et al.* 1976). *Cx. tarsalis* exhibits considerable variation in susceptibility to WEE virus (Hardy *et al.* 1976, Hardy *et al.* 1978), and *Culex tritaeniorhynchus* populations vary in their susceptibility for Japanese encephalitis (JE) virus (Takahashi 1980, Takahashi and Suzuki 1979) and West Nile virus (WNV) (Hayes *et al.* 1984). *Aedes triseriatus* varies in its susceptibility to LaCrosse (LAC) virus, and in its rate of transovarial transmission of LAC virus (Grimstad *et al.* 1977, Graham *et al.* 1999), and *Culicoides variipennis* populations varied in their susceptibility to bluetongue virus (Jones and Foster 1978). This was subsequently found to be associated with different species in the *Culicoides variipennis* complex (Tabachnick 1992).

More importantly, in regards to this work, *Ae. aegypti* is extremely polymorphic in its susceptibility to infection with dengue viruses (Gubler *et al.* 1979, Rosen *et al.* 1985, Tardieux *et al.*, 1990, Vazeille-Falcoz *et al.* 1999). Analysis of 13 geographic populations from around the globe showed that populations from the South Pacific and Southeast Asia had a higher susceptibility (9-62% DI) than did populations from East and West Africa (0-12%) (Gubler *et al.* 1979).

Interestingly, variation for flavivirus susceptibility and transmission is not limited to differences among distant populations, but has been documented even within *Ae. aegypti aegypti* and *Ae. aegypti formosus* families (Miller and Mitchell 1991). There were significant differences in DI (0-70%) and transmission (0-33%) of yellow fever virus among individual families within a population of *Ae. aegypti formosus* from Nigeria. Similar results were obtained for families within a population of *Ae. aegypti aegypti* from Puerto Rico (27-100% DI and 0-44% transmission) (Miller and Mitchell 1991). The fact that similar results were obtained when looking at the 4 dengue serotypes, Uganda S and Zika flaviviruses suggested that there may be a generalized genetic basis to flavivirus infection in these mosquitoes.

Despite the fact that *Ae. aegypti* is implicated as the primary vector of dengue viruses, and is associated with almost all urban epidemics, it is actually less susceptible to infection with dengue than many other vectors (Rosen *et al.* 1985). Indeed, a population of *Ae. aegypti* that was relatively resistant to yellow fever virus was found to be the main vector in a major yellow fever epidemic in Nigeria (Miller *et al.* 1989). Thus, vector competence for a virus is not the only factor involved in disease epidemics. There are several hypothesized reasons, then, that *Ae. aegypti* should be the primary vector of

dengue viruses. *Ae. aegypti* may select for dengue strains capable of replicating to a high viremia in humans (Gubler 1988). The high viremia associated with more severe disease may allow faster and more widespread transmission of the virus in urban epidemics. Additionally, the close association *Ae. aegypti* has with humans is likely instrumental in its imporance as the main vector of dengue viruses.

Aedes aegypti behavior may also play an important role in dengue transmission. *Aedes aegypti* females can take several blood meals per gonadotrophic cycle (Scott *et al.* 1993, 2000b), and may even forego other nutritional sources such as sugar when a blood meal is available (Edman *et al.* 1992, VanHandel *et al.* 1994). This dependence on blood, and the frequency with which female mosquitoes take a blood meal, increase *Ae. aegypti*'s potential to become infected or to transmit a virus (Scott *et al.* 1997). In addition, feeding only on human blood and not taking sugar meals may help promote virus transmission by increasing human contact and giving a fitness advantage to the mosquito (Harrington *et al.*2001). This behavior may even allow dengue transmission in areas where *Ae. aegypti* density is quite low (Kuno 1997, Focks *et al.* 2000). Results have shown that *An. gambiae s.l.* and *An. funestus* are also facultative sugar feeders and feed frequently on human hosts, thereby facilitating the transmission of malaria (Beier 1996).

Quantitative Genetics: Quantitative genetics is the study of quantitative traits that are controlled by more than one locus in the genome of an organism. In a population, the distribution of these traits often approximates a normal distribution. These characteristics are also often influenced by environmental factors, such as those described above. There are three categories of quantitative traits. Continuous traits are those for which there is a continuum of possible phenotypes. Within a given range, there is

potential for an infinite number of possible outcome phenotypes. The most common example of a continuous trait is height. Meristic traits are those for which there are discrete integer values, where the phenotype is within a given range and always represented by an integral value. A common example of this is the number of bristles on the abdominal sternites in *Drosophila* (Breese and Mather 1957), or the number of pectin teeth and comb scales on mosquito larvae (Wood 1976). Discrete traits are characteristics that are usually present or absent in an individual. Generally, a threshold must be met by a number of genotypic and environmental factors in order for a given phenotype to occur. An example would be disease susceptibility.

Quantitative traits are of interest in many fields of research. The study of quantitative traits is of extreme economic importance to plant and animal breeders, as many of the characters that determine crop/animal yield are quantitative. This includes such things as milk and egg production, and grain yield (Hartl and Clark 1989). Because of the economic importance that these characters have, plant and animal breeders have often beenthe first to use information gained from quantitative trait studies.

Quantitative genetics provides a means by which to determine how much of the variation in a phenotypic trait (phenotypic variance, V_P) is influenced by genetic (genotypic variance, V_G) and/or environmental (environmental variance, V_E) factors. Additionally, it can reveal how individual alleles contribute to a phenotype of interest. V_G can be further subdivided into additive (V_A), dominance (V_D), and epistatic (V_I) variance. Specific genotypes may even perform differently in different environments (genotype-environment interaction, V_{GxE}). Statistical methods have been developed to analyze all of

these factors in the context of a defined system (Mather and Jinks 1982, Becker 1992, Falconer and Mackay 1996). In general, the formula for genetic variance is:

$$V_G = V_A + V_D + V_I$$

And the formula for phenotypic variance is:

$$V_P = V_A + V_D + V_I + V_E + V_{GxE}$$

An understanding of these variance components is critical in determining heritability and for artificial selection. If variation in a character is due primarily to environmental, dominance, or epistatic effects, it will be a difficult character upon which to impose selection (Houle 1992). Broad sense heritability takes into account all the genetic variance components (V_G), and is not an accurate predictor of actual heritability. A better estimate is narrow-sense heritability, which takes into account only V_A and is calculated as:

$$h^2 = V_A / V_P$$

Heritability is also a function of allele frequencies, and can be expressed as:

$$h^2 = \frac{\Sigma 2pq[a+(q-p)d]^2}{\sigma^2}$$

where p and q are the frequencies of 2 alleles at a given locus, a and d are measures of the additive and dominance effects of the locus, and σ^2 is the variance of the trait in the population (Hartl and Clark 1989). These values are summed over all the loci that affect the character, with each locus having potential for different values of p, q, a, and d. Allele frequencies can also vary over distance and time, which makes heritability a population specific characteristic. The experimental unit should always be the population and not the species when trying to determine h^2.

Mapping Quantitative Trait Loci (QTL): As defined above, quantitative traits are phenotypic characters that are conditioned by more than one gene or set of genes. The approaches above address the collective value of all these genes/gene sets. When taken individually, each of the genes/gene sets that condition the phenotype of interest are considered a genetic locus, and are known as a quantitative trait locus (QTL). The ability to identify a QTL depends on the extent to which it actually affects the variation in the quantitative trait.

Often, the genes that condition a phenotype are not known. The idea of mapping QTL is to calculate the statistical probability that a give phenotypic trait is associated with a given marker or markers on the linkage map. In this way, regions of the genome not associated with the phenotype of interest can be identified, greatly limiting the area in which a candidate gene may be found. QTL mapping identifies genome areas important to the trait of interest when the actual genes are not known. In practice, QTL mapping requires a linkage map with polymorphic markers that cover the entire genome and a quantitative trait that shows variation within or between populations or strains (Falconer and Mackay 1996, Mackay 2001). Ideally, the marker loci are highly polymorphic so lines will likely carry different alleles at each locus, abundant enough to have complete coverage of the genome (for better resolution of the QTL), codominant to identify all possible genotypes at a marker locus, and neutral with respect to fitness and the quantitative trait (Falconer and Mackay 1996).

There are several statistical approaches to associate QTL and marker loci. The simplest is a t-test or a heterogeneity χ^2 test that directly analyzes phenotypes among genotypes at single markers (Soller *et al.* 1976, Darvasi *et al.* 1993). Although easy to

calculate, this method is overly simplistic in its determination of QTL, as it does not take into consideration all the potential interactions, or epistatic effects that might occur. Better methods for analyzing QTL coincided with the ability to create more fine-scale genetic maps based on molecular markers.

Methods for mapping QTL for discrete traits have been proposed (Visscher *et al.* 1996, Xu and Atchley 1996). In fact, most quantitative genetic models can be applied to liability even though liability is distributed as an unobservable continuous quantitative trait. A probit or liability model is used when the assumption is that liability is normally distributed. It uses an algorhithm that assumes an unobservable continuous variable is responsible for expression of the binary phenotype. In using this model for dengue susceptibility, it is assumed that below a certain liability threshold one phenotype is observed (DEN susceptibility), and above that threshold a different phenotype is seen (DEN refractory). Because dengue susceptibility is observed as a binary characteristic, the ability to map QTL depends on the liability model to distribute the character as an unobservable continuous quantitative trait. (Bosio *et al.* 2000). The computer program BINARYQTL (Xu *et al.* 1998) estimates liability using a single linear model with a heterogeneous residual variance and then uses standard interval mapping to determine the most probable location of QTL on the linkage map. This allows for calculation of the sampling variance surrounding each estimated parameter.

Interval mapping (IM) incorporates liability and involves a likelihood ratio test (LRT) at every position within a marker interval (Lander and Botstein 1989, Knapp *et al.* 1990). Intervals are created and tested along the entire genetic linkage map where markers are present. Linkage maps that have more densely spaced markers provide a

finer scale of resolution. Test statistics produce a LRT statistical profile for each chromosome. Intervals with the largest LRT statistics estimate QTL locations. IM is more powerful and requires fewer progeny than methods that directly analyze markers (Lander and Botstein 1989, Haley and Knott 1992, Zeng 1994). Although better than a simple t-test or a heterogeneity χ^2 test, IM is not perfect. IM considers only a single interval at a time. This can bias identification and estimation of a QTL when multiple QTL occur within a single linkage group or when there are epistatic effects among intervals.

To overcome bias due to epistatic effects among marker intervals, two independent research groups began to combine IM with multiple regression analysis (Jansen 1993, Jansen and Stam 1994, Zeng 1993, 1994). Zeng developed compostite interval mapping (CIM) as a means for testing for QTL within an interval while simultaneously using other markers as covariates. Methods have also been proposed for mapping quantitative traits using multiple regression and analysis of variance (ANOVA) (Knapp 1991, Kruglyak and Lander 1995).

Kao *et al.* (1999) described a method for mapping multiple QTL based on general formulas for statistical estimation developed by Kao and Zeng (1997). They termed this method "multiple interval mapping" or MIM. MIM looks at multiple marker intervals at the same time to construct multiple QTL in the model for QTL mapping. This method uses flanking marker genotypes to infer the most likely genotypes within the QTL interval based on recombination rates between the markers. Compared to IM and CIM, MIM is generally more powerful and precise in locating QTL. This is partially due to its ability to reduce genetic variation by taking into account linked QTL. It is also capable of analyzing epistatic QTL and giving individual genotypic values and heritabilities for the

quantitative traits. Additionally, V_G components (V_A, V_D, V_I) can be estimated, and used for marker assisted selection (MAS). MIM has been used successfully to look for QTL conditioning differences in *Drosophila* genitalia (Zeng *et al.* 2000) and *Drosophila* wing shape (Weber *et al.* 2001). A potential problem with MIM is that it requires powerful computers and/or lots of time. The benefits of using this method must be weighed against the computation time for the given system, but this is often worth the extra effort.

A problem common to all QTL mapping systems is definition of the threshold for identifying and precisely locating QTL (Lander and Kruglyak 1995, 1996, Curtis 1996, Witte *et al.* 1996). For most statistical testing, it is normal to reject the null hypothesis (in this case that there is no linkage between phenotype and genotype), if the probability of obtaining the observed results under the null hypothesis is less than a standard threshold. A typical threshold is 5% when a single test is performed. The problem arises when, as in QTL mapping, many tests, often thousands, are performed to look for linkage between a trait and the genome. With a threshold of 5%, it is expected that in 5% of the tests, there will be an observation that is significantly different than the null hypothesis by chance alone. These "false positives" can lead to the identification of false QTL at an unacceptable rate (Cheverud 2001).

Statisticians have suggested several different ways to address this problem. To date, there is still no single method of correction that is universally accepted, although all scientists agree that some method of correction must be applied. The Bonferroni correction (Lander and Botstein 1989, Cheverud 2001), the Davies approximation (Rebai *et al.*1994), and the use of permutation tests (Churchill and Doerge 1994) have all been recommended to determine significance thresholds. Each of these methodologies comes

with its own advantages and disadvantages, and it is left to the individual researcher to decide which methods are most appropriate for the given data set. Too stringent a threshold might ignore important correlations, while too relaxed a threshold could lead to the reporting of false QTL. Developers of the MIM method of QTL identification, have left it to others to determine an appropriate threshold. "Although the MIM method claims more QTL detection than the current methods in data analysis, it is not appropriate to conclude that the MIM method is better until the complicated problems of assessing the appropriate critical value for the multiple-QTL model has been solved" (Kao *et al.* 1999).

Another important consideration when determining threshold values is whether to use a genome wide threshold or to identify specific thresholds for each chromosome. In most cases, thresholds should be set for each chromosome individually. In most interval mapping studies, individual chromosomes differ in linkage length and marker density, both of which affect the number of tests that are performed. As more tests are performed, more false positive correlations are likely, and if there are large differences in chromosome length or marker density, the overall threshold may not be appropriate for the individual chromosome.

When using an F_1 intercross family for QTL mapping, one generally starts with inbred lines with phenotypes at either extreme of a trait of interest. Using inbred lines increases the likelihood that alleles at most loci are homozygous. This crossing design ensures that, even at loci that are heterozygous, there will be a maximum of 4 alleles for a given locus in a given cross. The power of such a cross in linkage mapping and QTL mapping comes from the ability to trace the origin of a given allele back to the inbred line from which it came, and to recognize points at which recombination have occurred. In

QTL mapping, such a cross allows one to associate phenotype in the F_2 generation with alleles originating from inbred lines and inherited from P_1 parents.

Once an F_1 intercross family has been created and phenotyped for the trait of interest, all individuals in the family are genotyped at several markers to determine which alleles they have inherited at each locus. Individuals are placed into treatment groups based on their genotype for each locus (Mackay 2001). These treatment groups are then compared using one of the methods described above. If no QTL is linked to the marker locus, then the phenotypes of the individuals in each treatment group will be equal. If a QTL is associated with a marker locus, the phenotypes of individuals in each of the treatment groups will vary depending on the recombination rate between the marker and the QTL, the additive effect of alleles at the QTL locus, and the degree of dominance between the QTL alleles.

"The number of QTL mapped in any one experiment is always a minimum number" (Mackay 2001). This is due to the fact that one crosses only 2 parent strains, which represent only 4 alleles at any locus and thus a limited sample of the genetic variation that actually exists in a population. Thus, different QTL may be found in different families. The number of QTL found is directly proportional to the effort that is put into finding them. When looking for QTL that have a very low magnitude of effect on the character, the limiting factor is sample size, the larger the sample size, the more QTL of small effect that can be detected. Also, a larger sample size allows more chance for recombination to occur, which, in turn, allows more precise mapping of QTL. This is also dependent upon having a sufficiently dense marker map (Mackay 2001).

In quantitative genetics, it is often assumed that genetic variation for quantitative traits is conditioned by a large number of QTL each with small and equal allelic effect (Falconer and Mackay 1996, Lynch and Walsh 1998). This is known as the infinitesimal model. The idea that there are many loci of small effect conditioning a quantitative trait is not accepted by all, and there is a growing number who believe that the distribution of allelic effects is actually closer to exponential. The exponential model believes that a few loci have large effects and account for most of the variation in the quantitative trait. Additional loci have increasingly smaller effects (Mackay 2001).

QTL mapping is complicated by the fact that the characteristics being mapped are usually affected by environmental conditions, and can also depend on the sex of the organism. Environment and sex specific QTL effects are often due to conditional neutrality. Thus, estimates of QTL position and effect are only relevant to the sex and environment in which the phenotypes were assessed (Mackay 2001). Epistatic effects can also complicate matters and are difficult to identify, because the need to minimize the number of tests in order to control the false positive error rate makes genome scans for epistasis among all markers impractical. Only large epistatic effects are usually detectable (Mackay 2001). Assuming that there are no epistatic interactions among QTL can cause incorrect estimates of main QTL effects.

The concept of linking marker loci to phenotypic characters was first shown successfully by Sax (1923) through crosses between inbred lines of bean plants (*Phaseolus vulgaris*). Since the advent of statistical methods for linking phenotypes with marker loci, QTL have been successfully associated with marker loci in many organisms and for many character traits, especially in agriculturally important organisms. In

tomatoes, for example, QTL have been associated with fruit size (Paterson *et al.* 1988, Frary *et al.* 2000), soluble solids content (Paterson *et al.* 1990), and pH (Paterson *et al.* 1991). In maize, several traits, including grain yield have been associated with QTL (Stuber *et al.* 1992, Graham *et al.* 1997). QTL have been associated with adaptive gene complexes in rice (Ford-Lloyd *et al.* 2001), and have even been used in barley to predict yield variation among recombinant inbred lines (Yin *et al.* 2000).

QTL have been demonstrated in nonagricultural species as well. For example: systolic and diastolic blood pressure in rats (Jacob *et al.* 1991), cognitive ability (McClearn *et al.* 1997) and male sexual orientation in humans (Hamer *et al.* 1993, 1999), floral traits in monkeyflowers (Bradshaw *et al.* 1995), bristle number in *Drosophila* (Long *et al.* 1995), and eyespot size in butterflies (Beldade *et al.* 2002).

Associations have been identified between genotype and phenotype in disease vectors for their susceptibility to infection with a pathogen. Mourya *et al.* (1994) showed linkage between the rosy eye locus on Chromosome *III* in *Ae. aegypti* and refractoriness to CHIK virus. QTL have been discovered for malaria susceptibility in anopheline mosquitoes (Zheng *et al.* 1997) and for susceptibility to avian malaria in *Ae. aegypti* (Severson *et al.* 1995, Kilama and Craig 1969). QTL associated with susceptibility to filarial worms have been mapped in *Ae. aegypti* (Macdonald 1962, Macdonald 1963, Severson *et al.* 1994). In addition, Bosio *et al.* (2000) identified QTL for susceptibility to DEN-2 in *Ae. aegypti*.

It is important to stress that none of these QTL studies could have been performed without the use of high resolution linkage maps. The *An. gambiae* linkage map is based on the integration of microsatellite and RAPD markers (Coluzzi and Sabatini 1967,

Zheng *et al.* 1996, Dimopoulos 1996). A linkage map based on enzyme loci was made for the *Ae. aegypti* genome in the late 1970's (Munstermann and Craig 1979). The resolution of this linkage map has been significantly improved through the use of molecular markers such as RFLPs (Severson *et al.* 1993), RAPDs (Antolin *et al.* 1996), and cDNA markers (Fulton *et al.* 2001, Severson *et al.* 2002).

QTL Mapping in *Aedes aegypti:* As defined above, quantitative traits are phenotypic characteristics that are under the influence of more than one gene or set of genes and are susceptible to environmental factors. Quantitative trait loci (QTL) are discrete locations on the genome that control the trait of interest. The set of QTL along with environmental factors will determine the phenotype. Susceptibility to infection with dengue is a quantitative, threshold trait in *Ae. aegypti*. Molecular, genetic and statistical advances have facilitated the mapping of QTL that condition vector competence of *Ae. aegypti* for dengue viruses.

Susceptibility to dengue viruses seems to be under the control of more than one genetic locus, and is influenced by environmental factors. Bosio *et al.* (1998) performed a quantitative genetic study of *Ae. aegypti* susceptibility to midgut and disseminated infection with DEN-2 to determine how useful quantitative genetics would be in segregating genetic and environmental components of vector competence. A half sib breeding design was used that included strains of *Ae. aegypti aegypti* and *Ae. aegypti formosus*. They found that genes that act additively to control midgut infection accounted for 41% of the total V_P in both subspecies. In *Ae. aegypti formosus*, genes that act dominantly accounted for an additional 9% of V_P. They also found that genes that control disseminated infection (DI) act additively in *Ae. aegypti formosus*, and accounted for

39% of V_P. In *Ae. aegypti aegypti*, genes that act additively accounted for only 14% of the variation in DI while genes that act dominantly accounted for 54% of V_P.

The results of Bosio *et al.* (1998) suggest that there are at least 2 genes or sets of genes that control vector competence for DEN-2 in *Ae. aegypti*. Previous studies also concluded that there must be several genes or gene sets controlling vector competence. Gubler *et al.* (1979) crossed *Ae. aegypti* lines that were susceptible and refractory to DEN-2 and found that the resistance phenotype was dominant. Wallis *et al.* (1985) found that several genes of major effect appeared to control vector competence for flaviviruses in *Ae. aegypti*, and Miller and Mitchell (1991) concluded that there were likely to be two loci of major effect involved in vector competence of *Ae. aegypti* for YF virus.

Bosio *et al.* (2000) then attempted to map QTL that control MIB and MEB for DEN-2 in *Ae. aegypti*. They found that alleles at 2 primary, independently segregating loci created a MIB. One QTL was located on chromosome *II* and the second on chromosome *III*. Alleles at these loci acted additively both within and among independently acting QTL. They were unable to distinguish any QTL for MEB. This was predominantly due to an insufficient sample size for analysis of MEB. Attempts to analyze MEB are complicated because mosquitoes with an MIB cannot be analyzed for MEB phenotype. In order to make statistically powerful conclusions when mapping QTL for MEB, one must have an extremely large sample size and carefully design the F_1 intercross to optimize the number of individuals exhibiting a MEB.

From QTL to Gene of Interest: Finding a QTL is only a starting point in determining the genes that actually condition a quantitative trait. Identifying a QTL serves to limit the search to a given genomic region, but other methods must be

implemented to discover which genes are actually responsible for a given phenotype. *Drosophila* geneticists have designed fairly effective ways of further limiting the genome region encompassed by a QTL, and characterizing the genes within that smaller region. The advantage they have over geneticist studying *Ae. aegypti* is access to the complete sequence of the genome, and with that, better understanding of genes in the QTL region. They also have the advantages of mutant and deletion stocks to work with and transposable elements that effectively and efficiently integrate into the *Drosophila* genome.

One method used by *Drosophila* geneticists to confirm the existence of QTL is to introgress putative QTL into a homozygous genetic background by multiple generations of backcrossing. Deficiency complementation is one technique that has been developed for fine-scale mapping of QTL. This is a technique by which multiple deletion stocks of *Drosophila* with deletions within the QTL region are crossed. The location of the actual QTL is determined by identifying the region of non-overlap between adjacent deficiencies that complement the QTL phenotype with those that fail to complement it (Mackay 2001). Even this technique cannot guarantee that a QTL is represented by a single gene (Graham *et al.*1997, Pasuykova *et al.* 2000).

The genes that affect the trait of interest can also be delineated, using stocks that are deficient in specific gene candidates (Pasuykova *et al.* 2000). Because this is time consuming, it is important to prioritize candidate genes by choosing those that are involved in development, physiology, and expression of the trait when possible. When there are no obvious candidate genes, all possible genetically defined loci must be tested. This method is limited by the availability of mutant stocks.

Drosophila geneticists also use mutagenesis to characterize candidate genes. They have been very successful with single *P* element transposon mutagenesis. Basically, *P* elements are more or less randomly inserted into a genome. Assuming that an insertion inactivates a gene that controls the phenotypes of interest, the gene can be identified in libraries of the mutagenized genome. *P* element insertion can also be used to study epistatic effects by interrupting gene interactions (Mackay 2001).

Identification of quantitative trait nucleotides (QTN) is gaining popularity. One method is linkage disequilibrium mapping, which can be applied to determine the nucleotide polymorphisms that define a QTL allele (Mackay 2001). Linkage disequilibrium is a measure of the correlation of allele frequencies at two polymorphic loci (Falconer and Mackay 1996). A molecular marker locus should be in linkage disequilibrium with a molecular variant that affects a quantitative trait only if they are tightly linked. The length of the genomic fragment surrounding the QTN depends on the amount of recombination per generation, and should become increasingly smaller with each generation.

Integration of Genetic Linkage and Physical Maps: Linkage maps do not give actual physical locations for given loci in the genome. They are a statistical representation of the rate of recombination between markers. In some cases, a single cM (the statistical measure associated with one recombinational event per 100 individuals) may cover a vast amount of genetic material (Mackay 2001). This is especially true in areas of reduced recombination such as telomeres, centromeres, and chromosomal inversions. Using linkage maps alone does not give an accurate depiction of the amount

of genetic material between markers. When this is taken into consideration, identifying a candidate gene from within a given linkage region can become a daunting task.

Physical maps have been generated in many organisms, but this has proven difficult in *Ae. aegypti* due to the lack of polytene chromosomes often used in other species. This difficulty has been overcome through the use of a technique known as fluorescence *in situ* hybridization (FISH). To identify the chromosomes and orient the chromosome arms, landmark probes for repetitive DNA that uniquely marks each chromosome have been developed (Brown and Knudson 1997). Physical maps localize genes on chromosomes by using known gene sequences as probes. Recently, the ability to generate probes based on cDNA, RFLP, and RAPD markers has increased the ability to generate physical maps that correlate to linkage maps.

Physical maps may be more useful than genetic linkage maps in determining the actual size of chromosomes, and how much material is located between markers. Physical and linkage maps have been integrated to test these correlations both in *Ae. aegypti* (Brown *et al.* 2001) and *An. gambiae* (Dimopoulos *et al.* 1996, Zheng *et al.* 1996). In *Ae. aegypti*, the linkage map represents 100% of chromosome *II*. Sixty-five percent of the chromosome *I* linkage map corresponds to only 36% of the short arm of the physical map. Eighty three percent of the chromosome *III* physical map contains the entire genetic linkage map (Brown *et al.* 2001).

The integration of physical and linkage maps is yet another step towards isolating specific genes that condition vector competence, and is one way to limit the number of candidate genes. This can be done through a technique known as map based positional cloning. Positional cloning is a process by which a phenotype of interest is linked to a

genetic map that is anchored to a physical map. Genomic libraries are screened for clones that fall into the defined region and a contig map is constructed. From this, a transcript map is produced, and from this, a gene in the area can be correlated to the phenotype of interest (Brown *et al.* 2001). Based on integration of the physical and linkage maps, Brown *et al.* (2001) estimated that as little as 45% of the *Ae. aegypti* genome is composed of euchromatin and that current genetic linkage maps are highly representative of these euchromatic regions. Although current linkage maps for *Aedes aegypti* have hundreds of markers, each cM still translates to several megabase pairs (Mbp). The *p*-arm of chromosome *I* has an estimated 1.7 Mbp/cM. The *p*-arm of chromosome *II* is resolved to 3.0 Mbp/cM while the *q*-arm is resolved to 2.2 Mbp/cM. For chromosome *III*, the *p*-arm is resolved to 2.8 Mbp/cM and the *q*-arm to 1.4 Mbp/cM (Brown *et al.* 2001). Based on these estimates, there remains a vast amount of genetic material within each linkage interval, and even with sophisticated techniques such as map based positional cloning, distinguishing genes responsible for vector competence within these regions is a daunting task.

Mosquito genomics includes the use of many new and exciting techniques in addition to QTL mapping. This field includes such approaches as genome mapping, whole genome sequencing, expressed sequence tag (EST) projects, and arrays. These approaches should not be seen as competing with QTL mapping but as complementing it. When several techniques are applied to the same question, the combined results often lead to a significantly improved understanding of the system.

The *Anopheles gambiae* genome project released its annotated sequence in the spring of 2002 (Aultman 2002, Holt *et al.* 2002). This will no doubt reveal candidate

genes in QTL regions associated with malaria transmission, as well as providing insight into the anopheline genome. This also paves the way for the sequencing of the *Ae. aegypti* genome (Atkinson and Michel 2002). This is a much greater task, as the *Ae. aegypti* genome is approximately 800 megabases, almost three times the size of the *An. gambiae* genome (Knudson *et al.* 1996).

Whole genome projects and the sequence identification of genes by EST have facilitated the use of transcriptional profiling through microarray techniques (Dimopoulos *et al.* 2000). This will allow the identification of cDNAs that are differentially expressed in specific groups of mosquitoes. For example, comparisons can be made between infected and uninfected mosquitoes, or mosquitoes in different stages of infection (Atkinson and Michel 2002). This technology has already been used for analysis of different developmental stages of *Plasmodium falciparum* in its vertebrate host (Hayward *et al.* 2000).

Marker Assisted Selection (MAS): Marker assisted selection (MAS) is a powerful tool that can be used to create organismal lines with desired phenotypes. MAS is much more powerful and efficient than traditional forms of selection, because it selects on genotype as well as on phenotype (Lande and Thompson 1990). Because selection is traditionally based on phenotype alone, it is subject to environmental factors that can influence a quantitative trait. MAS decreases the amount of environmental trait variation by selecting individual organisms based on markers that have been associated with the trait of interest. This allows selection to be based on genetic variation, and leads to stronger selection potential. As described previously, only V_G is heritable. By eliminating

non-heritable variation, the selection process becomes much more directed and thus more efficient and effective.

MAS has been and will continue to be of particular importance in agriculture, where it has been used quite extensively. For example, MAS has been used to increase yield in barley (Romagosa *et al.* 1999), and potato breeders are trying to use MAS to introgress virus resistance genes from wild potato species into species that have better agronomic characteristics (Solomon-Blackburn and Barker 2001).

Not all characters are ideal for MAS. Traits that are predominantly determined by environmental factors are not good candidates for MAS, or for any selection system. MAS also relies on the quality of the QTL maps that are generated. The more fine scale the QTL map, and the more closely associated the given markers are to the actual genes controlling the trait, the better the response to selection. Ideally, selection will be on the actual markers that condition the trait.

Although the use of MAS to introgress desirable genes is a major step forward, perhaps even more important is the potential to simultaneously eliminate deleterious alleles from the selected strain. Traditional forms of selection have often failed due to deleterious alleles that tend to be expressed in highly selected and often inbred lines. MAS allows for the detection of alleles that detract from the fitness of the selected line. These alleles can be selected against during the selection process, and eliminated as potential fitness hazards that may ultimately eliminate the selected strains.

The purpose of this dissertation is to determine genetic factors that condition variation in midgut and disseminated infection of dengue viruses in *Ae. aegypti*. The specific objectives of this dissertation are multiple. Integral to this work was the use of

field collected mosquitoes to define variation in vector competence among proximal natural populations. The second chapter focuses on determining natural variation in MIB MEB and VC for DEN-2 in collections of *Ae. aegypti* from throughout Mexico. The use of lab adapted strains to introgress genes from these natural populations was important in the development of selected strains. The third chapter focuses on the selection of mosquitoes with desired DEN susceptibility phenotype using lab adapted and field collected mosquitoes. These selected strains were essential in designing F_1 intercross families in which QTL for MEB could be mapped. The fourth chapter focuses on QTL mapping of MEB in *Ae. aegypti*.

Specific Aims:

1. Determine whether variation in MIB, MEB and VC occurs in natural populations of *Ae. aegypti* in Mexico. The hypothesis was that collections from Mexico would exhibit variation in susceptibility phenotype based on the three geographic areas defined by Gorrochotegui-Escalante *et al.* (2002). The null hypothesis was that there would be no variation in vector competence across Mexico (Chapter 2);
2. QTL mapping and selection of lines of mosquitoes with desired DEN susceptibility phenotypes. These included a line highly susceptible to infection with DEN-2 (designated *D2S3*) and a line with no MIB, but with a MEB (designated *D2MEB*). These lines were tested with other dengue serotypes and genotypes to determine if there is generalized genetic determination for dengue susceptibility or whether the genetics is serotype or genotype specific. The hypothesis was that these selected strains would maintain a consistent phenotype across dengue genotypes and serotypes, indicating the presence of general genetic factors responsible for

dengue, and perhaps even flavivirus susceptibility. The null hypothesis was that there would be no similarity in susceptibility phenotype across genotypes and serotypes (Chapter 3);

3. Cross these selected strains in order to map QTL associated with MEB for DEN-2 in *Ae. aegypti.* The hypothesis was that by using highly selected lines, an F_1 intercross designed for mapping MEB and a sufficient sample size, statistically significant QTL could be identified. The null hypothesis was that there are no correlations between genotype and MEB, and that even with increased sample size and selected lines, QTL would not be identified (Chapter 4).

CHAPTER 2

VARIATION IN VECTOR COMPETENCE FOR DEN-2 VIRUS AMONG 24 COLLECTIONS OF *AEDES AEGYPTI* FROM MEXICO AND THE UNITED STATES

Introduction

Dengue is a resurging disease with approximately 50-100 million cases of classical dengue fever (DF) and 500,000 cases of the more severe dengue hemorrhagic fever (DHF) occurring annually (Monath 1994, Gubler 1998). Between 1981 and 1995, there was a 4 fold increase in the incidence of DF as compared to the total incidence in the preceding 30 years (Gubler 1998). Dengue is normally thought of as a tropical disease, but in recent years, has been diagnosed in temperate regions such as the southern United States and northern Mexico (Pena *et al.* 1999).

Dengue viruses (genus *Flavivirus*, Family *Flaviviridae*) have a single stranded positive sense RNA genome (Rice 1996, Burke and Monath 2001). There are four antigenically distinct dengue virus serotypes. Infection with one serotype confers life-long immunity to infection with the homologous, but not heterologous serotypes. DHF occurs most commonly in patients suffering a secondary infection with a dengue serotype heterologous to the primary infection, suggesting a potential for immune enhancement (Halstead 1982, Halstead 1988). However, DHF has been documented in patients experiencing a primary infection, suggesting that certain viruses may be more virulent and capable of causing DHF without immune enhancement (Gubler 1998, Rico-Hesse *et*

*al.*1997). Host factors such as age, immune status and sex may also contribute to disease outcome (Monath and Heinz 1996, Burke and Monath 2001).

The primary vector of dengue is *Aedes aegypti*, a synanthropic, container-breeding mosquito that has adapted well to cohabitation with humans. *Ae. aegypti* has a global distribution in tropical and subtropical areas. Because of its public health importance in vectoring dengue and yellow fever flaviviruses, *Ae. aegypti* has been the subject of numerous vector competence and population genetic studies (Aiken *et al.* 1977, Tabachnick *et al.* 1985, Rosen *et al.* 1985, Gubler *et al.* 1979, Tabachnick and Powell 1979, Tardieux *et al.* 1990, Miller and Mitchell 1991, Apostol *et al.* 1996, Bosio *et al.* 1998, Bazeille-Falcoz *et al.* 1999, Gorrochotegui-Escalante *et al.* 2000, Bosio *et al.* 2000).

Vector competence (VC) is defined as the intrinsic permissiveness of a vector to infection, replication, and transmission of a virus (Hardy 1988). VC for arboviruses is associated with a number of anatomic barriers to productive vector infection. These include a midgut infection barrier (MIB), midgut escape barrier (MEB), and salivary gland barrier (Woodring *et al.* 1996). In potential vectors with a MIB, virus cannot infect and/or replicate in the mosquito midgut cells. This may be due to a lack of cell surface receptors for the virus or to midgut cells being nonpermissive for infection with the virus. Potential vectors with a MEB may allow virus replication in the midgut, even to high titers, but virus is then unable to exit the midgut to cause a disseminated infection. Barriers to infection can vary widely among *Ae. aegypti* populations, causing VC for dengue viruses to be variable as well. This, in part, may determine the epidemiology of dengue viruses.

Ae. aegypti populations exhibit considerable genetic variability in VC for flaviviruses (Aitken *et al.* 1977, Tabachnick *et al.* 1985, Rosen *et al.* 1989, Tabachnick and Powell 1979), including DEN-2 viruses (Rosen *et al.* 1989, Gubler *et al.* 1979, Tardieux *et al.* 1990, Bosio *et al.* 1998, 2000). VC for flaviviruses in *Ae. aegypti* is thought to be controlled by at least two genes or sets of genes; one controlling the MIB and the other controlling the MEB (Miller and Mitchell 1991, Bosio *et al.* 1998, 2000).

Many studies have focused on determining genetic differences in mosquitoes from a wide geographic distribution. More recently, significant genetic variation in *Ae. aegypti* on a smaller scale has been demonstrated in Puerto Rico (Apostol *et al.*, 1996) and in Mexico (Gorrochoetegui Esalante *et al.* 2000). In this study, we address potential variation in VC on a regional geographic scale in Mexico. The ultimate goal of this research is to determine if genetic variability in *Ae. aegypti* populations conditions the incidence and severity of DF and DHF outbreaks. If mosquito populations that condition more severe disease are found, there is potential for developing genetic biomarkers for populations that pose undue risk for severe dengue disease. This would allow dengue control programs to focus limited control resources on areas at the greatest risk for severe disease.

Materials and Methods

Mosquito Collections: Mosquitoes were collected as eggs from sites in Mexico and the USA (Figure 2.1). Table 2.1 lists collection sites, mosquito generations challenged orally with dengue-2 virus JAM1409 (DEN-2 JAM1409), the number of repetitions, and the sample size range and mean for each collection. Field collected eggs were hatched to begin colonies. Mosquitoes were raised at a constant temperature of

Table 2.1: *Aedes aegypti* collections assayed for VC. Mosquito strain designation, collection site, colony generations challenged, number of infected heads per total number tested (VC), number of repetitions, the sample size range and the mean sample size for each repetition. At least 60 mosquitoes were assayed for phenotype in all mosquito collections except Guaymas, Hermosillo and Coyuca de Benitez. All collections except Ciudad del Carmen and Campeche were challenged more than once. All mosquitoes challenged were less than 3 generations in the lab.

Country/State of Collection	City of Collection	Generations Used	# Head Infected/ # Tested	# Repetitions	Sample Size Range	Mean Sample Size
PR	Lab Colony	Unknown	460/568	12	18-63	47
Sonora	Hermosillo	F1	25/55	2	14-41	28
	Guaymas	F1	24/39	2	17-22	20
Sinaloa	Culiacan	F1,F2,F3	119/151	4	15-60	38
	Mazatlan	F1	54/84	5	10-25	17
Jalisco	Puerto Vallarta	F1	24/80	2	18-62	40
Colima	Manzanillo	F1	66/117	3	26-63	39
Michoacan	Lazaro Cardenas	F1	48/105	2	43-62	53
Guerrero	Ixtapa Zihuatanejo	F1	16/68	2	25-43	34
	Coyuca de Benitez	F1	33/45	3	10-19	15
Oaxaca	Puerto Escondido	F1,F2	89/148	4	15-63	37
Chiapas	Tapachula I	F1,F2	78/108	2	47-61	54
	Tapachula II	F2,F3	50/100	4	12-37	25
Quintana Roo	Chetumal	F1,F2	90/109	2	49-60	55
	Cancun	F1,F2	100/139	3	19-60	46
Yucatan	Merida	F1,F2	101/149	3	44-60	50
Campeche	Campeche	F1	26/60	1	60	60
	Ciudad del Carmen	F1	26/60	1	60	60
Tabasco	Villahermosa	F1,F2,F3	85/175	4	17-60	44
Veracruz	Moloacan	F1,F2	70/123	3	20-60	41
Tamaulipas	Miguel Aleman	F1,F2	117/196	4	20-60	49
	Nuevo Laredo	F1,F2	82/171	3	48-63	57
Nuevo Leon	Monterrey	F1,F2	69/121	3	19-60	40
USA/Texas	Houston	F1,F2	39/100	2	40-60	50
USA/Arizona	Tucson	F1,F2	64/99	2	39-60	50

Figure 2.1: Collection sites of *Aedes aegypti* used in this study.

27°C and 80% relative humidity in an insectary with a 12 hour photoperiod. All experiments were performed on mosquitoes of less than 3 generations in the laboratory in an attempt to avoid effects of colonization and inbreeding on vector competence (Lorenz *et al.* 1984). A laboratory colony of *Ae. aegypti aegypti* from Puerto Rico (PR) provided a highly susceptible mosquito control, (Bosio *et al.* 1998) which was used to monitor for consistency in the oral challenges.

Virus: DEN-2 JAM1409 virus, which was isolated in 1983 in Jamaica (Deubel *et al.* 1986), and had subsequently been passaged in C6/36 cells, was used in the studies. To prepare stock virus, DEN-2 JAM1409 was amplified in C6/36 cells. A 150cm^2 flask of confluent C6/36 cells was infected at a multiplicity of infection (MOI) of 1.5 plaque forming units (PFU) per cell. Cells were incubated for 14 days at 28°C in L15 medium supplemented with 2% heat inactivated fetal bovine serum (FBS), penicillin (0.01 mg/ml), streptomycin (0.01 mg/ml) and L-glutamine (0.01 mg/ml). Virus was harvested by scraping cells into the cell culture medium and placed in 0.5 ml aliquots, which were stored at -70°C. For *per os* challenges, 0.5ml aliquots were used to infect 75cm^2 flasks of confluent C6/36 cells at an MOI of 1.5. Infected cells were incubated for 14 days at 28°C in L15 medium as above, and the medium was changed on day 7. Virus and cells were harvested by scraping with a cell scraper, and medium, virus and cells were collected in a 50ml conical centrifuge tube. The virus suspension was mixed 1:1 with defibrinated sheep blood and placed in membrane feeders covered with mouse skin or parafilm membranes (Higgs and Beaty, 1996). Pre- and post-blood meal virus titers were determined by inoculating serial 10-fold dilutions of the respective meal onto C6/36 cells in the wells of a 96 well plate. Cells were assayed by immunofluorescence, and 50%

tissue culture infectious dose ($TCID_{50}$) titers were calculated using the Karber formula (Schoepp *et al.* 1990). Virus titers ranged from $10^{7.5}$ - $10^{8.5}$ $TCID_{50}$/ml in all oral challenges. Titers ranged from $10^{5.8}$ – $10^{7.7}$ $TCID_{50}$/ml in the dose response studies.

***Per os* Challenges**: For mosquito infection studies, 100-150 mosquitoes/1pint carton were sucrose-starved and water deprived 24 hours prior to blood feeding. Blood meals were maintained at a constant 37°C. Mosquitoes were allowed 45 min to 1 h to feed. Fully engorged mosquitoes were selected and held for 14 days at a constant temperature of 27°C and 80% relative humidity in an insectary with a 12 hour photoperiod.

Studies were conducted to determine the effect of viral dose on mosquito VC for certain collections. Sequential ten fold dilutions of virus were used to prepare infectious blood meals: 10^{-0}, 10^{-1}, and 10^{-2}. Fully engorged mosquitoes were selected and held for 14 days at a constant temperature of 27°C and 80% relative humidity in an insectary with a 12 hour photoperiod. Pre-blood meal and post-blood meal titers for each of the dilutions were determined as previously described (Schoepp *et al.* 1990).

Vector competence: Mosquitoes were phenotyped for VC using immunofluorescence at 14 days post oral challenge. Mosquito heads were severed, squashed onto acid washed slides, acetone fixed, and assayed for dengue virus antigen by indirect immunoflourescence. Slides were incubated for 40 minutes at 37°C with a mouse derived primary monoclonal antibody (86.13) directed against a flavivirus E gene epitope (Gould *et al.* 1985) diluted 1/200 in phosphate buffered saline (PBS). Slides were then washed 2 times in PBS. A mixture of biotinylated sheep anti mouse antibody (Amersham) and Evans blue (1%) as a counterstain was made at a dilution of 1/200 in

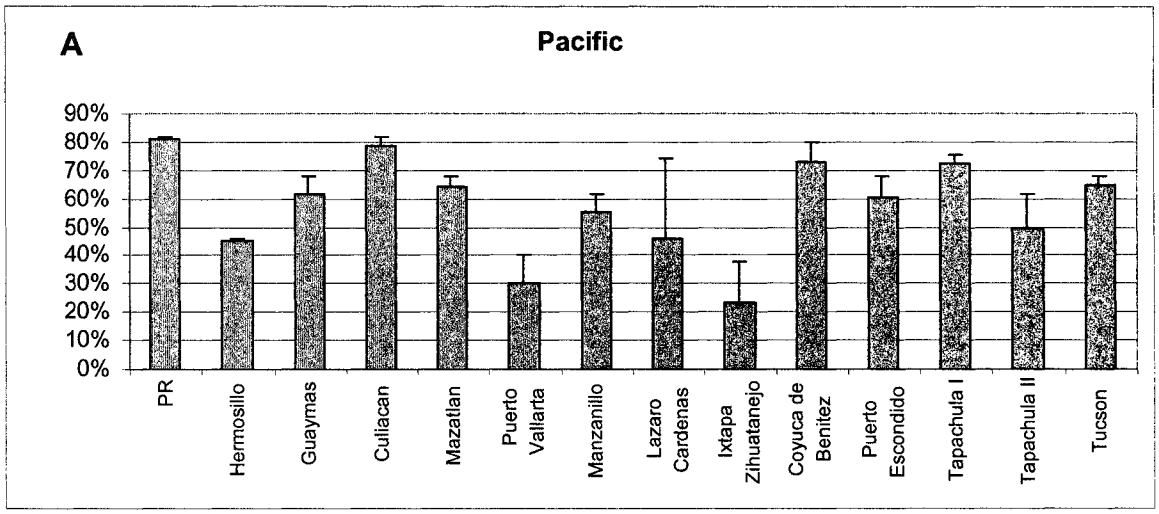
PBS. Slides were incubated for 40 minutes at 37°C followed by 2 washes in PBS. Slides were then incubated 10 minutes with streptavidin fluorescein (Amersham) diluted 1/200 in PBS to detect the primary-secondary antibody complexes. Slides were washed twice in PBS and once in water. Glycerin containing diazobicyclo(2,2,2)octane (DABCO Sigma D-2522) was applied, and a coverslip placed on each slide. Heads were examined at 200x using an Olympus BH2-RFCA microscope. If virus antigen was not detected in head tissues, the respective abdomens, which were stored at -70°C, were subsequently assayed by immunofluorescence for virus antigen.

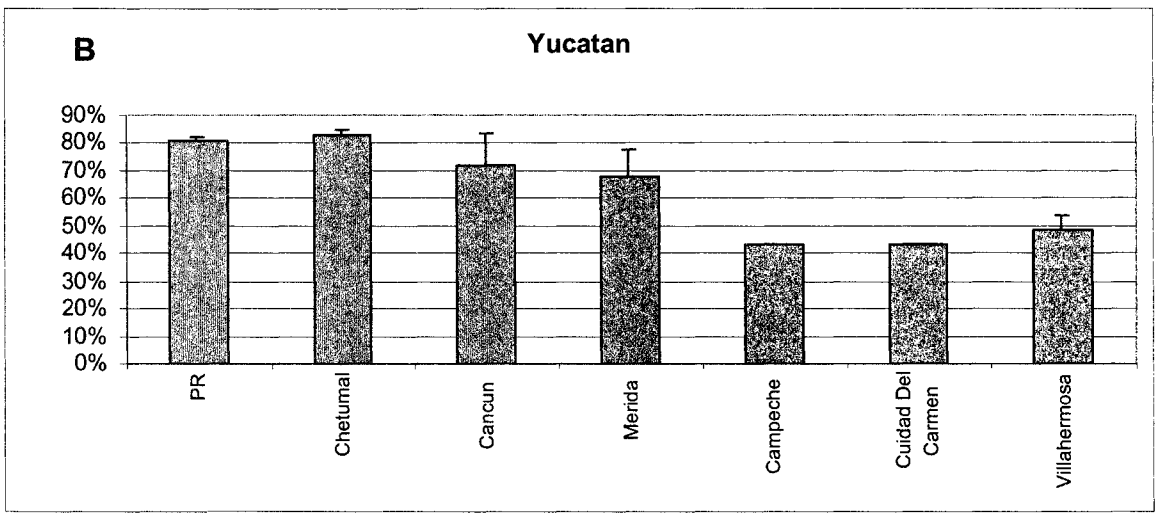
Detection of virus antigen in midgut tissue revealed a midgut infection (MI) and the MIR was calculated as the number of positive midguts divided by the number of mosquitoes exposed. Detection of virus antigen in head tissue revealed a disseminated infection (DI), and the DIR was calculated as the number of mosquitoes with virus antigen in the head tissue divided by the number of mosquitoes with virus antigen in the midgut. VC was calculated as the number of mosquitoes with DI divided by the number of mosquitoes exposed. Mosquitoes were not assayed for salivary gland infection or for actual virus transmission; rather VC was assumed to be the same as the rate of virus dissemination to the head tissues. Once mosquitoes were phenotyped for MI and DI, the percent mosquitoes exhibiting a MIB was calculated as 1-MIR. The percentage of mosquitoes with a MEB was calculated as the MIR - DIR.

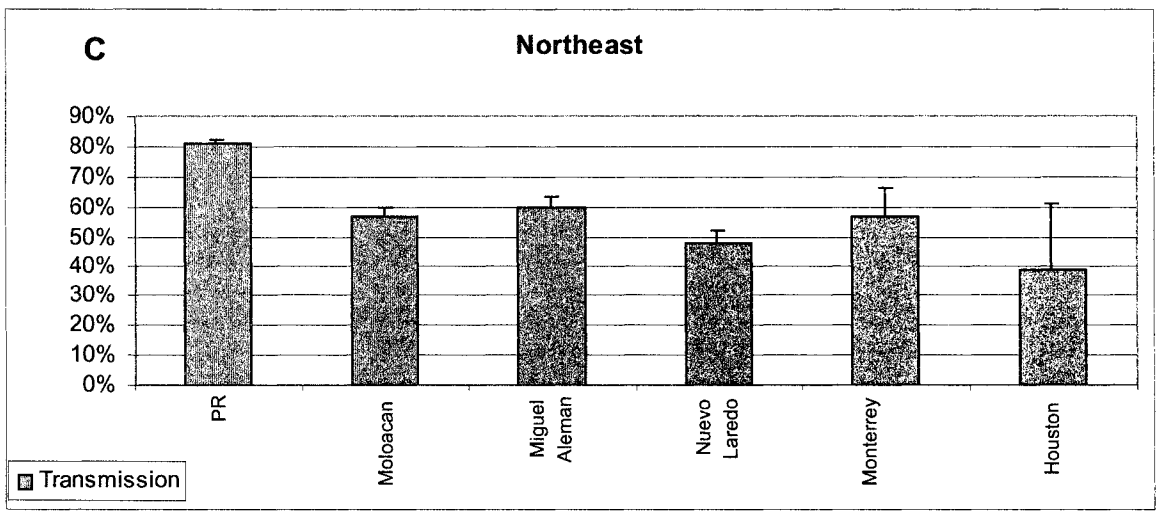
Results

Variation in VC: The VC of mosquitoes from the 24 collections sampled are shown in Figure 2.2. These populations were previously grouped geographically as Pacific, Yucatan or Northeast by Gorrochotegui-Escalante *et al.* (2001).

Figure 2.2: The VC of *Ae. aegypti* collections for DEN-2 JAM1409 virus expressed as the percentage of the total number of individuals tested that displayed disseminated infection. Collections are arranged according to geographic areas designated by Gorrochotegui *et al.* 2002. These include collections from the Pacific coast (A), collections from the Yucatan Peninsula (B), and collections from the Northeast of Mexico (C). Error bars indicate standard error of challenge experiments. Where there are no error bars, collections were challenged only once (Campeche and Ciudad del Carmen) or there was no difference in VC among challenges. Table 2.1 contains information on the number of challenges for each collection.







Error bars indicate the standard error among replicates of oral challenges for the respective collection. All results are based on a sample size of greater than 60 mosquitoes per collection, with the exception of collections from Guaymas, Hermosillo, and Coyuca de Benitez, and most are based on composite results of more than one oral challenge (Table 2.1). Oral infection rates were consistent between challenges. The VC for the PR strain ranged from 72 to 96%, and did not differ significantly by χ^2 analyses ($p < 0.05$). The VC ranged from 24% in the Ixtapa Zihuatenejo collection to 83% in the Chetumal collection. Interestingly, the mosquito populations from the Yucatan exhibited a greater overall VC (62%) than mosquito populations from the Northeast (53%) and the Pacific (57%) (Figure 2.2) This difference was significant ($p < 0.005$). The VC of the Yucatan populations appeared less variable than that of the other geographic collections. The Yucatan collections clustered into two groups, which differed significantly in VC by χ^2 analysis ($p<0.001$); 73% (291/397) of mosquitoes from Quintana Roo and Yucatan states and 46% (137/295) of mosquitoes from Campeche and Tabasco states were capable of transmitting virus.

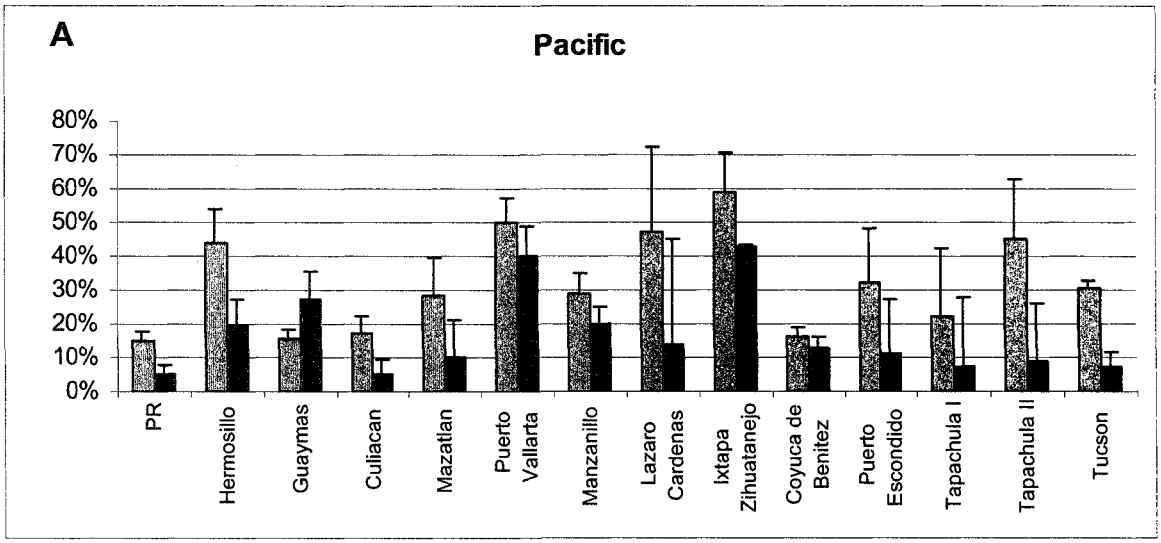
VC was more variable in the Northeastern and Pacific collections, and significant differences in VC were detected in geographically proximal collections. For example, the VC of mosquitoes from Coyuca de Benitez was 73%, while mosquitoes from Ixtapa Zihuatanejo had a VC of 24% (Figure 2.2). These rates differed significantly by χ^2 analysis ($p<0.01$) despite the fact that the two collections are proximally close and in the same state (Figure 2.1). Thus, geographic location was not necessarily correlated with VC.

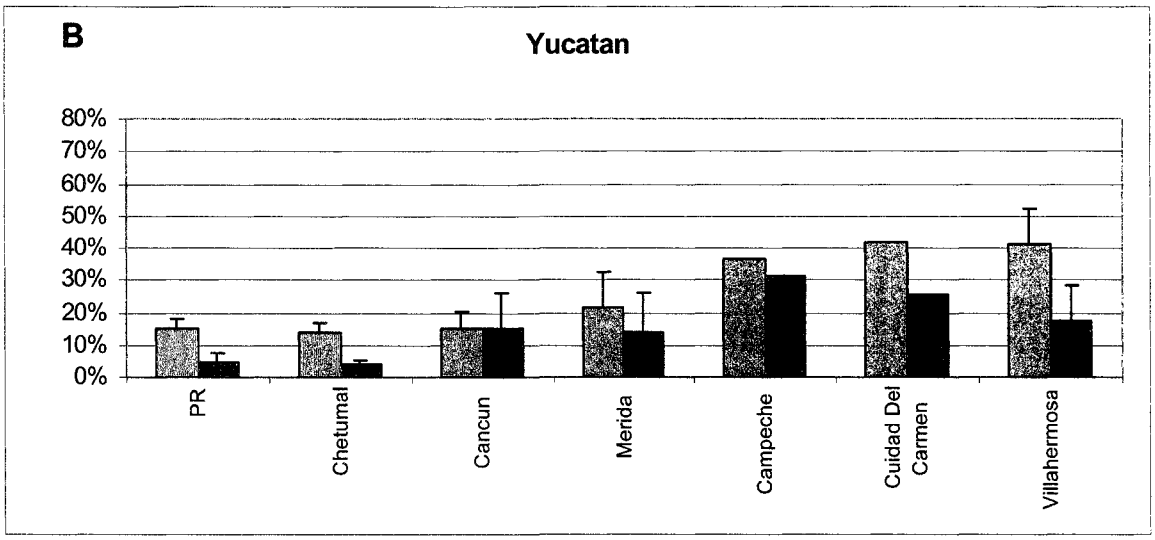
Anatomic basis of transmission potential: To determine anatomic barriers that condition transmission potential, mosquitoes from the 24 experimental *Ae. aegypti* collections and the PR (susceptible) control were phenotyped for the presence of MIB and MEB. The collections exhibited a wide range of expression in MIB and MEB for DEN-2 JAM1409 (Figure 2.3). The Ixtapa Zihuatanejo mosquitoes exhibited the largest MIB (59%) as well as the largest MEB (43%). Mosquitoes from Chetumal had the lowest MIB (14%) and the lowest MEB (4%).

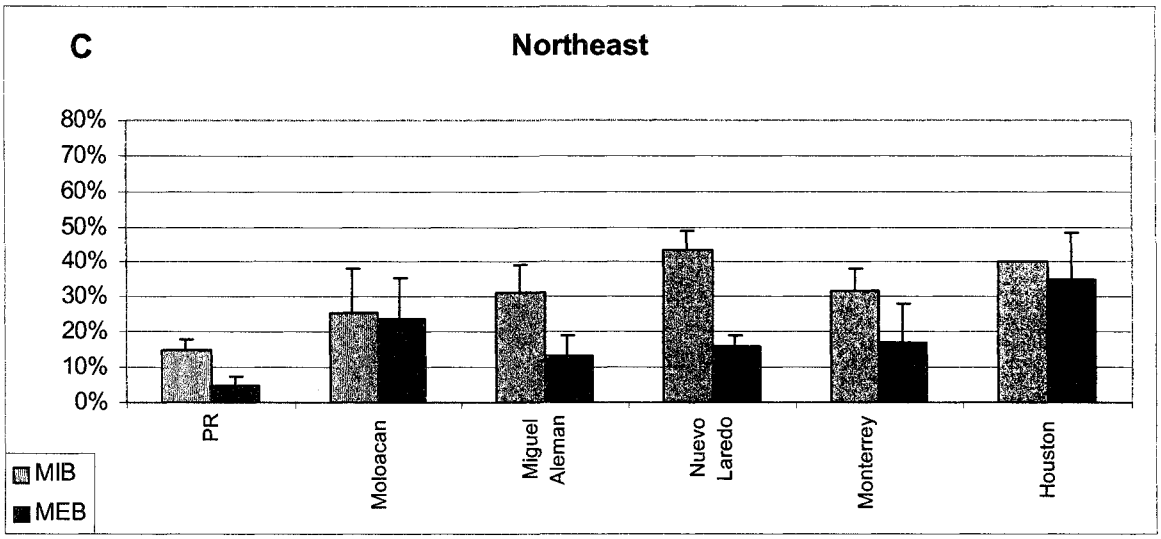
Correlation between MIB and MEB: Figure 2.4 is a plot of the percentage of mosquitoes exhibiting a MIB versus those exhibiting a MEB. This was performed to determine whether MIB and MEB are completely independent of one another or whether there is a correlation between them. Regression analysis gives r values for the different geographic groupings that indicate a weak correlation between MIB and MEB. This correlation, however, is only significant by Pearson's χ^2 goodness of fit test in the Pacific grouping ($p<0.05$) and for all sites grouped together ($p<0.01$).

Dose response: The effect of virus dose on MIR was determined for selected mosquito collections (Figure 2.5). MIR were directly correlated with virus dose. In each collection, infection rates increased as virus titer increased. However, the dose response was not consistent among collections - some infection rates decreased rapidly with lowered virus titer. The Chetumal collection exhibited a gradual decrease in MIR (90%, 88%, 61%), as did Tucson (67%, 50%, 36%). In contrast, the Nuevo Laredo MIR (60%, 27%, 14%) and Merida MIR (57%, 27%, 14%) rapidly decreased. DIR were not correlated to virus dose in infectious feeds (data not shown). The undiluted virus that was used to challenge mosquito collections from Miguel Aleman and Nuevo Laredo had a

Figure 2.3: The percentage of mosquitoes tested that exhibit a MIB and MEB for each collection. Collections are arranged according to geographic areas designated by Gorrochotegui *et al.* 2002. These include collections from the Pacific coast (A), collections from the Yucatan Peninsula (B), and collections from the Northeast of Mexico (C). Error bars indicate standard error of challenge experiments. Where there are no error bars, collections were challenged only once (Campeche and Ciudad del Carmen) or there was no difference in VC among challenges. Table 2.1 contains information on the number of challenges for each collection.







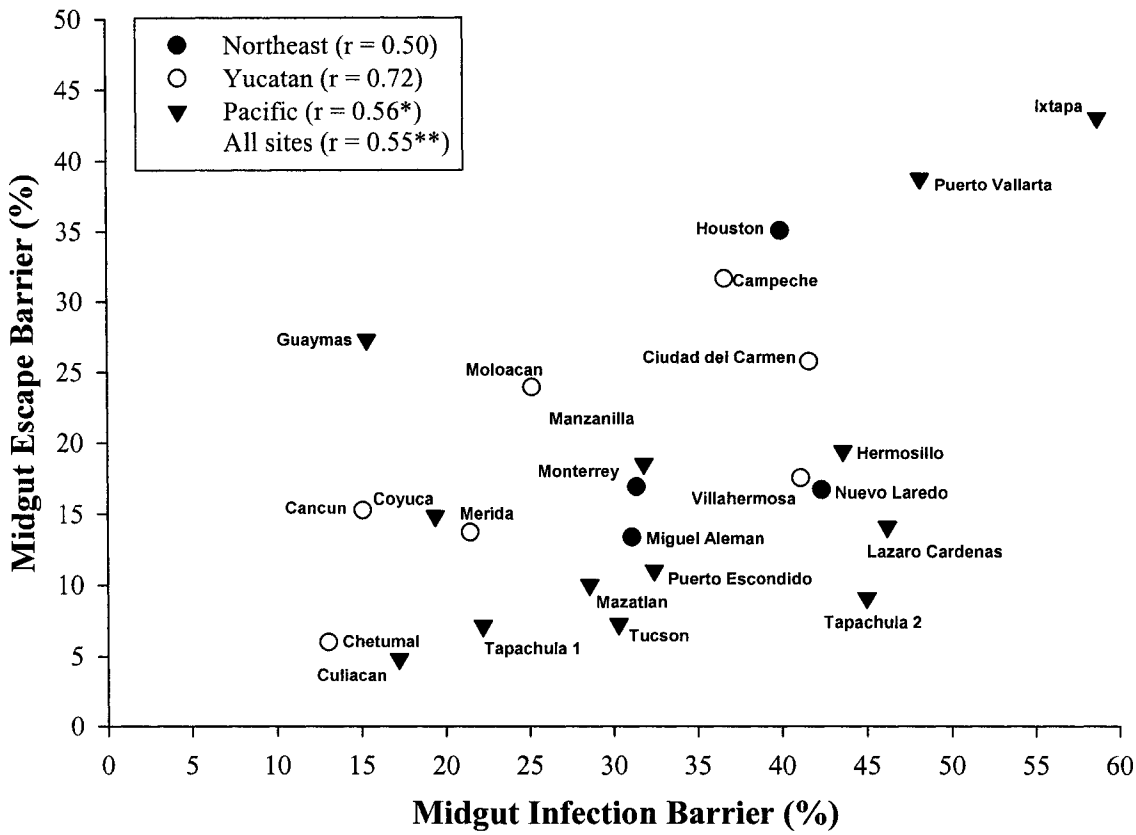


Figure 2.4: Regression analysis of MIB vs MEB. Collections were plotted based on the percentage of individuals exhibiting a MIB or MEB. Regression analysis was performed for all collections within the designated geographic locations as well as for all collections combined. One asterisk denotes a significance value of $p<0.05$ and two asterisks denotes a significance value of $p<0.01$ using Pearson's χ^2 goodness of fit test.

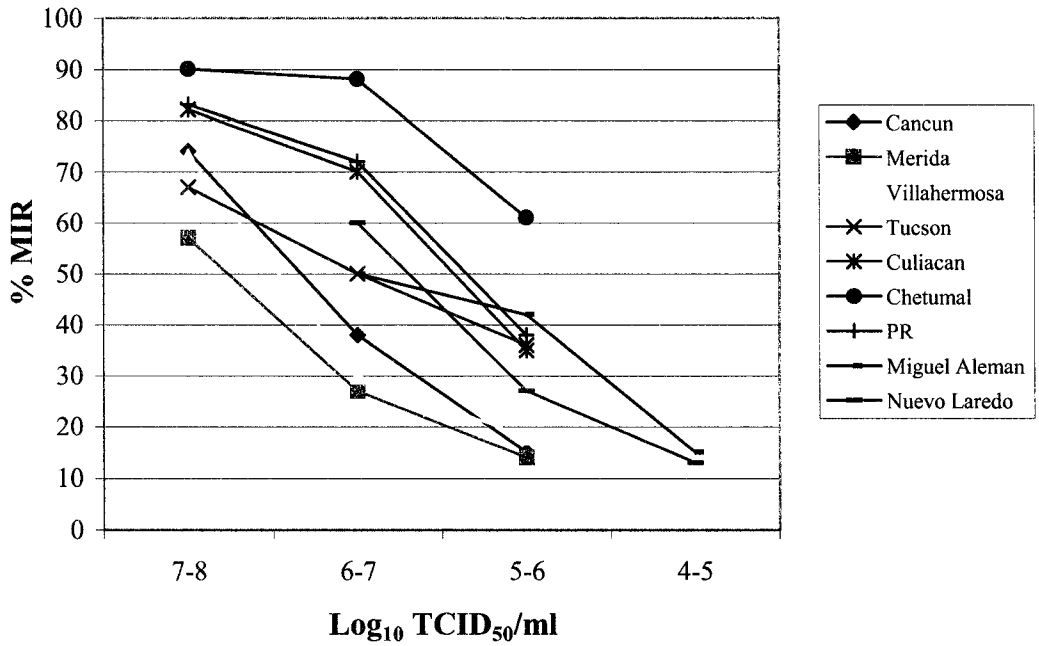


Figure 2.5: Mosquito midgut infection rates with DEN-2 virus are dose dependent. Mosquitoes were exposed to serial 10-fold dilutions of DEN-2 JAM1409 virus in blood meals, and subsequently assayed for midgut infection. The X-axis denotes virus titers in $\log_{10}$ $TCID_{50}$

lower titer than that used for the other challenges. For this reason, Figure 2.5 shows infection rates based on titer of virus in the infectious blood meal rather than based on the dilution factor.

Discussion

In general, the *Ae. aegypti* collections from throughout Mexico exhibited considerable variability in VC, and collections from the Yucatan Peninsula were generally more vector competent than those from other geographic regions (Figure 2.2). Interestingly, collections from the Eastern Yucatan Peninsula exhibited greater VC than those from the Western Yucatan Peninsula. The anatomic and genetic determinants of these phenotypic differences are being investigated. *Ae. aegypti* collections from throughout Mexico differ significantly in their midgut susceptibility to infection with DEN-2 JAM1409 (Figure 2.3). Mosquito collections exhibited MIB that range from 59% in the Ixtapa Zihuatanejo collection to 14% in the Chetumal collection, MEB that range from 43% in the Ixtapa Zihuatanejo collection to 4% in the Chetumal collection, and VCs that range from 24% in the Ixtapa Zihuatanejo collection to 83% in the Chetumal collection (Figure 2.2). In general the Eastern Yucatan Peninsula collections, which exhibited high VC, had low MIB and generally low MEB.

Results shown in Figure 2.3 seem to suggest that VC in the majority of these collections is primarily conditioned by a MIB. This may be due in part to the fact that those mosquitoes with a MIB cannot be tested for the presence of a MEB, as there is no midgut infection. Mosquito collections from Chetumal and Culiacan have neither a strong MIB nor a strong MEB. Extremely high VC in both of these collections is evidence for this. Ixtapa Zihuatanejo had a strong MIB and MEB, and its VC is significantly lower

than that of Chetumal and Culiacan ($p<0.001$). Overall, the VC can be thought of as being inversely proportional to the level of MIB and MEB found in the mosquito collection: strong MIB and MEB reduce the potential for transmission.

The perceived range of susceptibilities suggests that the ability to overcome the MIB and MEB in order to transmit DEN-2 JAM1409 is a quantitative trait with multiple genes that likely condition VC and collectively determine the infection rates of mosquito populations. This concept is not new, and has been studied using crosses of susceptible and refractory mosquito lines (Miller and Mitchell 1991, Bosio *et al.* 1998).

It is interesting that the geographic origin of the *Ae. aegypti* collections is not necessarily indicative of infection rates (Figure 2.2). Certain collections in close proximity do not exhibit similar infection rates. This can be seen in collections from Ixtapa Zihuatanejo and Coyuca de Benitez, which are geographically close but have significantly different MIB, MEB and VC ($p<0.01$). A detailed genetic analysis is needed to determine if these collections are actually isolated and constitute independent populations which differ in VC. Some areas exhibit similar infection rates. Collections from Villahermosa, Ciudad del Carmen, and Campeche are located near one another and do not differ in MIB, MEB, and VC. To determine whether these are distinct populations, an analysis of gene flow similar to that discussed below should be completed on these collections.

Patterns of gene flow in *Ae. aegypti* populations could influence infection rates of proximally close populations. Gorrochotegui-Escalante *et al.* (2002) recently showed that gene flow among *Ae. aegypti* populations in Mexico varied by region as determined by the mitochondrial gene ND4. They found high rates of gene flow in populations from the

Pacific coast. These populations also had a large genetic diversity. Populations from the Yucatan Peninsula had variable genetic diversity. Many of the mosquito collections used were the same as those characterized for VC in this study, however, there does not seem to be an overall correlation between the mosquito phylogenies determined in Gorrochotegui-Escalante *et al*, 2002, and the infection rates reported in this paper. Other genes are currently being analyzed to determine potential correlates with VC.

Previous studies have revealed quantitative trait loci (QTL) that condition differences in VC and MIB and MEB (Bosio *et al.* 1998, 2000). Whether or not these condition dengue transmission in these natural populations remains to be determined. It is important to remember that there is potential for different loci to affect VC in different populations. That is, QTL discovered in previous studies based on crosses of laboratory colonies may not be pertinent to field populations, which may have their own QTL acting on VC for dengue. Thus, the importance of looking for novel QTL and genetic variation is evident.

The regression analyses (Figure 2.4) show that MIB and MEB do have some degree of correlation (r values ranging from 0.50 to 0.72). This correlation may suggest the presence of an overall antiviral response from the mosquito that inhibits both midgut infection and midgut escape. If, for example, a mosquito responded to virus by inducing apoptosis of infected cells, this could potentially affect both midgut infection and escape, and would help explain why the two are correlated. MIB and MEB are not tightly correlated, however, and this leaves room for independently acting factors to affect either midgut infection or midgut escape. This again stresses the importance of mapping QTL associated with MIB, MEB, or both.

In each collection, MIR were dose dependent (Figure 2.5). The dose response curves that reflect the thresholds of infection differed slightly for each collection (Woodring *et al.* 1996). DIR did not correlate with virus dose in infectious blood meals (data not shown). This is not surprising, as Bosio *et al*, (1998), previously reported that virus dissemination rates in *Ae. aegypti* do not correlate to midgut virus titer. The dose response results suggest that the high titers used in the VC studies do not obscure differences in susceptibility among the collections. In most cases, the collections retain the same VC ranking through the different virus titers (Figure 2.5). Where they do change order, the differences are not great.

In future studies, we will determine if different DEN-2 virus genotypes and other DEN serotypes yield the same vector phenotypes as DEN-2 JAM1409 virus. This would allow us to determine whether mosquito populations differ in their susceptibility to different DEN virus strains or genotypes. If mosquitoes vary in their ability to transmit different DEN genotypes or serotypes, VC may be a major determinant of which genotypes/serotypes do circulate and thus are more prevalent in a given area. Specific genotypes or serotypes would, in this case, be selected for by the vector.

These studies with DEN-2 JAM1409 and *Ae. aegypti* mosquito collections lay the groundwork for genetic analysis of mosquito determinants of VC. Identified QTL that condition VC can be used to perform marker assisted selection (MAS) (Bosio *et al.* 1998, 2000, Lande and Thompson 1990, Romagosa *et al.* 1999). MAS would greatly facilitate studies on the mosquito genetics aspect of virus-vector interactions.

CHAPTER 3

SELECTION OF *D2S3*, AN *AEDES AEGYPTI* STRAIN WITH HIGH ORAL SUSCEPTIBILITY TO DENGUE TYPE 2 VIRUS (DEN-2) AND *D2MEB*, A STRAIN WITH A MIDGUT ESCAPE BARRIER TO DENGUE TYPE 2 VIRUS

Introduction

Dengue Fever (DF) is one of the most rapidly expanding diseases in the tropics with over 2 billion people at risk. All four serotypes of the virus are now circulating in the Americas and an estimated 100 million human infections occur annually. DEN virus infections typically result in DF, an acute, self limiting, febrile illness (Clarke 2002, Guzman and Kouri 2002), however several hundred thousand cases of the severe form of the disease, DEN hemorrhagic fever-shock syndrome (DHF-SS) occur now annually (Halstead *et al.* 2002, Isturiz *et al.* 2000, Kantachuvessiri 2002, Sharma *et al.* 2000). Projected demographic trends suggest that with increased urbanization, the numbers of DF and DHF cases will increase (Barbosa da Silva *et al.* 2002, Cho Min 2000, Condon *et al.* 2000, Endy *et al.* 2002, Flisser *et al.* 2002, Gubler 2002, Guzman and Kouri 2002, Isturiz *et al.* 2000, Kantachuvessiri 2002, Nogueira *et al.* 2000). The recent introduction of Southeast Asian genotypes of DEN viruses has dramatically increased DHF in the Americas. Indeed, DEN emergencies have been declared almost every year during the past decade in tropical areas of the Americas.

Vector competence refers to the intrinsic permissiveness of an arthropod vector to infection, replication, and transmission of a virus (Hardy 1988, Woodring *et al.* 1996).

Transmission of DEN involves a complex interaction between the virus and *Aedes aegypti,* the most prevalent vector of DEN (serotypes 1-4) in a human-mosquito cycle in tropical and subtropical regions. When *Ae. aegypti* takes a DEN viremic bloodmeal, the virus encounters several barriers to infection. First, the virus must establish an infection in the *Ae. aegypti* midgut (MI) by overcoming a midgut infection barrier (MIB). Following replication in the midgut epithelium, virus must pass through a midgut escape barrier (MEB) and replicate in other tissues to establish a disseminated infection (DI). Finally, virus must infect the salivary glands and be shed in the saliva to be transmitted to the next vertebrate host.

The genetics of vector competence for flaviviruses in *Ae. aegypti* has been studied extensively using strains selected for susceptibility and resistance. Previous crosses of *Ae. aegypti* strains exhibiting high and low susceptibility to DEN-2 virus have suggested that the resistant phenotype was dominant (Gubler and Rosen 1976, Gubler *et al.* 1979). Wallis *et al.* (1985) concluded that only a few genes of major effect control flavivirus vector competence in *Ae. aegypti*. Miller and Mitchell (1991) selected lines of *Ae. aegypti* that were completely refractory or highly susceptible to Yellow Fever virus (YFV). F_1 progeny were intermediate in susceptibility, suggesting that alleles at vector competence loci act additively. F_2 backcrosses suggested the involvement of multiple loci. A quantitative genetic study of DEN-2 MIB and MEB in *Ae. a. aegypti* and *Ae. a. formosus* showed that the heritability was 0.41 for MI titers and 0.39 for DI in both subspecies (Bosio *et al.* 1998). Results suggested that at least two genes or sets of genes control vector competence for DEN-2, one set controlling MIB, the other controlling MEB. Bosio *et al.* (2000) mapped Quantitative Trait Loci (QTL) affecting the ability of *Ae.*

aegypti to become infected with DEN-2 using an F_1 intercross. *Aedes a. aegypti* and *Ae. a. formosus* were crossed and MIB and MEB were tested for in the F_2 offspring. Two MIB QTL were detected with standard and composite interval mapping on chromosomes *II* and *III*. These accounted for ~30% of the phenotypic variance in dengue infection and respectively accounted for 44 and 56% of the overall genetic variance. Alleles at loci on both chromosomes acted additively to determine MI rates.

QTL mapping has been instrumental in identifying the number of loci that control a trait, the relative contributions of these loci in determining the overall expression of a trait, and the ways that alleles at a QTL and alleles at different QTL interact to determine a phenotype. QTL mapping has also lead to a new procedure known as Marker Assisted Selection (MAS) for more rapidly breeding individuals with desired phenotypic traits. In contrast to traditional selection in which individuals are selected for breeding based on phenotype alone, MAS selects for breeding those individuals with the desired phenotype and the genotypes at DNA markers linked to a QTL that controls the phenotype of interest. MAS yields a much stronger and more rapid response to selection than traditional phenotypic selection because quantitative traits are frequently subject to random and/or environmental effects such that an individual may or may not exhibit a particular phenotype due to non-genetic effects. However, with MAS fewer of these individuals are selected as breeding stock for the next generation because the breeder can identify progeny that carry the desired genes. An abundance of genetic markers flanking QTL provide the basis for MAS and allow genetic strains to be rapidly constructed that are homozygous for specific alleles at QTL. MAS is currently used in animal and plant breeding programs to increase the rate and efficiency of selection (Gimelfarb and Lande

1994a, Gimelfarb and Lande 1994b, Lande and Thompson 1990). In several cases, MAS has allowed breeders to generate breeding stocks with much higher or lower values of a phenotype and with a lower variance than was possible with traditional selection (Stuber 1995, Williams *et al.* 1995).

Here we describe the use of phenotypic selection with Dengue serotype 2 virus (DEN-2) strain JAM1409 to breed mosquito lines that are either highly susceptible to DEN-2 infection (the *Dengue 2 Susceptible on 3 chromosomes* (*D2S3*) strain) or have an efficient DEN-2 MEB (the *Dengue 2 Midgut Escape Barrier* (*D2MEB*) strain). We intended to use MAS to cull deleterious and lethal recessive genes and thereby increase survivorship and fecundity. Because different DEN serotypes and genotypes differ in their ability to infect *Ae. aegypti* populations (Gubler and Rosen 1976, Tabachnick *et al.* 1985, Hardy 1988), the second goal of this work was to test whether the MIR and DIR selected in both *D2S3* and *D2MEB* were consistent with other DEN-2 genotypes and the three other dengue serotypes.

Materials and Methods

Mosquito strains: The *Aedes aegypti aegypti* PR strain was established from eggs collected in field ovitraps in the spring of 1995 in San Juan, Puerto Rico. PR mosquitoes used in these experiments were in the 20th generation in the laboratory. The *Aedes aegypti aegypti* Houston strain was established from eggs collected in field ovitraps in the summer of 1998 in Houston, Texas. Houston mosquitoes used in these experiments were all third and fourth generation mosquitoes and were previously determined to have a MIR of 61% and a DIR of 65% (Bennett *et al.* 2002). The *Aedes aegypti formosus* Ibo strain was established from eggs collected in Ibo village, Nigeria

(Ballinger-Crabtree *et al.* 1992) and had been maintained through at least 20 generations in the lab.

Oral infection of mosquitoes: All initial mosquito phenotyping and selection was done using DEN-2 JAM1409 virus, which was isolated in 1983 in Jamaica (Deubel *et al.* 1986) and subsequently passaged in C6/36 cells. Oral feeding, and DEN-2 infection protocols follow Bennett *et al.* (2002). Once mosquito lines of specific phenotype were created, they were challenged with the other dengue serotypes and genotypes listed in Table 3.1 to determine whether dengue susceptibility in the selected lines was genotype or serotype specific, or whether common genetic factors conditioned susceptibility to all genotypes and serotypes.

Vector competence: Fourteen days after feeding on the infectious bloodmeal, mosquitoes were frozen, heads were severed, squashed onto acid washed slides, acetone fixed, and assayed for DEN-2 by indirect immunofluoresence following Bennett *et al.* (2002). When viral antigen was not detected in head tissues, the respective abdomens, which had been stored at -70°C, were assayed by immunofluoresence for virus antigen. Detection of virus antigen in midgut tissue revealed a midgut infection (MI) and the MI rate (MIR) was calculated as the number of positive midguts divided by the number of mosquitoes exposed. Virus antigen in head tissue indicated a disseminated infection (DI), and the DI rate (DIR) was the number of mosquitoes with virus antigen in the head tissue divided by the number of mosquitoes with virus antigen in the midgut. VC was calculated as the number of mosquitoes with DI divided by the number of mosquitoes exposed. Mosquitoes were not assayed for salivary gland infection or for actual virus transmission, rather VC was assumed to be the same as the rate of virus dissemination to the

Table 3.1: Viruses used to test susceptibility phenotypes of the *D2S3* and *D2MEB* lines. Attempts were made to obtain viruses of more genotypes within each serotype, but they were not readily available.

Dengue serotype	Dengue genotype	Strain	Collection location and passage history	Viral titer in blood meal [Ψ] $log_{10}TCID_{50}$/ml
1	**III** (Caribbean)	Hawaii	Hawaii, 28 passages in mice and 5 in C6/36 cells	6.83-7.17
1	**V** (American, African and SE Asian)	BC-136	Yucatan, 6 passage in C6/36 cells	7.17-7.5
2	**II** (South East Asian)	JAM1409[a]	Jamaica, lab adapted in C6/36 cells	7.17-8.0
2	**II** (South East Asian)	JAM1409[b]	Jamaica, lab adapted in C6/36 cells	6.83-7.17
2	**I** (South Pacific, American)	PR159	Puerto Rico, 5 passages in C6/36 cells	6.17-6.83
2	**V** (Sri Lanka, Seychelles)	SLK-1592	Sri Lanka, 5 passages in C6/36 cells	5.5-5.17
3	**I** (Philippines, Malaysia, Indonesia)	H-87	28 passages in mice and 5 in C6/36 cells	7.5-7.17
4	**I** (Thailand, Philippines, Sri Lanka)	H-241	Philippines, 28 passages in mice and 6 in C6/36 cells	5.17-5.83
4	**II** (American, Pacific, SE Asian)	BC-9*	Yucatan, passed 5 times in C6/36 cells	6.5-6.83

[Ψ]Denotes range of virus titer from pre and post blood meal samples
*No results in the *D2MEB* line
[a] Titer of virus used for selection of a line and for testing different generations
[b] Titer of virus used for testing different genotypes and genotypes

head tissues. Once mosquitoes were phenotyped for MI and DI, the rate of mosquitoes exhibiting a MIB was calculated as 1-MIR and the MEB rate was calculated as MIR - DIR.

Mating design for selection of the *D2S3* line: An F_1 intercross mating design was implemented for family based phenotypic and MAS. Twenty-five families were generated by crossing individual P_1 females of the PR line with individual P_1 males of the Ibo line. An additional 25 families were generated as reciprocal crosses. Mosquitoes were allowed to mate 6-7 days, and males were then collected and frozen at –70°C. Females were given an infectious blood meal and eggs were collected from individual females. After the 14-day EIP, P_1 females were tested for DI. Families of PR P_1 females that exhibited a DI, and families of Ibo P_1 females that were DI and MI negative were maintained. F_1 females from families with these characteristics were intercrossed with F_1 brothers. After 6-7 days, males were frozen at –70°C and females were fed several times on uninfected mice. Eggs were collected from individual females. The resulting F_2 families were hatched, intercrossed, and F_2 females were given a DEN-2 JAM1409 infected blood meal. DIR and MIR were determined. F_2 families with high MI and DI rates were maintained.

Mating design for selection of the *D2MEB* line: P_1 parents were from the Houston strain (Bennett *et al.* 2002) and *D2S3* lines. Twenty-five P_1 females of both strains were phenotyped for susceptibility to DEN-2 JAM1409 as above. Crosses in which *D2S3* females were positive for MI and DI and in which Houston females were positive for MI but negative for DI became the P_1 generation. Eggs collected from individual P_1 females were hatched for 3 families (HD2S3 10, HD2S3 18, and HD2S3

21), and the F_1 offspring were intercrossed. Females were provided several noninfectious blood meals, and eggs were collected. Eggs from the most fecund F_1 females were hatched (HD2S3 10.4, HD2S3 18.9 and HD2S3 21.1). F_2 individuals of each family were allowed to intermate, males were removed and frozen, and females were given an infectious blood meal and placed in individual cartons for egg collection. After the 14-day EIP, DIR and MIR were determined in F_2 females. HD2S3 21.1 had a high MI rate and a low DI rate and was maintained.

DNA Extraction, PCR and SSCP analyses: Following phenotypic selection, SSCP genotypes were examined at loci distributed throughout the *Aedes aegypti* genome. Genomic DNA was extracted from individual mosquitoes (Black and DuTeau 1997) PCR was performed in P_1, F_1 and F_2 mosquitoes using each of the primer pairs listed in Table 3.2 (Fulton *et al.* 2001). Table 3.2 also indicates whether or not these primers were polymorphic and used subsequently in the F_2 generation. These primers were selected based on previous linkage maps (Bosio *et al.* 2000, Fulton *et al.* 2001, Severson *et al.* 2002) and were chosen to scan all three of the *Ae aegypti* chromosomes. Single strand conformation polymorphism (SSCP) was performed and fragments were detected with silver staining (Bosio *et al.* 2000, Black and DuTeau 1997). Our intention was to identify genome regions in which genotypes were not inherited in a Mendelian fashion, presumably due to cosegregation with proximate deleterious and lethal recessive alleles. A χ^2 goodness of fit test was used to determine if genotypes were inherited in Mendelian proportions.

Table 3.2: *Aedes aegypti* genetic markers used to genotype mosquito families during selection of the *D2S3* and *D2MEB* lines.

Primer Name	*A aegypti* linkage location	Accession no.	T_a	Size	Forward/reverse primer sequence	*D2S3*	*D2MEB*
Hemepoly (hemolymph polypeptide)	I, 0	U11235	60	385	cgacaagcgccagcagaagg agttggccgggtccacttcg	- m	+
Cathbp (cathepsin b-like thiol protease)*	I,5	L41940	60	343	caaattcggaacctcaccag tatccacccttgcatccatc	+	- m
Tfs (transferrin precursor)	I,10	AF019117	60	309	atgcggccatccaggttcag cccgccgacttcagtttcgt	+	- m
LF198	I, 20	T58319	48	284	ctggcgtagattccgtgctg tccgtgttgacgtaggtggc	- m	+
BMIOP (blood meal-induced ovarian protein)	I, 30	U84248	60	337	ttgaagtcgtcgttgctgtt ctgcctttgtcatgtttgct	- m	+
AmyII (α-amylase 2)	I,45	U01208	60	330	atgacgttggagtgcgaatc accaggttgccgtagatgaa	+	- m
AbdA (abdominal a)	I,70	X67132	48	367	agcggtagatcctgttgtct aacctccaactcacctgttc	- m	+
TrypEarl (trypsin-early)	II, 23	X64362	60	459	ccaacggtggcatcatagtgaaag gatccattggcgaacagtggagac	+	+
CarboxA (carboxypeptidase A)	II,24	AF165923	54	378	ttgaattgtaatgggttgag ttatgataggaatcgctttg	+	+
TrypAbun (trypsin-abundant)	II, 32	M77814	60	339	acggctaccctcggtcagtt ctacctcgcggtcggtaaag	+	+
Malt (maltase)	III, 21	M30442	48	234	ggactggtgggaacatggaa cttatcggacaaccgctgga	+	+
Elastase (Trypsin-elastase)	III, 25	X64363	60	325	tggctttgaagtgcccgttgag caagttccttcgttgaccggagtg	+	+

Apolipo2 (apolipophorin 2)	III, 25	AF038654	54	329	gctggaatcggtcaaactcg ccggccttaacttgctggta	+	- m
Hsp70 (Heat shock protein 70)	III, 46	AI658418	56	342	cccgtcctacgtggcgttca ggtggcctgacgttgcgagt	- m	+
AspSyn (asparagines synthetase)	III, 52	U84118	60	297	ggctgaacaatgtgcggtat tgatttcctgtgccatcagc	- m	+
Apyr2 (apyrase 2)	III, 57	L41391	54	317	tgattgcatcgtcgttgatt caacttgcgctgtttgtttt	+	+

Listed with each gene is the GenBank accession number, optimal annealing temperature (T_a) and the approximate size of the amplified product. Also listed are the sequences and locations of the oligonucleotide primers, relative to the GenBank sequence, and the location of the genes in the *Ae. aegypti* linkage map. The last 2 columns indicate whether the primer was used in each of the selected mosquito lines.

Results

Generation of the *D2S3* line: A single F_2 family, 12C23, exhibited a MIR of 98% and a DIR of 95% in the F_2 generation. F_2 males (n = 87) and females (n = 41) were genotyped (Table 3.3). With the exception of the *Sex* and *ACE* loci, which are linked (~1 cM), genotypes fit Mendelian expectations. There was an excess of males at the *Sex* locus and an excess of *ACE* heterozygotes and homozygotes for alleles inherited from the PR P_1 mother but a pronounced deficiency of homozygotes for alleles inherited from the Ibo P_1 father. This suggests the presence of a sex-linked locus with deleterious recessive alleles inherited through the father. No heterozygote deficiency occurred because the deleterious allele is recessive. We could not use MAS on *ACE* homozygote males because the females had already been mated. However, when we attempted to hatch eggs from the 41 F_2 females, only eggs from 12 genotyped females hatched. These were combined to form the *D2S3* line. No marker assisted culling was required in this strain, and no obvious sex ratio distortion has been detected since the F_2 generation. It is possible that the 29 F_2 egg liners, which did not hatch, were homozygous for deleterious and lethal recessives.

Subsequent generations F_3, F_4, F_6, F_{10}, F_{13} were monitored for their susceptibility to infection with DEN-2 JAM1409 (Table 3.4). Throughout these generations, there has been no obvious decline in the survivorship and fecundity of the mosquito line and the susceptibility phenotype has been retained. There are no significant differences ($p<0.05$) between generations for MIR or MIB as determined by heterogeneity χ^2 analysis.

Table 3.3: Segregation of genotypes at marker loci

Strain Chromosome Marker		F2 genotypes aa	ab	bb	χ^2 (df)
			D2S3		
Chromosome *I*					
Cathbp	Obs.	14	18	8	2.20 (2)
	Exp.	10.00	20.00	10.00	
Tfs	Obs.	14	17	8	2.49 (2)
	Exp.	9.75	19.5	9.75	
ACE	Obs.	42	77	4	31.29 (2)***
	Exp.	30.75	61.5	30.75	
Sex	Obs.	87	41		16.53 (1)***
	Exp.	64.00	64.00		
AmyII	Obs.	17	21		0.42 (1)
	Exp.	19.00	19.00		
AbdA	Obs.	15	24		2.08 (1)
	Exp.	19.50	19.50		
Chromosome *II*					
TrypEarl	Obs.	13	27		1.20 (1)
	Exp.	10.00	30.00		
CarboxA	Obs.	21	14		1.40 (1)
	Exp.	17.50	17.50		
TrypAbun	Obs.	13	17	11	1.39 (2)
	Exp.	10.25	20.50	10.25	
Chromosome *III*					
Malt	Obs.	6	24	6	4.00 (2)
	Exp.	9.00	18.00	9.00	
Elastase	Obs.	6	24		0.04 (1)
	Exp.	10.00	20.00		
Apolipo2	Obs.	5	28	7	6.60 (2)*
	Exp.	10.00	20.00	10.00	
Apyr2	Obs.	6	18	8	0.75 (2)
	Exp.	8.00	16.00	8.00	
			D2MEB		
Chromosome *I*					
Hemepoly	Obs.	20	20	6	8.38 (1)**
	Exp.	11.50	23.00	11.50	
LF198	Obs.	25	14		3.10 (1)
	Exp.	19.50	19.50		

Table 3.3 (continued).

Strain Chromosome Marker		F2 genotypes aa	ab	bb	χ^2 (df)
BMIOP	Obs.	41	5		28.17 (1)***
	Exp.	23.00	23.00		
AbdA	Obs.	23	20	3	18.17 (2)***
	Exp.	11.50	23.00	11.50	
Chromosome *II*					
TrypEarl	Obs.	10	31		0.01 (1)
	Exp.	10.25	30.75		
CarboxA	Obs.	9	35		0.48 (1)
	Exp.	11.00	33.00		
TrypAbun	Obs.	11	27	6	3.41 (2)
	Exp.	11.00	22.00	11.00	
Chromosome *III*					
Malt	Obs.	17	21	6	5.59 (2)
	Exp.	11.00	22.00	11.00	
Elastase	Obs.	20	13		1.48 (1)
	Exp.	16.50	16.50		
Hsp70	Obs.	17	21	7	3.92(1)*
	Exp.	11.25	22.50	11.25	
AspSyn	Obs.	16	21	7	3.77 (2)
	Exp.	11.00	22.00	11.00	
Apyr2	Obs.	13	23	5	3.73 (2)
	Exp.	10.25	20.50	10.25	

* $p<0.05$ **$p<0.01$ ***$p<0.001$

Table 3.4: Infection rates of the *D2S3* and *D2MEB* lines with DEN-2 JAM1409 over several generations.

Generation MEB	Total # Mosquitoes	#(MIR)	#(DIR)	VC	MIB	
		D2S3				
F_2	41	40(0.98)	39(0.97)	0.95	0.02	0.03
F_3	35	34(0.97)	30(0.88)	0.86	0.03	0.12
F_4	37	-	36(-)	0.97	-	-
F_6	32	-	30(-)	0.94	-	-
F_{10}	65	61(0.94)	56(0.91)	0.86	0.06	0.09
F_{13}	48	48(1.00)	48(1.00)	1.00	0.00	0.00
		D2MEB				
F_2	50	39(0.78)	11(0.28)	0.22	0.22	0.72
F_3	24	21(0.88)	7(0.33)	0.29	0.12	0.67
F_4	76	56(0.74)	7(0.13)	0.09	0.26	0.87
F_5	65	55(0.85)	15(0.27)	0.23	0.15	0.73

- Indicates that abdomen of these mosquitoes were not assayed or that this calculation could not be completed because the MI was not determined.

Generation of the *D2MEB* line: One F_2 family, HD2S3 21.1, (P_1 Houston mother, P_1 *D2S3* father), exhibited a MIR of 78% and a DIR of only 22%. The large differential between MIR and DIR suggested the presence of a fairly strong MEB, and this family was maintained. Fifty HD2S3 21.1 female F_2 mosquitoes were genotyped (Table 3.3). Because F_2 females were already mated, no selection could be performed on males and thus F_2 males were not genotyped. Genotypes at marker loci on chromosomes *II* and *III* conformed to Mendelian expectations (Table 3.3). The deficiencies in heterozygotes and homozygotes for the alleles inherited from the *D2S3* P_1 father are a result of only genotyping females. Eggs from 21 F_2 females that were midgut positive but head negative were combined to create the *D2MEB* line.

Subsequent generations F_3-F_5 were monitored for MEB with DEN-2 (Table 3.4). There was no decline in survivorship and fecundity and each generation has maintained a large MIR and a small DIR. There are no significant differences ($p<0.05$) between generations for MIR or MIB as determined by heterogeneity χ^2 analysis.

Oral challenge with other DEN serotypes and genotypes: Because mosquitoes were selected using only the laboratory adapted virus strain, DEN-2 JAM1409, it was important to see if dengue susceptibility phenotypes in the selected lines were consistent for other dengue serotypes, and among different genotypes within serotypes. This was accomplished through *per os* infection of mosquitoes from the F_{13} generation in the *D2S3* line and the F_6 generation in the *D2MEB* line. The viruses used, as well as their titers and passage histories are listed in Table 3.1.

Figure 3.1 shows the MIR for *D2S3* and *D2MEB* when they were fed on different dengue serotypes and genotypes. While MIR were fairly consistent across different

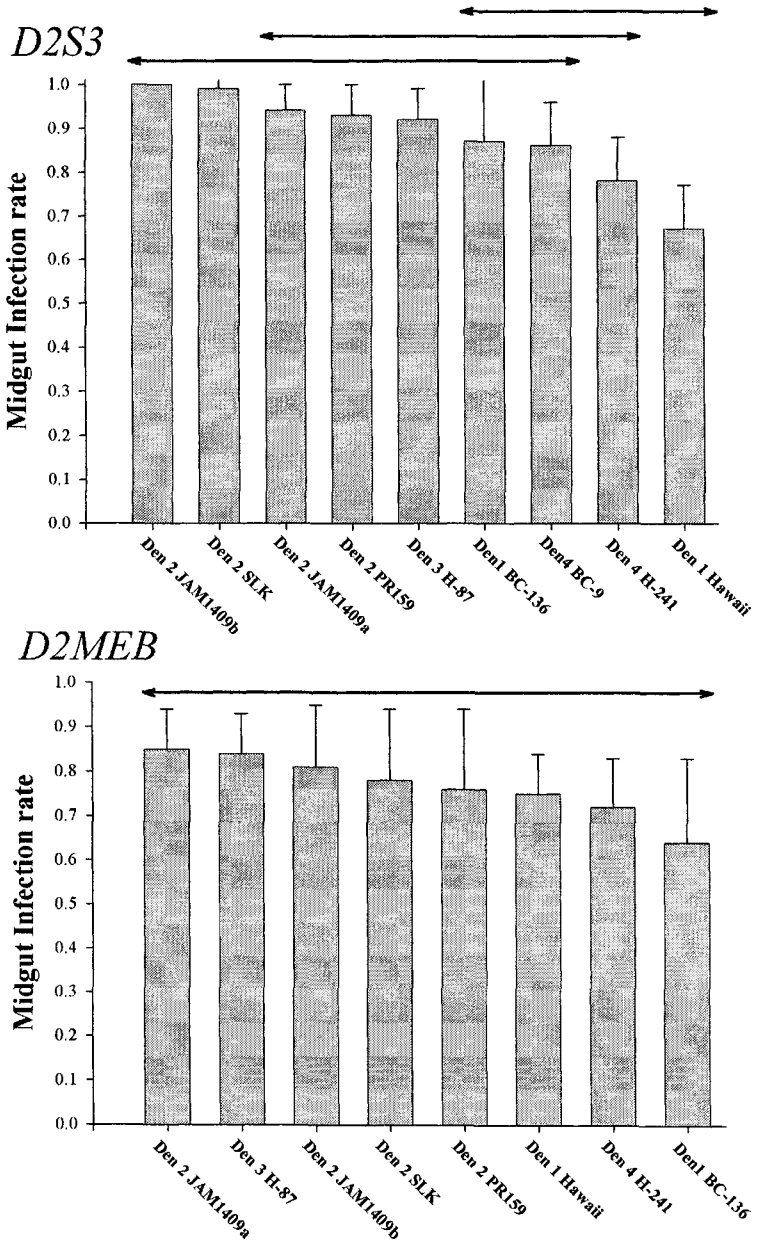


Figure 3.1: Midgut Infection Rates in *D2S3* and *D2MEB* infected with various DEN genotypes and serotypes. Statistically homogeneous groups as determined by pairwise 2x2 contingency χ^2 analyses (Table 3.5) are covered by double arrow lines.

DEN-2 genotypes in the *D2S3* line, there were distinct differences among the different serotypes. However, no significant variation in MIR was seen in the *D2MEB* line. Figure 3.2 shows DIR for *D2S3* and *D2MEB* when exposed to different DEN serotypes and genotypes. In *D2S3*, DIR was consistent across DEN-2 genotypes but not among serotypes. In contrast, in *D2MEB*, DIR was not consistent across DEN-2 genotypes. Specifically, DIR was greater in the second trial (DEN-2 JAM1409b) than in the first. In addition DIR varied greatly among serotypes. Table 3.5 contains the individual χ^2 analyses and associated MIR and DIR for each pairwise comparison.

Discussion

We have successfully bred two genetic strains of *Aedes aegypti* that differ in their susceptibility to infection with dengue serotype 2 virus (DEN-2) strain JAM1409. We used a combination of individual and family-based selection in our breeding design. Only F_1 families arising from individual P_1 mothers with the desired phenotype were maintained. However, F_2 families were selected based upon their MIR and DIR.

We chose to intercross *Ae. aegypti aegypti* and *Ae. aegypti formosus* P_1 parents to generate *D2S3* because earlier attempts to select mosquitoes with a high DIR directly from the PR strain failed. Most of the families died in the F_1 generation, had low fecundity and survivorship or died in the F_2 generation. Similar failures occurred when trying to breed low MIR families from the Ibo *Ae. aegypti formosus* line. One line, *Ibo11*, has been maintained with an MIR of 10-15%. However, *Ibo11* eggs must be hatched every 2-3 weeks and adults typically live for only ~14 days and lay few eggs. *Ibo11* has been outcrossed to *D2S3* to construct a QTL mapping family and to maintain the refractory MIB *Ibo11* alleles in an outbred strain. We encountered similar problems with

Table 3.5: MIR, DIR and VC in *D2S3* and *D2MEB* infected with various DEN genotypes and serotypes.

		MI+	DI+	Total	MIR	DIR	VC
		D2S3					
Den-1 Hawaii	Den-1 BC-136	52	35	78	0.67	0.67	0.45
		20	17	23	0.87	0.85	0.74
Den-1 Hawaii	Den-2 JAM1409a	52	35	78	0.67*	0.67*	0.45*
		61	56	65	0.94	0.92	0.86
Den-1 Hawaii	Den-2 JAM1409b	52	35	78	0.67*	0.67*	0.45*
		48	48	48	1.00	1.00	1.00
Den-1 Hawaii	DEN-2 PR159	52	35	78	0.67*	0.67	0.45*
		52	48	56	0.93	0.92	0.86
Den-1 Hawaii	DEN-2 SLK	52	35	78	0.67*	0.67*	0.45*
		69	69	70	0.99	1.00	0.99
Den-1 Hawaii	DEN-3 H-87	52	35	78	0.67*	0.67	0.45
		48	31	52	0.92	0.65	0.60
Den-1 Hawaii	DEN-4 H-241	52	35	78	0.67	0.67*	0.45
		51	15	65	0.78	0.29	0.23
DEN-1 Hawaii	DEN-4 BC-9	52	35	78	0.67	0.67	0.45
		37	23	43	0.86	0.62	0.53
DEN-1 BC-136	DEN-2 JAM1409a	20	17	23	0.87	0.85	0.74
		61	56	65	0.94	0.92	0.86
DEN-1 BC-136	DEN-2 JAM1409b	20	17	23	0.87	0.85*	0.74*
		48	48	48	1.00	1.00	1.00
DEN-1 BC-136	DEN-2 PR159	20	17	23	0.87	0.85	0.74
		52	48	56	0.93	0.92	0.86
DEN-1 BC-136	DEN-2 SLK	20	17	23	0.87	0.85*	0.74*
		69	69	70	0.99	1.00	0.99
DEN-1 BC-136	DEN-3 H-87	20	17	23	0.87	0.85	0.74
		48	31	52	0.92	0.65	0.60
DEN-1 BC-136	DEN-4 H-241	20	17	23	0.87	0.85*	0.74*
		51	15	65	0.78	0.29	0.23
DEN-1 BC-136	DEN-4 BC-9	20	17	23	0.87	0.85	0.74
		37	23	43	0.86	0.62	0.53
DEN-2 JAM1409a	DEN-2 JAM1409b	61	56	65	0.94	0.92	0.86
		48	48	48	1.00	1.00	1.00
DEN-2 JAM1409a	DEN-2 PR159	61	56	65	0.94	0.92	0.86
		52	48	56	0.93	0.92	0.86
DEN-2 JAM1409a	DEN-2 SLK	61	56	65	0.94	0.92	0.86
		69	69	70	0.99	1.00	0.99
DEN-2 JAM1409a	DEN-3 H-87	61	56	65	0.94	0.92*	0.86*
		48	31	52	0.92	0.65	0.60
DEN-2 JAM1409a	DEN-4 H-241	61	56	65	0.94	0.92*	0.86*
		51	15	65	0.78	0.29	0.23

Table 3.5 (continued)

DEN-2 JAM1409a	DEN-4 BC-9	61	56	65	0.94	0.92*	0.86*
		37	23	43	0.86	0.62	0.53
DEN-2 JAM1409b	DEN-2 PR159	48	48	48	1.00	1.00	1.00
		52	48	56	0.93	0.92	0.86
DEN-2 JAM1409b	DEN-2 SLK	48	48	48	1.00	1.00	1.00
		69	69	70	0.99	1.00	0.99
DEN-2 JAM1409b	DEN-3 H-87	48	48	48	1.00	1.00*	1.00*
		48	31	52	0.92	0.65	0.60
DEN-2 JAM1409b	DEN-4 H-241	48	48	48	1.00*	1.00*	1.00*
		51	15	65	0.78	0.29	0.23
DEN-2 JAM1409b	DEN-4 BC-9	48	48	48	1.00	1.00*	1.00*
		37	23	43	0.86	0.62	0.53
DEN-2 PR159	DEN-2 SLK	52	48	56	0.93	0.92	0.86
		69	69	70	0.99	1.00	0.99
DEN-2 PR159	DEN-3 H-87	52	48	56	0.93	0.92*	0.86
		48	31	52	0.92	0.65	0.60
DEN-2 PR159	DEN-4 H-241	52	48	56	0.93	0.92*	0.86*
		51	15	65	0.78	0.29	0.23
DEN-2 PR159	DEN-4 BC-9	52	48	56	0.93	0.92*	0.86*
		37	23	43	0.86	0.62	0.53
DEN-2 SLK	DEN-3 H-87	69	69	70	0.99	1.00*	0.99*
		48	31	52	0.92	0.65	0.60
DEN-2 SLK	DEN-4 H-241	69	69	70	0.99*	1.00*	0.99*
		51	15	65	0.78	0.29	0.23
DEN-2 SLK	DEN-4 BC-9	69	69	70	0.99	1.00*	0.99*
		37	23	43	0.86	0.62	0.53
DEN-3 H-87	DEN-4 H-241	48	31	52	0.92	0.65*	0.60*
		51	15	65	0.78	0.29	0.23
DEN-3 H-87	DEN-4 BC-9	48	31	52	0.92	0.65	0.60
		37	23	43	0.86	0.62	0.53
DEN-4 H-241	DEN-4 BC-9	51	15	65	0.78	0.29	0.23*
		37	23	43	0.86	0.62	0.53
		D2MEB					
DEN-1 Hawaii	DEN-1 BC-136	61	9	81	0.75	0.15	0.11
		16	7	25	0.64	0.44	0.28
DEN-1 Hawaii	DEN-2 JAM1409a	61	9	81	0.75	0.15	0.11
		55	15	65	0.85	0.27	0.23
DEN-1 Hawaii	DEN-2 JAM1409b	61	9	81	0.75	0.15*	0.11*
		25	16	31	0.81	0.64	0.52
DEN-1 Hawaii	DEN-2 PR159	61	9	81	0.75	0.15*	0.11*
		16	9	21	0.76	0.56	0.43
DEN-1 Hawaii	DEN-2 SLK	61	9	81	0.75	0.15	0.11
		21	9	27	0.78	0.43	0.33

Table 3.5 (continued)

DEN-1 Hawaii	DEN-3 H-87	61	9	81	0.75	0.15	0.11
		58	13	69	0.84	0.22	0.19
DEN-1 Hawaii	DEN-4 H-241	61	9	81	0.75	0.15	0.11
		43	5	60	0.72	0.12	0.08
DEN-1 BC-136	DEN-2 JAM1409a	16	7	25	0.64	0.44	0.28
		55	15	65	0.85	0.27	0.23
DEN-1 BC-136	DEN-2 JAM1409b	16	7	25	0.64	0.44	0.28
		25	16	31	0.81	0.64	0.52
DEN-1 BC-136	DEN-2 PR159	16	7	25	0.64	0.44	0.28
		16	9	21	0.76	0.56	0.43
DEN-1 BC-136	DEN-2 SLK	16	7	25	0.64	0.44	0.28
		21	9	27	0.78	0.43	0.33
DEN-1 BC-136	DEN-3 H-87	16	7	25	0.64	0.44	0.28
		58	13	69	0.84	0.22	0.19
DEN-1 BC-136	DEN-4 H-241	16	7	25	0.64	0.44	0.28
		43	5	60	0.72	0.12	0.08
DEN-2 JAM1409a	DEN-2 JAM1409b	55	15	65	0.85	0.27*	0.23
		25	16	31	0.81	0.64	0.52
DEN-2 JAM1409a	DEN-2 PR159	55	15	65	0.85	0.27	0.23
		16	9	21	0.76	0.56	0.43
DEN-2 JAM1409a	DEN-2 SLK	55	15	65	0.85	0.27	0.23
		21	9	27	0.78	0.43	0.33
DEN-2 JAM1409a	DEN-3 H-87	55	15	65	0.85	0.27	0.23
		58	13	69	0.84	0.22	0.19
DEN-2 JAM1409a	DEN-4 H-241	55	15	65	0.85	0.27	0.23
		43	5	60	0.72	0.12	0.08
DEN-2 JAM1409b	DEN-2 PR159	25	16	31	0.81	0.64	0.52
		16	9	21	0.76	0.56	0.43
DEN-2 JAM1409b	DEN-2 SLK	25	16	31	0.81	0.64	0.52
		21	9	27	0.78	0.43	0.33
DEN-2 JAM1409b	DEN-3 H-87	25	16	31	0.81	0.64*	0.52*
		58	13	69	0.84	0.22	0.19
DEN-2 JAM1409b	DEN-4 H-241	25	16	31	0.81	0.64*	0.52*
		43	5	60	0.72	0.12	0.08*
DEN-2 PR159	DEN-2 SLK	16	9	21	0.76	0.56	0.43
		21	9	27	0.78	0.43	0.33
DEN-2 PR159	DEN-3 H-87	16	9	21	0.76	0.56	0.43
		58	13	69	0.84	0.22	0.19
DEN-2 PR159	DEN-4 H-241	16	9	21	0.76	0.56*	0.43*
		43	5	60	0.72	0.12	0.08
DEN-2 SLK	DEN-3 H-87	21	9	27	0.78	0.43	0.33
		58	13	69	0.84	0.22	0.19
DEN-2 SLK	DEN-4 H-241	21	9	27	0.78	0.43	0.33
		43	5	60	0.72	0.12	0.08

Table 3.5 (continued)

DEN-3 H-87	DEN-4 H-241	58	13	69	0.84	0.22	0.19
		43	5	60	0.72	0.12	0.08

*Rates were significantly different in a 2x2 contingency χ^2 analysis with a Bonferonni adjusted $\chi' = \chi / k$ where k = number of pairwise comparisons (36 comparisons $\chi'_{(0.05)} = 0.001389$ for *D2S3*; 28 comparisons $\chi'_{(0.05)} = 0.001786$ for *D2MEB*).

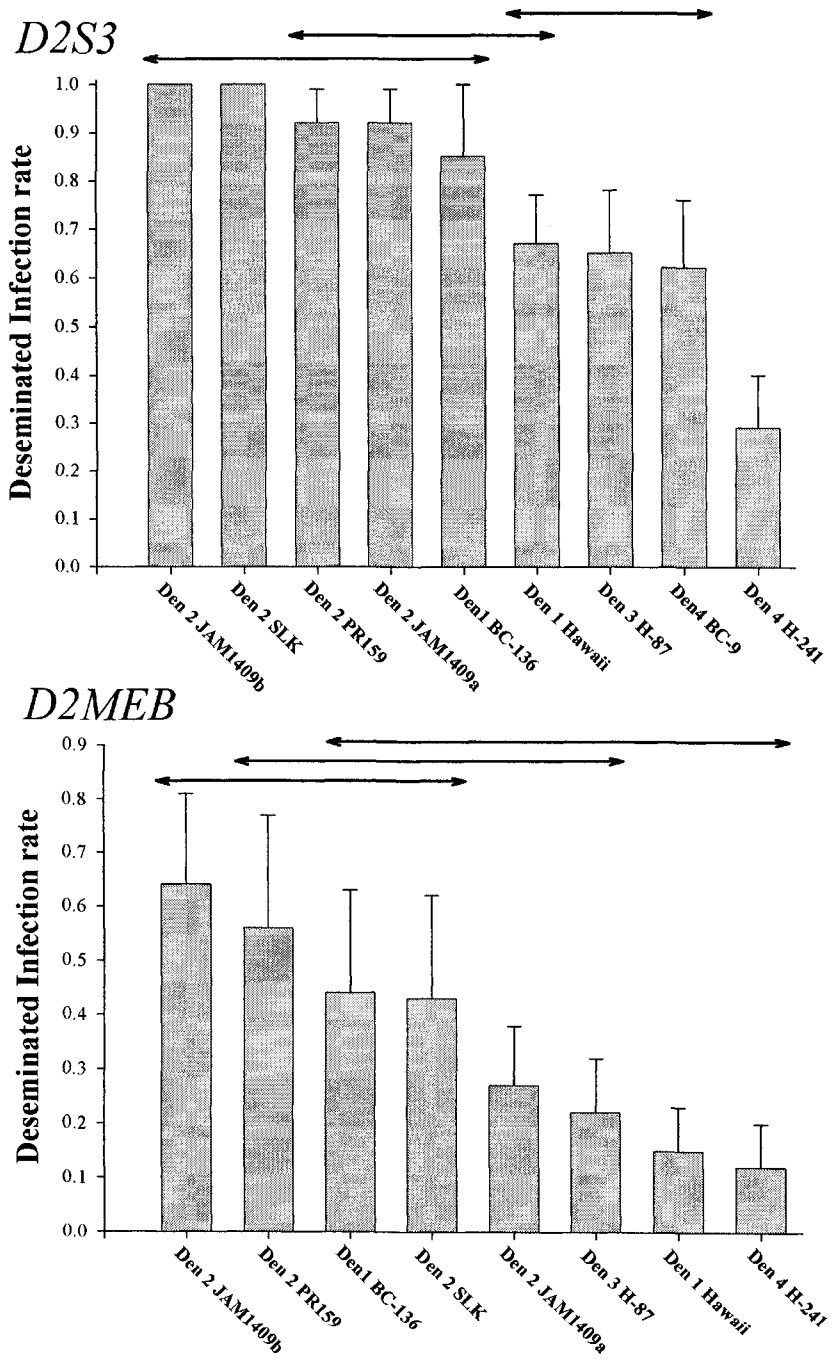


Figure 3.2: Disseminated infection rates in *D2S3* and *D2MEB* infected with various DEN genotypes and serotypes. Statistically homogeneous groups as determined by pairwise 2x2 contingency χ^2 analyses (Table 3.5) are covered by double arrow lines.

low survivorship and fecundity in attempts to breed families with low DIR from the Houston *Ae. aegypti aegypti* line. Problems with inbreeding and colonization are common in this and other *Aedes* species (Munstermann 1994, Munstermann 1997) and probably reflect a high load of deleterious and lethal recessive alleles in natural populations (Craig and Hickey 1967).

We initially generated 50 F_1 families in the *D2S3* selection and ended up only maintaining one F_2 family (12C23). Similarly, during selection of the *D2MEB* line, 50 F_1 families were generated, of which only three families (HD2S3 10, HD2S3 18, and HD2S3 21) were subsequently maintained for selection in the F_2. This demonstrates the difficulty in creating and maintaining mosquito lines of specific phenotype. These same difficulties have arisen in attempts to use refractory lines such as *Ibo11* to create F_1 intercross families.

Because of the problems with survivorship and fecundity discussed above, we initially intended to use MAS to cull deleterious or lethal recessive alleles from the selected F_2 families and thereby increase their survivorship and fecundity. However, with the exception of sex ratio distortion on Chromosome I in *D2S3* (Table 3.3) no significant departures from Mendelian expectations were detected and thus MAS was unnecessary. Both PR and Ibo strains have been inbred for at least 20 generations in the laboratory. This may have culled large numbers of mosquitoes that were homozygous for deleterious or lethal recessive alleles. It is also likely that brother-sister mating during the F_1 intercross made deleterious or lethal recessive alleles homozygous and that these individuals died before mating. It may be that *D2S3* and *D2MEB* still carry deleterious and lethal recessive alleles maintained in a balanced state in heterozygotes as was seen by

Matthews and Craig (1987) and Munstermann (1994). However, both strains continue to exhibit high egg, larval, and adult survivorship, good adult longevity and high fecundity.

The effort put into genotyping loci across the *Ae. aegypti* genome was not wasted. This information insures that any future contamination of the line can be detected. Knowledge of the genotypes specific to a line, and the ability to track them over many generations will facilitate future attempts at MAS to select MIR and DIR QTL. We have in fact crossed *D2S3* and *D2MEB* P_1 parents to map MEB QTL (Bennett *et al.*, in preparation) and found that DEN-2 MEB is predominantly controlled by three QTL, one on each chromosome. On Chromosomes *I-III,* these respectively account for 51%, 34% and 8% of the total genotypic variance in DIR. These results demonstrate that three MEB loci were co-selected in breeding the *D2MEB* line. These F_1 intercross QTL mapping families have been maintained and are now in generation F_9. We now plan to use MAS to isolate each of the three QTL into separate lines.

In further characterizing *D2S3* and *D2MEB,* we estimated MIR and DIR when they were orally challenged with other DEN-2 genotypes and the other three DEN serotypes. We anticipated that DEN susceptibility phenotypes would be consistent across serotypes and genotypes and that a generalized set of genetic factors would condition susceptibility of *Aedes aegypti* to infection with DEN serotypes. This would be consistent with previous work suggesting a generalized susceptibility to other flaviviruses (Miller and Mitchell 1991). Instead, the MIR and DIR in *D2S3* and the DIR in *D2MEB* varied among DEN serotypes.

There are a variety of explanations for the observed variance in MIR and DIR among serotypes. MIR is associated with $TCID_{50}$ of DEN-2 JAM1409 in the bloodmeal

(Bennett *et al.* 2002). Furthermore, the method of DEN-2 PR159 preparation and amplification greatly influenced MIR (Bosio *et al.* 2000). However, in the current experiments all viruses were prepared in an identical fashion and MIR was independent of $TCID_{50}$ in *D2S3* ($r = 0.003$, $P = 0.99$) and in *D2MEB* ($r = 0.184$, $P = 0.66$). The F_1 intercross design used to select both strains only allowed four alleles (2 from each P_1 parent) at a given locus. Furthermore, the PR and Ibo strains are highly inbred. Given these factors and the consistency of phenotype across generations (Table 3.4), it is unlikely that the variance in MIR or DIR is associated with differences among mosquitoes in different experiments. It is still possible that different loci or different alleles at the same loci in natural *Ae. aegypti* populations condition susceptibility to the four DEN serotypes. However, ultimately testing this possibility will require selecting strains for susceptibility to alternate DEN serotypes (e.g. a strain with high DEN-4 MIR) and then testing these against the other DEN serotypes (e.g. DEN-1-3).

Having considered these alternatives, it is most likely that the large variance in MIR and DIR among serotypes reflects qualitative differences in the ability of the different DEN isolates to infect the mosquito midgut. Subtle differences in viral requirements such as proteolytic processing or receptor binding might be factors contributing to differences in susceptibility. Certain virus serotypes might require more or less proteolytic processing in the mosquito midgut, or have different affinities for the receptors present in the mosquito midgut.

CHAPTER 4

QUANTITATIVE TRAIT LOCI (QTL) ASSOCIATED WITH MIDGUT INFECTION AND MIDGUT ESCAPE BARRIERS TO DENGUE 2 VIRUS INFECTION IN *AEDES AEGYPTI*

Introduction

Quantitative trait locus (QTL) mapping is a way to statistically identify areas of a genome associated with a phenotype of interest. QTL have been identified in both plants and animals for quantitative traits from fruit size (Paterson *et al.* 1990, Frary *et al.* 2000) to susceptibility to malaria (Severson *et al.* 1995, Zheng *et al.* 1997). In cases where the trait of interest is disease susceptibility or the ability to transmit a pathogen, the ultimate objective is normally to identify genes within the QTL region that might be involved. QTL mapping is based on the use of a dense linkage map, and thus units are measured in cM. The ability to limit the QTL region to specific genes is not a simple task, as each cM is often comprised of hundreds of kilobase pairs of DNA, and a single QTL region often spans several cM of the linkage map.

While Bosio *et al.* (2000) detected the presence of QTL for MEB, the results were inconclusive because of reduced statistical power due to the small sample size once all MIB positive individuals were removed. The previous chapter set the stage for mapping QTL for MEB in *Aedes aegypti* through the selection of two lines of mosquitoes, one highly susceptible to infection with DEN-2 (the *D2S3* line) and the second refractory to DEN-2 and having a strong MEB (the *D2MEB* line).

This chapter describes an experimental design used to map QTL for MEB in *Ae. aegypti*. The hypothesis is that by crossing a highly susceptible line with a line exhibiting little MIB, but a strong MEB, the confounding effects of MIB will be avoided. Additionally, by creating advanced intercross lines and testing phenotypes and genotypes in the F_5 generation, sample size will be increased for statistical power in QTL analysis. In this way, areas of the *Ae. aegypti* genome associated with MEB to dengue susceptibility can be identified.

Materials and Methods

Mosquito Strains: *Ae. aegypti* mosquito lines generated in Chapter 3 were used to create F_1 intercross families. The *D2S3* line was at the F_6 generation and the *D2MEB* line was at the F_4 generation.

DEN-2 Virus: DEN-2 JAM1409 was used for all experiments. Virus was prepared and infectious feeds were performed following Bennett *et al.* (2002) using an artificial membrane feeding system and a hog gut membrane. Pre and post blood meal virus was titrated by quantal assay in C6/36 cells. Virus titer was consistently between 7-8 $\log_{10}$ $TCID_{50}$/ml.

Breeding and Experimental Design: Parents from the *D2S3* and *D2MEB* lines were used to create reciprocal F_1 intercross families and map genomic areas that contribute to MEB. Ten crosses were made in each direction. After 4-7 days, females were given an infectious blood meal as described by Bennett *et al.* (2002) and males were collected and frozen at -70°C. Eggs were collected from individual females.

After the 14-day extrinsic incubation period (EIP), P_1 females were tested for disseminated infection (DI) using an indirect immunofluorescent assay (IFA) of head

tissues following Bennett *et al.* (2002). Those mosquitoes without a DI were tested for midgut infection (MI) using an indirect IFA of midgut tissues. Remnants of the thorax and abdomen of the P_1 females were retained and stored at -70°C after being phenotyped for dengue infection.

P_1 females of the *D2S3* line positive for DI and MI and P_1 females of the *D2MEB* line positive for MI but negative for DI were used. Eggs from each of these females were hatched. Individual F_1 females from this hatch were paired with full sibling F_1 males. After 6-7 days, males were removed and stored at -70°C. Females were given several noninfectious blood meals and eggs were collected from individual females. After 3-4 weeks, the F_1 females were also stored at -70°C.

Resulting F_2 families from the most fecund F_1 females were hatched and reared to adult. These included *D2S3*x*D2MEB* (Y) families 1.2, 2.2, 4.2, 7.1, 8.1, and 9.1 and *D2MEB*x*D2S3* (P) families 1.1, 2.2, 3.2, 6.1, 8.1, 9.2, and 10.3. Full siblings of these F_2 individuals were allowed to intermate. After 4-7 days F_2 females were provided an infectious blood meal containing DEN-2 JAM1409. The F_2 males and unfed females were removed and stored at -70°C. Fed females were maintained through the EIP and assayed for MIB and MEB phenotype. Thoraces and remnants of the abdomen were stored at -70°C. Phenotypes were scored on a 0/1 system, where 0/0 denotes MI(-)/DI(-), 1/0 denotes MI(+)/DI(-) and 1/1 denotes MI(+)/DI(+).

DNA Extraction, PCR and SSCP Analysis: DNA was extracted from each individual as described by Black and DuTeau (1997) and resuspended in 300 µl TE buffer. A 50 µl aliquot was stored at 4°C for daily use in PCR and the remainder was archived at -70°C in screw top vials.

PCR was performed on the P_1 and F_1 males and females using each of the primer pairs listed in Table 4.1 (Fulton *et al.* 2001). This table also indicates which primers were used for each group (Y2.2 F_2s, P7.1 F_2s or P7.1 F_5s). Primers were selected based on linkage maps created by Bosio *et al.* (2000), Fulton *et al.* (2001) and Severson *et al.* (2002), and were chosen to scan all three chromosomes of the *Ae. aegypti* genome.

PCR was performed following Bennett *et al.* (in preparation) with the following modifications. A cold start was used as follows. A single large PCR mixture sufficient for 100 - 50 µl reactions was prepared to ensure homogeneity of PCR conditions. This reaction mixture consisted of: 4,350 µl sterile double glass distilled water (ddH_2O), 500 µl 10x Taq buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1% gelatin (w/v) and 1.0% Triton-X-100], 5 µl of 200 mM dNTPs, and 5000 pmoles of the appropriate primer. This reaction mixture, 1.7 ml eppendorf tubes of sterile mineral oil, and empty 96 well plates were exposed to a UV light source (260nm) for 10 minutes (UVP CL-1000 Ultraviolet Crosslinker) to destroy any contaminating DNA. Fifty µl of Taq was added to the reaction mixture and mixed well. This was distributed in the 96 well plate at 49 µl per well and overlaid with 2 drops of sterile mineral oil (~25 µl). Template DNA from individual mosquitoes was then added at 0.5 µl per well. Each set of PCR reactions was checked for contamination by the use of a negative control containing all reagents except template DNA. The plate was then subjected to 30 cycles of: 1.) 1 minute at 95°C for denaturation, 2.) 1 minute at the annealing temperature appropriate for the primer, 3.) 2 minutes at 72°C for strand extension. After the 30 cycles, a final extension period of 7 minutes at 72°C was used. The plate was then cooled to and held at 4°C.

Table 4.1: *Aedes aegypti* cDNA genes analyzed in this study

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
Chromosome *I*									
Hemepoly (hemolymph polypeptide)	U11235	60	385	CGACAAGCGCCAGCAGAAGG	39	I, 0	Poly	Poly	Poly
				AGTTGGCCGGGTCCACTTCG	423				
LF090 (Ribosomal protein S14)	T58320	48	145	AGCAGAATGGCTCCCCGTAA	31	I, 0	Mono	Mono	Poly
				ATGGTTTCCTTGCCGGACAG	175				
LF188a (Translation elongation factor 1-gamma)	BM005472	50	431	GGAGGAGCAGCCCAGCAGAA	32	I, 6.4	Poly/ND	Poly/ND	Poly
				GCGGGAAATTGGTTGTTGTTTT	462				
TSFM (transferrin precursor)	AF387489	59	512	GATGAAGTGAACCGATTGC	607	I, 9	ND	ND	Poly
				CTTGGCGTCCTGGTAGAA	1118				
Transfer (transferrin precursor)	AF019117	60	309	ATGCGGCCATCCAGGTTCAG	146	I, 10.2	Mono	Mono	Poly
				CCCGCCGACTTCAGTTTCGT	454				
VitgRecp (vitellogenin receptor)	AI650188	60	308	CCTCTGCAAGAAGCCGATGT	246	I, 12	Mono	Mono	Poly
				ACCCAGTCGTGGCTGTTGAT	553				
LF198	T58319	48	284	CTGGCGTAGATTCCGTGCTG	52	I, 20.0	Poly	Poly	Poly
				TCCGTGTTGACGTAGGTGGC	235				
Hexam2 (hexamerin 2)	U86080	56	436	TTCCTGGTGAAGCAGAAACA	100	I, 24	Poly	Poly	Poly
				TCATGCCATAGAATCCTTGC	535				
White	U88851	59		TACCTGACSGCACTGCTGATTG		I, 29.7	Poly	Mono	Poly
				TGATGACMGGCGGCCCAAC					
BMIOP (blood meal-induced ovarian protein	U84248	60	337	TTGAAGTCGTCGTTGCTGTT	196	I, 30	Poly	Poly	Poly
				CTGCCTTTGTCATGTTTGCT	532				
Riboptl-1 (ribosomal protein L1)	AI658439	56	285	AGAATCTTGCGTTGCCGTAC	137	I, 30	Poly	Poly	Poly
				GCTTCAGGGTTCACGTTGAT	421				
Actin	U20287			CTCTATCTACCTTCCAGGCTAT		I, 31	ND	ND	Poly
				GACGCTGACAAGTATCACAA					

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
LF178 (QM protein)	T58309	60	266	TAAGAATAAGCCTACCCGAAGT	26	I, 32.8	ND	ND	Poly
				TTGTTGATGCGGATGACG	291				
LF189b (ribosomal protein L29)	BM005474	50	237	ACAACCTGCGATTCTCCA	165	I, 33.4	Mono	Mono	Poly
				TGAACTTATTATGCCCACTT	401				
LF159 (Translation elongation factor 1-delta)	T58315	50	365	TCTTGAGCGAATGGATGGA	1	I, 34.3	Mono	Mono	Poly
				GGGCGGGTTTCTTGGACT	365				
LF314 (ADP-ribosylation factor 2, Arf2)	BM005509	60	214	GAGGAAATCGGCGGTCAG	27	I, 34.5	Poly/ND	Poly/ND	Poly
				CAGCAGTCATTGCGTTGG	240				
Ace (acetylcholineesterase)	AAB35001					I, 38.0	Mono	Mono	Poly
Ferritin	L37082	60	321	AGGAACAAACAGTAGGAGCAA	185	I, 44.6	Poly	Poly	Poly
				CCAGAGCGGATTCACCAG	505				
Aamy2 (midgut α-amylase 2)	U01208	60	330	ATGACGTTGGAGTGCGAATC	40	I, 45.1	Poly	Poly	Poly
				ACCAGGTTGCCGTAGATGAA	369				
Peroxnc (peroxinectin)	AI657546	58	305	GCATTTCAGCAGGGTAGA	130	I, 47	Poly	Poly	Poly
				AAGATCGGCAAGAACTCA	434				
Chitin1 (chitinase 1)	AF026491	60	327	AAACTGACCTACGCCCAAAG	687	I, 50	Poly	Poly	Poly
				GTCTACGCCGATGAACGAAT	1013				
APN (aminopeptidase N)	AF378117	50	203	TCCATCACGGCAATCACA	1852	I, 53.6	Poly	Poly	Poly
				AGATCCAGCCAGCATTCG	2054				
AbdA (abdominal a)	X67132	48	367	AGCGGTAGATCCTGTTGTCT	868	I, 59	Poly	ND	Poly
				AACCTCCAACTCACCTGTTC	1234				
AEGI22	BI099650	53	381	TTGAACGCTTGAAGGCATCT	384	I, 59.0	Poly	Poly	ND
				GACGGAATGGATTGTTGTCG	764				
Chromosome *II*									
AEGI18 (meiotic recombination protein REC14)	AF326340	45	228	CGCTGAGCATTGCCTACA	607	II, 0	ND	Poly	ND
				TGCCAACAACATCCGAGT	834				

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
LF250 (ribosomal protein L14)	T58311	55	102	TTGTCGCTATCGTCAATGTG	70	II, 16.5	Poly	Poly	ND
				TCAGGTGCAGGTGGTTCA	171				
LF338 (Cuticle protein Lcp65Ac)	BM005508	52	154	TTAGAAATGCTGGAACCG	49	II, 18.6	Poly	Poly	Poly
				TGCCGTAACTACTTCTTGG	202				
TrypEarly (trypsin-early)	X64362	60	459	CCAACGGTGGCATCATAGTGAAAG	295	II, 23	Poly	Poly	Poly
				GATCCATTGGCGAACAGTGGAGAC	753				
LF233	T58327	48	218	AAAGCGCCCACCTTTGCC	38	II, 29.4	Poly	Poly N/D	Poly
				ATCGCCCGTCAGCTTCAGTC	255				
TrypAbun (trypsin- Barillas Mury)	M77814	60	339	ACGGCTACCCTCGGTCAGTT	93	II, 31.8	Poly	Poly	Poly
				CTACCTCGCGGTCGGTAAAG	431				
PGK (Phosphoglycerate kinase)	AY043171	53	342	TGTTGGGTCGTGATGTGA	363	II, 37.7	Poly	Poly	Poly
				CCAGAGCTTGCGAGAAGT	704				
Fxa (Fxa-directed anticoagulant precursor)	AF050133	46	200	TTAGCACCAATCCAGCCTCA	214	II, 41	Poly	Mono	Poly
				TGGCACAACTGTTGGGAAGA	413				
Chym2		60		GACCGCATACTCCAATACC		II, 43	Mono	Mono	Poly
				GAGCACTGTCGTTGTCCC					
MtATPSyn (mitochondrial ATP synthase subunit-α)	AI650137	58	251	CCACAGCCGTCGAAGAAACC	83	II, 47	Poly	ND	Poly
				CGTCACCGATGCTCAACACG	333				
Mle (male-less)	AI650222	59	303	GTTTGATGACCCACCAGAA	40	II, 50	Mono	Mono	Poly
				ACAGACCAACGCAGAGCA	342				
D7 (D7 salivary gland protein)	M33156	60	896	AACTCAGTGTTTCGGCAAAT	2627	II, 54.1	Mono	Mono	Poly
				TCTTCTGGCGAATGTCTTTT	3524				
EF2 (elongation factor 2)	AI658391	59	340	ATGAACTACAAGACCGATGAG	951	II, 59.8	Mono	Mono	Poly
				CAAATACACGACCGAAAGC	1290				
Gst-2 (glutathiones-transferase-2)	S43311	56	282	TTATGCTGCTGTCGCTCCG	467	II, 60	Mono	Mono	Poly
				ATGCTGGCACCCTCTTCG	748				

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
D6L600	BH214535	59	307	CAACAAGTCGTCCTAATCG	143	II, 61.8	Mono	Mono	Poly
				TGGAGCAGTAGAACCGTAT	449				
F17M590	BH214537	59	314	CGGGAGGTTGCTGGAGT	159	II, 63.6	Mono	Mono	Poly
				CGGTTTGTGGCTTTTCG	472				
Sin3 (transcriptase factor, sin3)	AI561370	58	454	GTATCTGTTCCTGCGGTTGC	46	II, 70	Mono	Mono	Poly
				CCTGAAGTGCTCTGCT	499				
Chromosome *III*									
LF347	T58329	60	178	TATGATACGGACCGAACA	225	III, 0	Poly	Poly	Poly
				AAGCACAAGACTGGGACT	402				
LF253 (ribosomal protein L30)	T58331	60	182	CGCCAGGGAAAGGCTAAG	22	III, 16.7	ND	Poly	Mono
				GCATCGGTGATCGACAGG	203				
Malt (maltase)	M30442	48	234	GGACTGGTGGGAACATGGAA	72	III, 21.2	Mono	Mono	Poly
				CTTATCGGACAACCGCTGGA	305				
LF396 (CG9134 gene product)	BM005498	60	241	AACTCGGCTGAGGAGCAA	165	III, 21.3	Poly	Poly	Mono
				ACGAACGCCAATCCAAAA	405				
Apolipo2 (apolipophorin 2)	AF038654	54	329	GCTGGAATCGGTCAAACTCG	24	III, 25.0	Poly	Poly	Mono
				CCGGCCTTAACTTGCTGGTA	352				
Elastase (Trypsin-Late)	X64363	60	325	TGGCTTTGAAGTGCCCGTTGAG	44	III, 25	Poly	Poly	Poly
				CAAGTTCCTTCGTTGACCGGAGTG	368				
VitgConv (vitellogenin convertase)	L41842	60	296	TGCACAGAAGACCACCAATG	30	III, 26	Poly	Poly	Mono
				TCGACTGTTCCGCTGAGTTA	316				
Vitg (vitellogenin)	L41842	60	296	AGATGGCGTCTTCGGTAAAG	41	III, 40	Uninf	ND	Poly
				AGTGAGCACGGAACCTTTGT	336				
RNAHelic (ATP-dependent RNA helicase 46)	AI650162	58	399	TTTGACTTCATGGACCCTCC	109	III, 44	Poly	Poly	Poly
				AACTGTGACGCACATTGTCG	507				
Hsp70 (Heat shock protein 70)	AI658418	56	342	CCCGTCCTACGTGGCGTTCA	257	III, 46	Poly	ND	Poly
				GGTGGCCTGACGTTGCGAGT	598				

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
UGALS (UGALS vitellogenin)	U02548	60	328	AGGGCTACAATCCTGGCTAT	80	III, 46	Poly	ND	Poly
				GTATTVTGGCTGCTTGACGT	407				
DefA1 (defensinA1)	AF156088	54	193	CATTTGTTTCCTGGCTCTGT	88	III, 47.2	Poly	ND	Poly
				GAGCAGCACAAGCACTATC	280				
AspSyn (asparagine synthetase)	U84118	60	297	GGCTGAACAATGTGCGGTAT	88	III, 52	Poly	Poly	Poly
				TGATTTCCTGTGCCATCAGC	384				
Apyrase2 (apyrase 2)	L41391	54	317	TGATTGCATCGTCGTTGATT	103	III, 57.1	Poly	Poly	Poly
				CAACTTGCGCTGTTTGTTTT					
Primers attempted but not used									
Rdl (dieldren resistance)	U28803	48	441	GGGGTGACCAATAAAGCAAG	97	II, 17.8	Mono	Mono	Mono
				TGCGTCTAAATGGATGG	537				
ANTC		54	258	GATGCTAATGGGAAACAAAT	250	I, 70	Mono	Mono	Mono
				TGATACCGTTTAAGGCAAAG	507				
AEGI28	BI096849	46	195	GGGTGATGAAGTTGACGG	119	III, 14.6	Mono	Mono	Poly/ND
				GATTTCCTTTGTTGGGTTT	313				
LF128 (malate dehydrogenase precursor)	BM005494	51	189	TGCCGATTGCCTGCGAAAC	117	III, 1.0	Mono	Mono	Poly/ND
				GCGACAGGACGGGAATGA	305				
LF138 (RNA helicase)	T58332	60	192	AATCTGTTCGACGTGGTATG	1	II, 47.9	Poly/ND	Mono	Poly/ND
				CCGGCAATTCGGTGATGTT	192				
LF227 (CG11522 gene product)	T58323	51	189	AAAGTTCGTCCGGTGTCCAA	43	III, 17.4	Mono	Mono	Mono
				TCTTCTTCAGGAGAACCGTG	231				
Dynein (cytoplasmic dynein heavy chain)	AI618900	56	276	ATGGGATGCTTTGGTTACTC	110	III, 48	Mono	Mono	Mono
				TACTTTCTCAGCGTTGTTCG	385				
LF99a (heat shock cognate protein 79 protein)	BM005477		163	ATCTGTTCCGTTCCACCAT	187	III, 20.6	No amp	No amp	No amp
				ACTTGTTCAGCTCCCTTGC	349				
LF188b (Translation elongation factor 1-gamma)	BM005472		431	GGAGGAGCAGCCCAGCAGAA	32	III, 20.1	No amp	No amp	No amp
				GCGGGAAATTGGTTGTTGTTTT	462				

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
LF96 (opsin)	BM005491		220	TGGCCTGGACTCCGTATC	99	III, 20.1	No amp	No amp	No amp
				TGGTTATCGTGGCTGCTTT	318				
LF92 (ribosomal protein CEP52)	BM005493	60	243	TGTCCGACTACAACATCCA	57	III, 16.4	Mono	Mono	Poly/ND
				TGAAACACCTCCCAAGAAT	299				
LF111 (ribosomal protein L13)	BM005492	56	158	CAGCAAGATGGTGAAGGGAA	46	III, 19.0	Mono	Mono	Mono
				CAGCAGGACGTGGGAACA	203				
LF334 (ATP synthase G chain, mitochondrial)	BM005506	46	154	TTTAACCGTGAAGGAGGC	262	II, 68.3	Mono	Mono	Mono
				TTCAATGATCCGACCAATAC	415				
LF223 (ribosomal protein L9)	BM005515	55	249	TTTCTTCCACTCCTGCTCT	18	II, 68.8	Mono	Mono	Mono
				GTGACCGTTGTGAACTCG	266				
LF211(cytidine deaminase)	BM005514		294	TTTTCAACCCTTGATGCG	104	II, 68.9	No amp	No amp	No amp
				AGAGTCTGGCGACAGTTCC	397				
LF150 (*Pol* protein)	BM005476		222	ATGTGGCGTATGTCAGTA	201	I, 40.7	No amp	No amp	No amp
				CAGTGTTCAACGGGAATT	422				
LF115 (ribosomal protein L18)	R67978	55	146	CCGCAGAACGCACAAGAA	67	II, 7.3	Mono	Mono	Mono
				TCCGACAACGACGGCAAT	212				
nAcBP (nucleic acid binding protein)	AY040341		322	AACAATCCTGTCGTGGTC	108	I, 44.5	No amp	No amp	No amp
				CATCAACTTACAATTCCCT	429				
B8L720	BH214539		126	CGGAGCTGGAGTGAAACT	402	I, 19.4	No amp	No amp	No amp
				GCCTACAGGACCCGATTA	527				
LF99b (heat shock cognate protein 79 protein)	BM005477	50	163	ATCTGTTCCGTTCCACCAT	187	I, 39.2	Mono	ND	Poly/ND
				ACTTGTTCAGCTCCCTTGC	349				
LF231 (ribosomal protein L36)	BM005478	56	155	CCCTGAAGTTCCTGAAGC	42	I, 38.6	Mono	Mono	Poly/ND
				AGAGCGAACGGTGTAAAG	196				
LAP (lysosomal aspartic protease)	M95187	56	188	CACAACAAATACAATGCCAAGA	440	I, 36.6	Mono	Mono	Mono
				GCCGCAACGAATACCAATC	627				

Gene name	Accession no.[ξ]	T_a[*]	Size[ϕ]	Forward/reverse primer sequence	Primer location[Ψ]	*Ae. aegypti* linkage location[ω]	P7.1 F_2s	P7.1 F_5s	Y2.2
ARC3	BH214542		302	GGACGGTGTCGGTAGAGGA	51	I, 38.2	No amp	No amp	No amp
				TTGGCATGAAACGAGAAGC	352				
LF284 (ribosomal protein L4)	BM005502		280	CATCTACGCCAAGGACGAG	31	I, 38.0	No amp	No amp	No amp
				CAGGACCTTACGGATTTCAT	310				
LF377	BM005496	60	166	CTGCAAAATCTTGGAAAT	53	III, 21.3	Mono	Mono	Mono
				GACATTGTGCTGGCTGAT	218				
LF204 (CG15697 gene product)	BM378050		110	CCACCACCATGTCTCAACC	146	I, 35.2	No amp	No amp	No amp
				CAAGCGTCGCATCCAGTA	255				
Sialokinin (salivary vasodilatory protein-sialokinin 1)	AF108099	60	298	CCCGATAAATCCTTTCCTTC	83	I, 4	Mono	Mono	Mono
				AAATGGGTATCCCTTTCCTG	380				
Immuno (immunophilin)	AI618957	60	360	AAATCTCGCACCCGTAAA	22	I, 4	No amp	No amp	No amp
				GCCCTCGTCAAAGTTCAG	381				
Cathbp (cathepsin b-like thiol protease)	L41940	60	343	CAAATTCGGAACCTCACCAG	133	I, 7	Mono	Mono	Mono
				TATCCACCCTTGCATCCATC	475				
PPAllost (preproallatostatin)	U66841	60	326	AAGAAGAAGATTACGACGAT	16	I, 36	No amp	No amp	No amp
				CAATTTCCCACTATCACTTA	341				
ADP/ATPtla, ADP/ATPtlb (ADP/ATP translocase)	AI657540	57	284	CTGGCGCTACTTCATGGGTA	14	II, 9	Mono	Mono	Mono
AI650113	AI650176			ATCGAGGTGTTCTTCGGGTC	297	II, 27			
Erudi (enhancer of rudimentary)	U66869	46	444	CGGACGAAGCTGAATGAAGA	13	I, 4	Mono	Mono	Mono
				TCCGCTAACTGATCCACGAA	456				
InsRec (insulin receptor)	U72939	46	313	AGCAGCGGCAGGATCGGTAG	24	II, 19	Mono	Mono	Mono
				CGCGGAACAGCAGCAGGTAC	336				
LF101 (ribosomal protein L34)	BM005475	50	277	AACTCGCCAAGATGGTCC	38	I, 38.0	Mono	Mono	Mono
				ATGCGTTCACGGAGACAA	314				

[ξ] accession number for NCBI; [*] Temperature in degrees celsius; [ϕ] size in bp; [Ψ] location within the given gene in bp; [ω] location by chromosome followed by cM; Poly, marker was polymorphic; Mono, marker was monomorphic; ND, marker was not done; Uninf, marker was uninformative.

The negative control and several randomly selected wells of the plate were tested for the presence of product using a 1.2% TBE agarose gel containing 0.004% ethidium bromide. For this, 4 µl PCR product were combined with 4 µl DNA loading buffer and added to the wells of the agarose gel. Electrophoresis was performed for 15-20 minutes at 112 V and PCR product was visualized under a UV transilluminator.

For the Y2.2 F_2 individuals a total of 44 primer pairs were used, for the P7.1 F_2 individuals a total of 32 primer pairs were used, and for the P7.1 F_5 individuals, a total of 26 primer pairs were used (Table 4.1 and Figure 4.1 A, B, and C respectively).

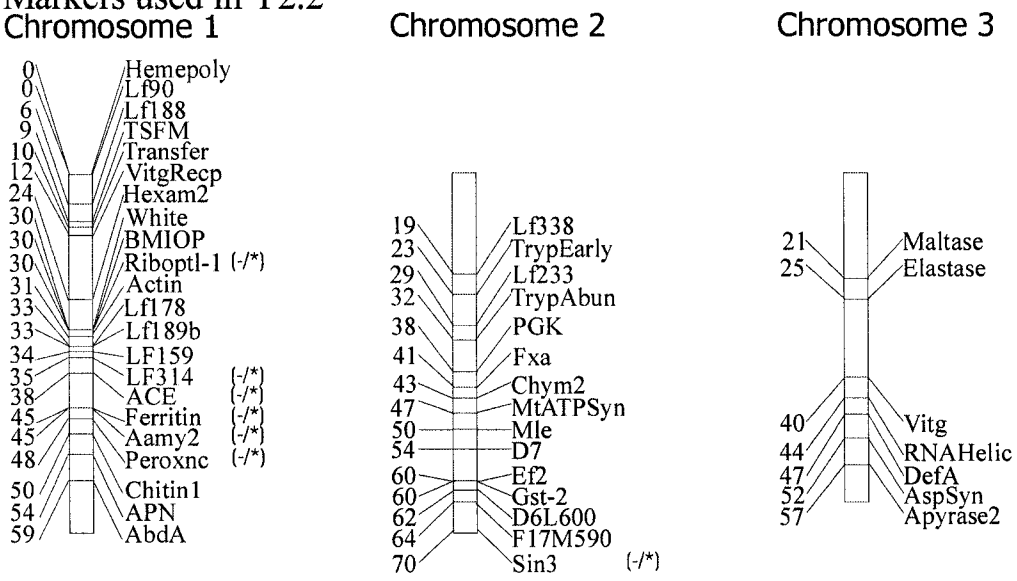
Single strand conformation polymorphism (SSCP) was performed and fragments were detected using silver staining (Black and DuTeau 1997, Bosio *et al.* 2000).

QTL mapping: Each marker was subjected to a χ^2 goodness of fit test to look for conformity to expected Mendelian segregation ratios of alleles for the F_2 generations (Y2.2 and P7.1). This test was not appropriate for the P7.1 F_5 individuals, because all matings beyond the F_2 generation were random. An initial scan for potential QTL was performed among marker loci using the χ^2 goodness of fit test to detect markers linked to QTL. At each marker locus, the null hypothesis (H_0) was that infection rates were equal in each genotypic class. Markers that deviated significantly from expected values were considered to be linked to a putative QTL. The direction of inheritance of the phenotype was also evaluated for each marker with a significant χ^2 value to determine if the marker allele putatively linked to susceptibility originated from the susceptible parent.

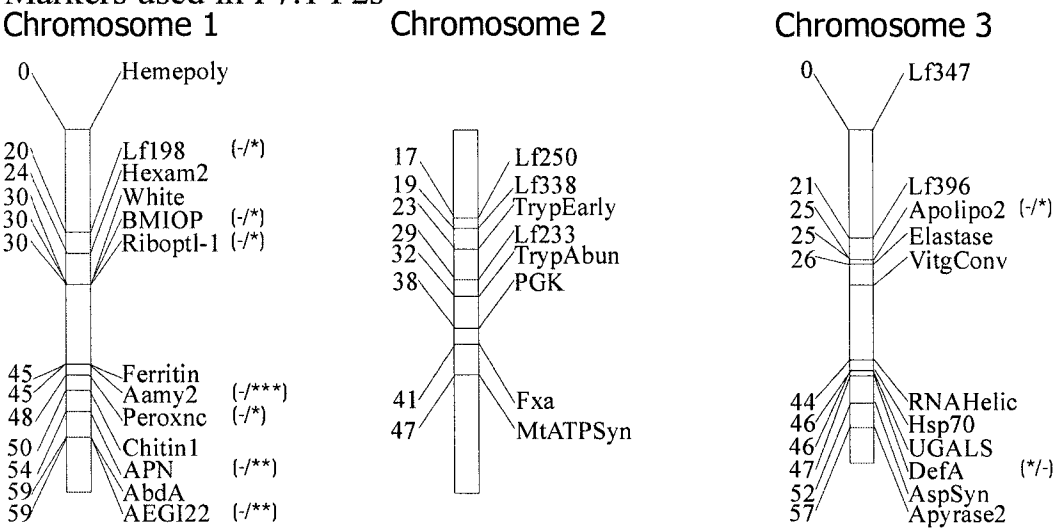
The computer program BINARYQTL (Xu *et al.* 1998) was used for interval mapping (Lander and Botstein 1986). Binary QTL is a FORTRAN program used to map QTL associated with binary traits. The algorithm used assumes that there is an

Figure 4.1: Linkage maps used for QTL mapping in each of the families: Y2.2 F_2's (A), P7.1 F_2's (B), and P7.1 F_5's (C). Markers with significant association to MIB/MEB as determined by χ^2 goodness of fit tests are denoted by asterisks at the following significance levels. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. The right side of each chromosome is labeled by markers used in each cross and the left side is labeled with corresponding linkage distances measured in cM. Linkage distances are based on linkage maps created by Severson *et al.* (2000) and Fulton *et al.* (2001).

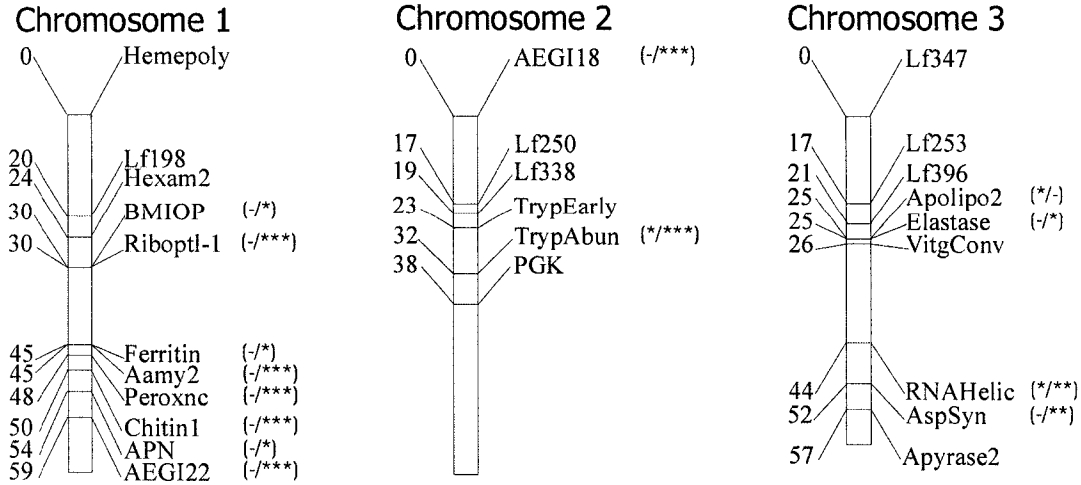
A. Markers used in Y2.2



B. Markers used in P7.1 F2s



C. Markers used in P7.1 F5s



unobservable continuous variable (liability) that is responsible for expression of the binary phenotype. That is, there is a certain liability threshold above which one phenotype is observed (dengue susceptibility) and below which another phenotype is observed (dengue refractoriness). In the case of dengue susceptibility, one only observes that mosquitoes become infected or not, and in this sense, the liability is distributed as an unobservable continuous quantitative trait that is seen as a binary characteristic. BINARYQTL estimates the liability using a single linear model with heterogeneous residual variance and then uses standard interval mapping to determine the most probable location of QTL on a linkage map. This model allows for calculation of the sampling variance for each estimated parameter. BINARYQTL calculates the 90, 95, and 99% comparison-wise thresholds at each centimorgan interval, and also calculates the 90, 95, and 99% experiment-wise thresholds (Churchill and Doerge 1994).

A log of odds ratio, or LOD score, is used to express the strength of evidence for a QTL at a given location. The LOD is calculated as follows:

$$LOD = -\log_{10}(L_0/L_1)$$

where L_1 is the maximum likelihood function estimate as described by Lander and Botstein (1989) and L_0 is the maximum likelihood estimate of the null hypothesis (H_0) The H_0 is that phenotypic effect at a given location is 0, meaning that there is no effect on the phenotype of interest at that location. If the presence of a QTL is estimated to be 1,000 times more likely than the H_0, the LOD score would be 3.

The computer program QTL Cartographer 1.16 (Basten *et al.,* 1994, Basten *et al.*, 2002) was also used to analyze the data for QTL. In addition to using IM, QTL Cartographer is able to perform composite interval mapping (CIM). Because LOD at

different map intervals may covary with one another depending on the magnitude of their effect and on their linkage relationships, they may be subject to an upward bias at individual intervals. To at least partially correct for this, CIM (Zeng 1994) adjusts LOD scores at individual intervals. This is accomplished through the use of a variable number of markers to control for effects of other intervals in the map and a variable window size to adjust for the effects of intervals in proximity to the interval under analysis.

QTL Cartographer 1.16 uses a sequential order of modules to characterize and rank QTL. The first module is called Qstats and calculates basic statistics and summarizes missing data. Qstats also tests for adherence to Mendelian segregation at all marker loci. The second module is LRmapqtl. LRmapqtl fits the data to a simple linear regression model one marker at a time. It is a first approximation of where QTL may be found on the genome using the linear model:

$$y_i = b_0 + b_1 x_i + e$$

where y_i is the phenotype of the ith individual and x_i is the indicator variable for the marker genotype. b_0 and b_1 are regression parameters that can be estimated and e is assumed to have a normal distribution.

The third module is SRmapqtl, which performs a forward stepwise regression to test each marker in turn for its effect on the quantitative trait using linear regression. The marker with the largest F-statistic is assigned a rank of one and included in all subsequent analyses. This module then tests the remaining markers and assigns the second rank to the marker with the largest F-statistic. This is repeated until all markers have been assigned a rank. This process determines which markers are most closely associated with the QTL, and establishes values for the strength of the phenotypic effect at these markers.

In other words, this module ranks the degree to which individual markers explain the phenotypic variance (σ^2_p). Because of this stepwise procedure, if marker genotypes at a QTL on chromosome *III* are correlated to marker genotypes at a QTL on chromosome *II*, the effects of the chromosome *III* QTL may be underestimated. The output of SRmapqtl is used by the Zmapqtl module.

Zmapqtl is the fourth module and can perform Interval Mapping (IM) and Composite Interval Mapping (CIM) as well as performing a permutation test (Churchill and Doerge 1994 and Doerge and Churchill 1996). CIM combines interval mapping with multiple regression. The statistical model is defined as:

$$\mathbf{Y} = \mathbf{x}^* b^* + \mathbf{z}^* d^* + \mathbf{XB} + \mathbf{E}$$

where $\mathbf{Y}$ is a vector of trait values, b^* and d^* are the additive and dominance effects of the putative QTL being tested, $\mathbf{x}^*$ and $\mathbf{z}^*$ are indicator variable vectors specifying the probabilities of an individual being in different genotypes for the putative QTL constructed by flanking markers, $\mathbf{B}$ is the vector of effects of other selected markers fitted in the model, $\mathbf{X}$ is the marker information matrix for those selected markers and $\mathbf{E}$ is the error vector. Estimates of the parameters are obtained by maximum likelihood.

Zmapqtl offers 6 models for mapping. CIM is model 6 and was used in most analyses. When no genetic background (no associations with a QTL) was detected in SRmapqtl, Zmapqtl defaults to model 3, which basically performs IM and does not use other markers to control for genetic background. This is the method developed by Lander and Botstein (1989).

Model 6 requires 2 parameters not needed for IM. One is the number of markers (n_p) used to control for the genetic background and the other is a window size (w_s). Model

6 reads the output of the SRmapqtl to determine which markers to use to control for the genetic background. The w_s blocks out a region of the genome on either side of the markers flanking the test site. Flanking sites are closely linked to the test site, and cannot be used as background markers, as they would eliminate the signal from the test site itself. For this analysis, the default settings of $n_p = 5$ and $w_s = 10$ cM were used.

Within Zmapqtl, it is possible to specify the use of a permutation test to determine the experiment-wise significance levels and comparison-wise probabilities (Churchill and Doerge 1994, Doerge and Churchill 1996) by shuffling phenotypes against genotypes. The comparison-wise probability is the proportion of permuted datasets that have test statistics less than the observed data set test statistic. The experiment-wise significance level is determined by taking the highest test statistic in each permutation, and ordering these at the end of the permutations. In this work, all analyses were permuted 1000 times to estimate the comparison-wise and experiment-wise thresholds.

Estimation of variance components: For the P7.1 F_5 individuals, marker genotypes were scored as 0, 1, or 2 according to the number of alleles inherited from the *D2MEB* parent. Pearson's correlation coefficients between MIB, MEB or VC phenotypes and marker genotypes were computed using PROC CORR in SAS 8.1 (SAS Institute 1999). MIB, MEB or VC phenotypes were regressed on marker genotypes. RSQDELTA in SAS 8.1 was used to compute the change in R^2 and the associated F-statistics and p values as genotypes are added to a linear regression model. The F-statistic and p values represent a partial F-statistic for the general linear model.

Results

Families used for QTL mapping: Table 4.2 shows the phenotypes for the different F_1 intercross families. *D2S3*x*D2MEB* (Y2.2) had 76 phenotyped females with a MIR of 82% (62/76) and a DIR of 73% (45/62), and a *D2MEB*x*D2S3* cross (P7.1) had 40 phenotyped females with a MIR of 83% (33/40) and a DIR of 52% (17/33). Because the P7.1 F_2 family size was not statistically powerful, this family was expanded as a recombinant inbred line through the F_5 generation. In generation F_5, 296 females were phenotyped and genotyped and had a MIR of 81% (241/296) and a DIR of 41% (99/241).

Genotype - Phenotype Associations: χ^2 goodness of fit tests indicated that several marker genotypes were not distributed independently of MIB and MEB phenotypes (Figure 4.1). Loci that are statistically associated ($p < 0.05$) with a midgut infection barrier (MIB) or a midgut escape barrier (MEB) by χ^2 goodness of fit tests are denoted by asterisks in parenthesis for MIB/MEB (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). In the Y2.2 F_2 individuals (Figure 4.1A), chromosome *I* exhibited a significant association with MEB for genotypes at 30 cM and 35-48 cM. In the P7.1 F_2 individuals (Figure 4.1B), genotypes at loci on chromosome *I* were significantly associated with MEB at regions 20-24, 30, 45-48, 54, and 59 cM. On chromosome *III*, MEB was correlated to genotypes at 25 cM and MIB was associated with genotypes at 47 cM. In the P7.1 F_5 individuals, genotypes at loci on chromosome *I* were significantly associated with MEB at 30 and distributed at loci 45-59 cM, on chromosome *II* at 0 and 32 cM, and on chromosome *III* at 25 and 44-52 cM. Significant associations were also found between MIB and genotypes at loci on chromosome *II* at 32 cM and chromosome *III* at 25 and 44 cM.

Table 4.2: Phenotypes of F_2 individuals of the intercross families generated in this study. Selection of families for QTL mapping was based on family size and phenotype. MIR was calculated as the total number of individuals with a midgut infection divided by the total number tested. DIR was calculated as the total number of individuals with a disseminated infection divided by the total number of individuals with a midgut infection. VC was calculated as the total number of individuals with a disseminated infection divided by the total number of individuals tested.

	Family Size	VC %(#)	MIR %(#)	DIR %(#)
D2S3xD2MEB				
Y1.2	30	77%(23/30)	N/D	N/D
Y2.2	76	59%(45/76)	82%(62/76)	73%(45/62)
Y4.2	69	87%(60/69)	94%(65/69)	92%(60/65)
Y7.1	67	75%(50/67)	87%(58/67)	86%(50/58)
Y8.1	16	56%(9/16)	N/D	N/D
Y9.1	42	71%(30/42)	N/D	N/D
D2MEBxD2S3				
P1.1	24	71%(17/24)	N/D	N/D
P2.2	11	36%(4/11)	N/D	N/D
P3.2	48	88%(42/48)	96%(46/48)	91%(42/46)
P6.1	3	33%(1/3)	N/D	N/D
P7.1	40	43%(17/40)	83%(33/40)	52%(17/33)
P8.1	12	42%(5/12)	N/D	N/D
P9.2	9	67%(6/9)	N/D	N/D
P10.3	11	91%(10/11)	N/D	N/D

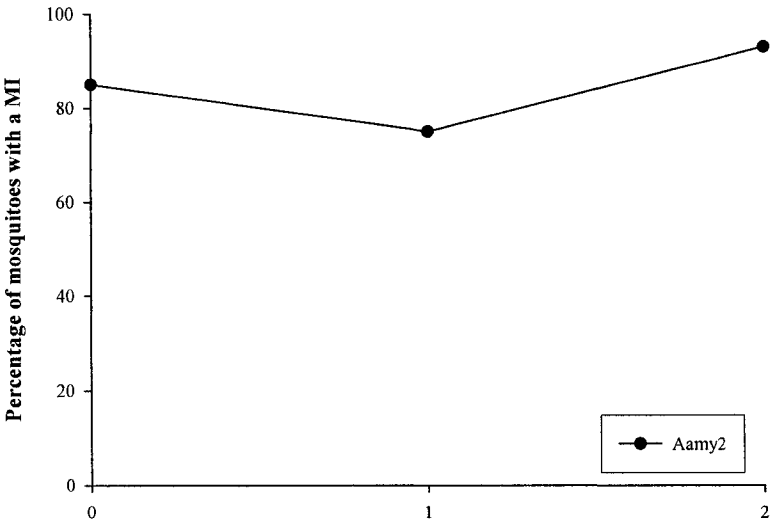
N/D indicates that this was not done for that family

The crossing design implemented focused on identifying QTL associated with MEB. It was not possible to distinguish which parent contributed alleles associated with MIB, because both parents were expected to be highly midgut susceptible. Figure 4.2 shows that only a small difference in MIR was observed between individuals homozygous for alleles from the *D2MEB* parent and those homozygous for alleles from the *D2S3* parent. The number of alleles from the *D2S3* parent is reported to maintain consistency among the figures. At all loci except *Apyrase2*, having more alleles inherited from the *D2S3* parent conferred a higher MIR. More copies of alleles from the *D2S3* line at the *Apyrase2* locus were actually associated with a decrease in infection rate.

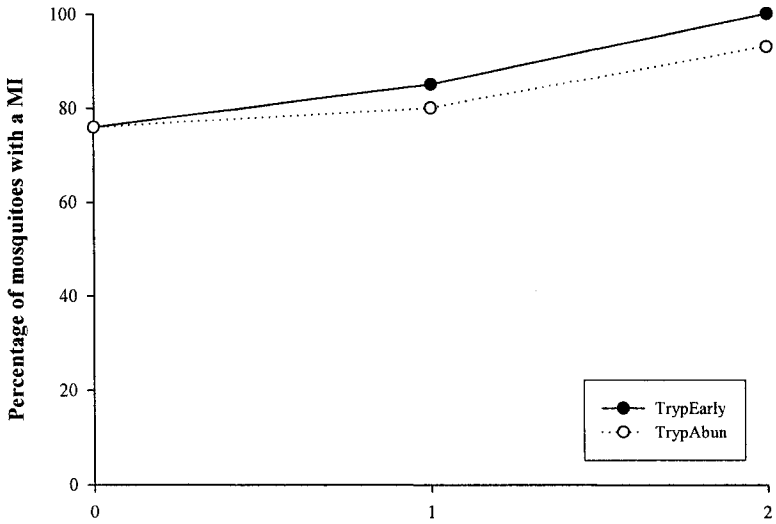
In contrast, there was an expected pattern of inheritance for MEB. The *a priori* assumption was that alleles significantly associated with MEB would be inherited from the *D2MEB* P_1 parent. For the P7.1 F_2 and F_5 individuals, alleles at the majority of markers associated with MEB were inherited as expected, (individuals homozygous for alleles from the *D2MEB* parent expressed a significantly higher rate of MEB) than individuals homozygous or heterozygous for alleles from the *D2S3* parent. Results for the P7.1 F_5 individuals are shown in Figure 4.3. Exceptions to expected results occured at markers *BMIOP*, *Aamy2*, *AEGI18*, and *Elastase*. In the Y2.2 F_2 individuals, MEB was associated with alleles inherited from the *D2MEB* parent with the exception of genotypes at *LF314* and *ACE* in which individuals with alleles inherited from the *D2S3* P_1 parent had a higher rate of MEB. DI of individuals in the P7.1 F_5 family with 0 copies of alleles inherited from *D2S3* at a single locus on chromosome *I* ranged from 29-35%, while DI of individuals with 2 copies of alleles inherited from *D2S3* ranged from 50-70%. Similar results were seen for chromosomes *II* and *III* (Figure 4.3).

Figure 4.2: Plot of the inheritance of susceptibility alleles at marker loci linked to QTL for MIB on chromosomes *I*, *II* and *III* in the P7.1 F_5 family.

MIB Chromosome 1



MIB Chromosome 2



MIB Chromosome 3

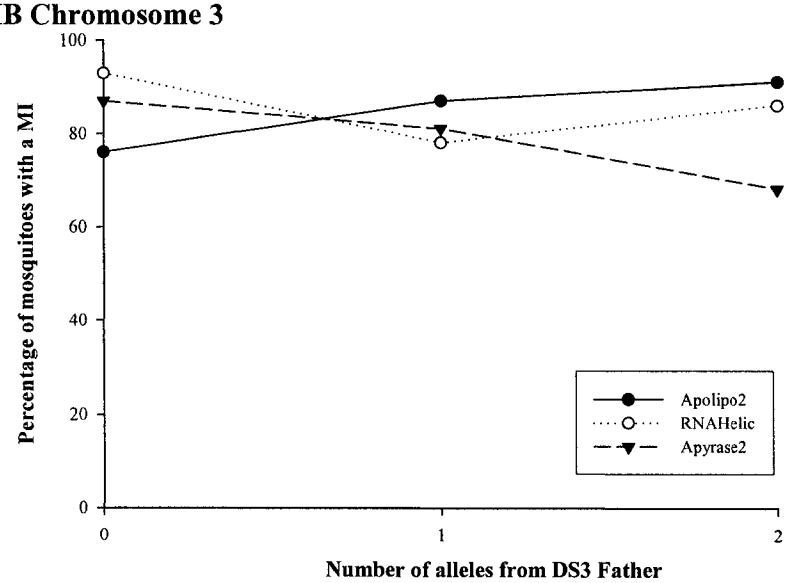
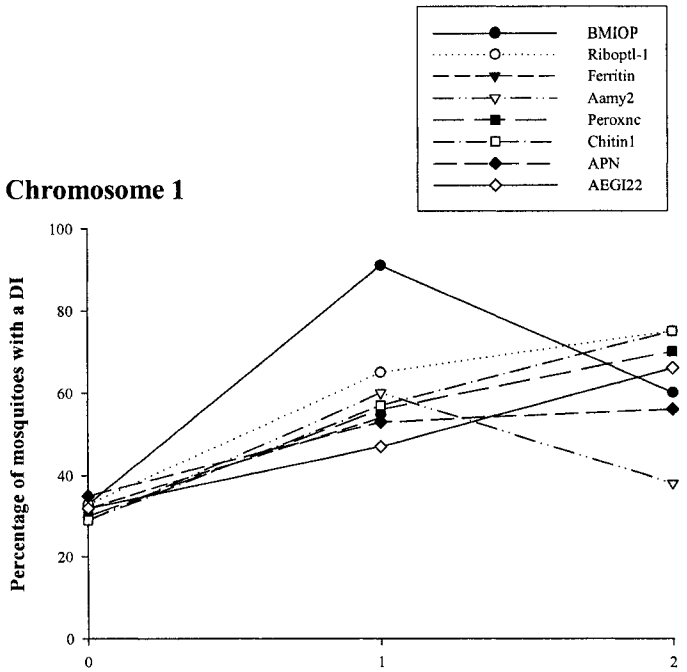
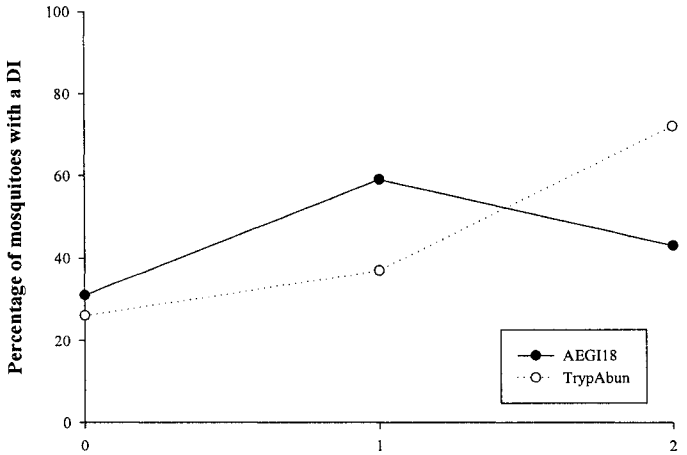


Figure 4.3: Plot of the inheritance of susceptibility alleles at marker loci linked to QTL for MEB on chromosomes *I*, *II* and *III* in the P7.1 F_5 family.

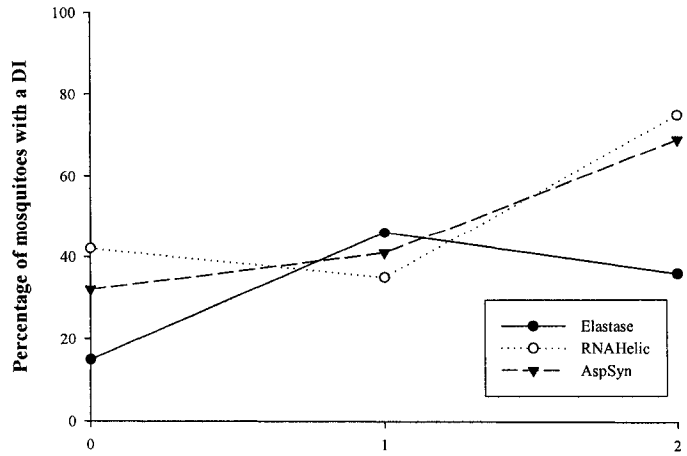
MEB Chromosome 1



MEB Chromosome 2



MEB Chromosome 3



Number of alleles from DS3 father

In general, the more copies of alleles inherited from the *D2S3* parent, the more susceptible to disseminated infection the mosquitoes tended to be. When the number of alleles inherited from the *D2S3* parent was regressed against the percentage of mosquitoes exhibiting a MI (Figure 4.2) or a DI (Figure 4.3), a linear relationship was not always observed. In some cases it was fairly linear, as with *Chitin1* and *AEGI22* on chromosome *I* of Figure 4.2. In other cases, as with *APN* (chromosomes *I*), having 1 or 2 copies of an allele inherited from the *D2S3* parent gave similar DIR, while having 0 copies from the *D2S3* parent gives a reduced DIR. This can be interpreted as the *D2S3* susceptible allele being dominant to the *D2MEB* refractory allele. At the *TrypAbun* (chromosome *II*) locus, having 0 or 1 copy of alleles from the *D2S3* parent gives a similar susceptibility pattern while having 2 copies causes an increase in the DIR. This can be interpreted as the *D2MEB* refractory allele being recessive to the *D2S3* susceptible allele.

Results showed a basic additivity when the number of alleles inherited from the *D2S3* parents was regressed against the DIR (Figure 4.4). In general, the greater the number of alleles inherited from the *D2S3* parent, the higher the DIR. Small sample sizes may skew results. When considering only groups with sample sizes of 20 individuals or more, DIR increased with the number of alleles from the *D2S3* parent in a fairly linear pattern, beginning at 19% DIR with 4 alleles and increasing to greater than 80% DIR with 10 alleles. This suggests that alleles for MEB act additively across loci.

QTL Mapping: The most probable locations of QTL conditioning MIB and MEB are estimated using standard IM for Y2.2 F_2 individuals (Figure 4.5) using BINARYQTL (Xu *et al.* 1998). The 95% comparison-wise and the 99% experiment-wise thresholds were also estimated (Churchill and Doerge 1994). LOD scores for MIB or MEB did not

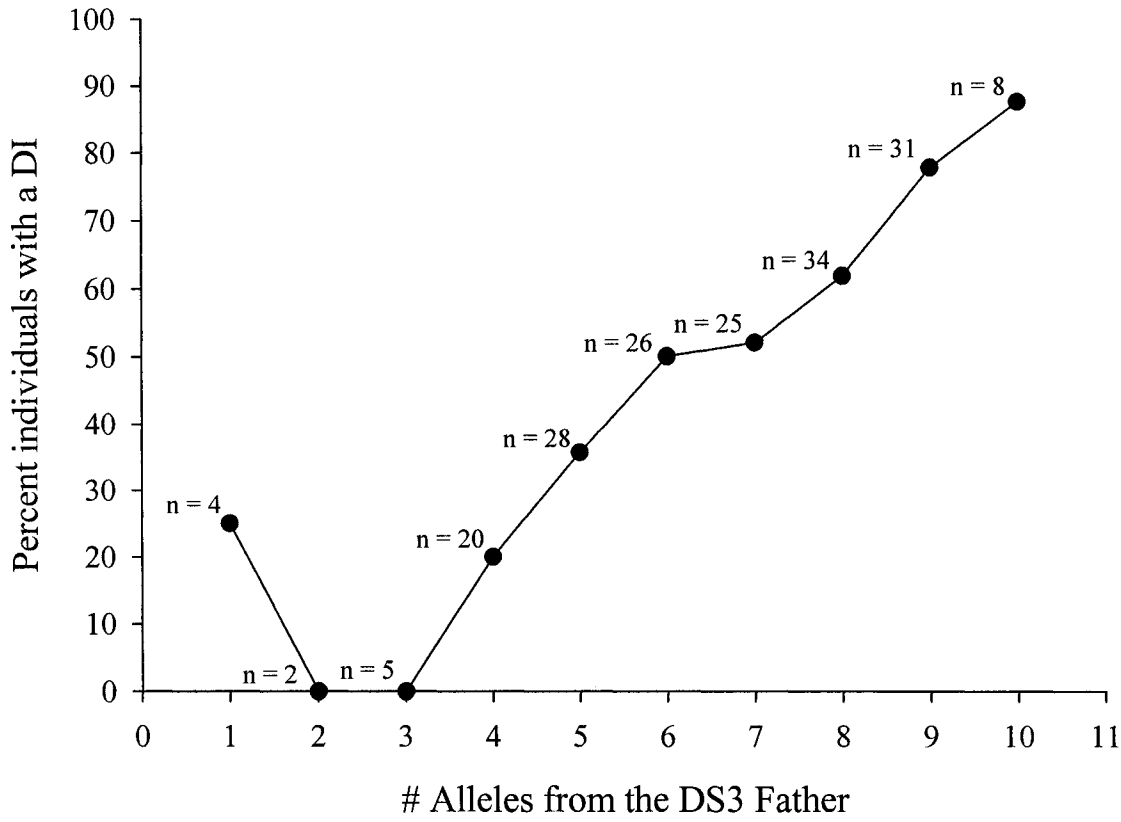
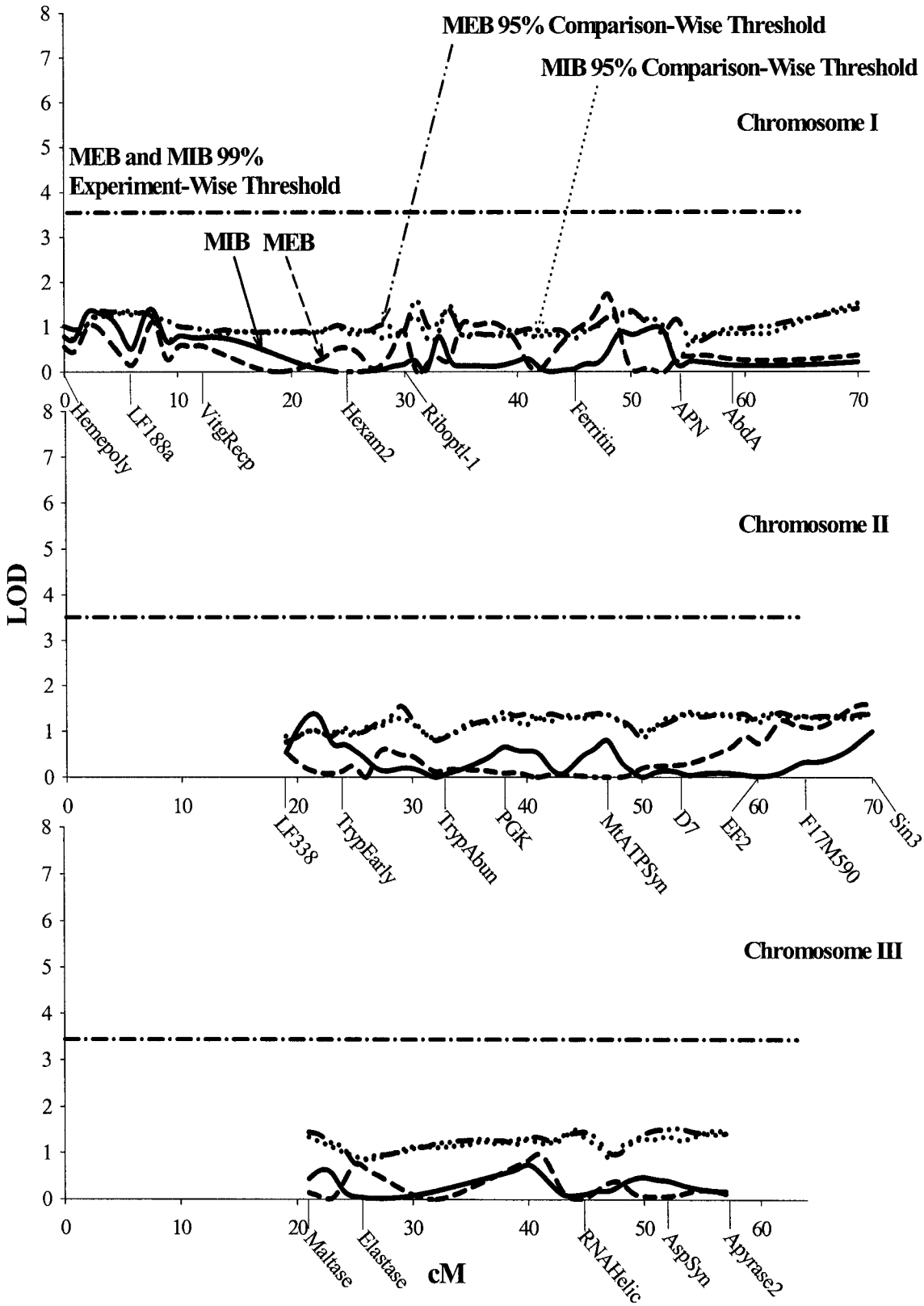


Figure 4.4: Number of alleles inherited from the DEN susceptible father at markers associated with MEB plotted against the percentage of individuals exhibiting a disseminated infection. Sample size is given at each point.

Figure 4.5: Plot of LOD values associated with MIB and MEB along chromosomes *I*, *II* and *III* in the Y2.2 F_2 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1A. The LOD estimated for interval mapping (IM) using a heterogeneous residual variance model for binary traits (Xu *et al.* 1998) appears as a solid line for MIB and as a dashed line for MEB. The comparison-wise 95% threshold is a dotted line for MIB and a dot-dot-dash for MEB. The experiment-wise 99% threshold is a dot-dash line across the top of each graph.



exceed the 99% experiment-wise threshold. The experiment-wise threshold tended to underestimate the number of QTL while the comparison-wise threshold overestimated the number of QTL, and often identified QTL of minor effect (Churchill and Doerge 1994, Xu *et al.* 1998). LOD scores for MIB exceeded the 95% comparison-wise threshold on chromosome *I*, at 3 and 9 cM, and on chromosome *II* at 23 cM. LOD scores for MEB also exceed the 95% comparison-wise threshold on chromosome *I* at 30, 33-39 and 46-49 cM and on chromosome *II* at 68-70 cM. In no case were the 95% comparison-wise thresholds exceeded by much. Most of the locations where QTL were identified through IM for MEB, agree with the locus-wise χ^2 goodness of fit tests (Figure 4.1).

In the Y2.2 family, CIM was performed using QTL Cartographer 1.16 (Basten *et al.* 2002) (Figure 4.6). The only significant QTL ($p = 0.001$) found in this family was for MEB and was located on the bottom of chromosome *II* at 64-68 cM. This corresponded with results of the χ^2 goodness of fit tests (Figure 4.1A), as the marker *Sin3* at the bottom of chromosome *II* was found to be significantly associated with MEB. Although there was a large spike in the LOD score for MIB on chromosome *III*, this was not significant ($p = 0.07$). CIM gives a more conservative estimation of the number of QTL for MIB and MEB in the Y2.2 F_2 family, if one uses the 95% comparison-wise threshold for IM. The magnitude of the QTL found on the bottom of chromosome *II* was slightly larger for CIM (LOD~2) than for IM (LOD~1.8).

IM was completed for the P7.1 family F_2 individuals (Figure 4.7) and F_5 individuals (Figure 4.8) using BINARYQTL (Xu *et al.* 1998). In this family, no polymorphic markers have been found on the bottom of chromosome *II*, and it was not possible to test for the MEB QTL identified in the Y2.2 intercross. In the F_2 individuals

Figure 4.6: Plot of LOD values associated with MIB (solid line) and MEB (dotted line) along chromosomes *I*, *II* and *III* for the Y2.2 F_2 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1A. The LOD score was estimated using composite interval mapping (CIM) (Zeng 1994, Basten *et al.* 1994, 2002). Intervals at which the CIM LOD estimated in ZmapQTL (QTL Cartographer 1.16) is significant are bracketed and labeled with the *p*-value.

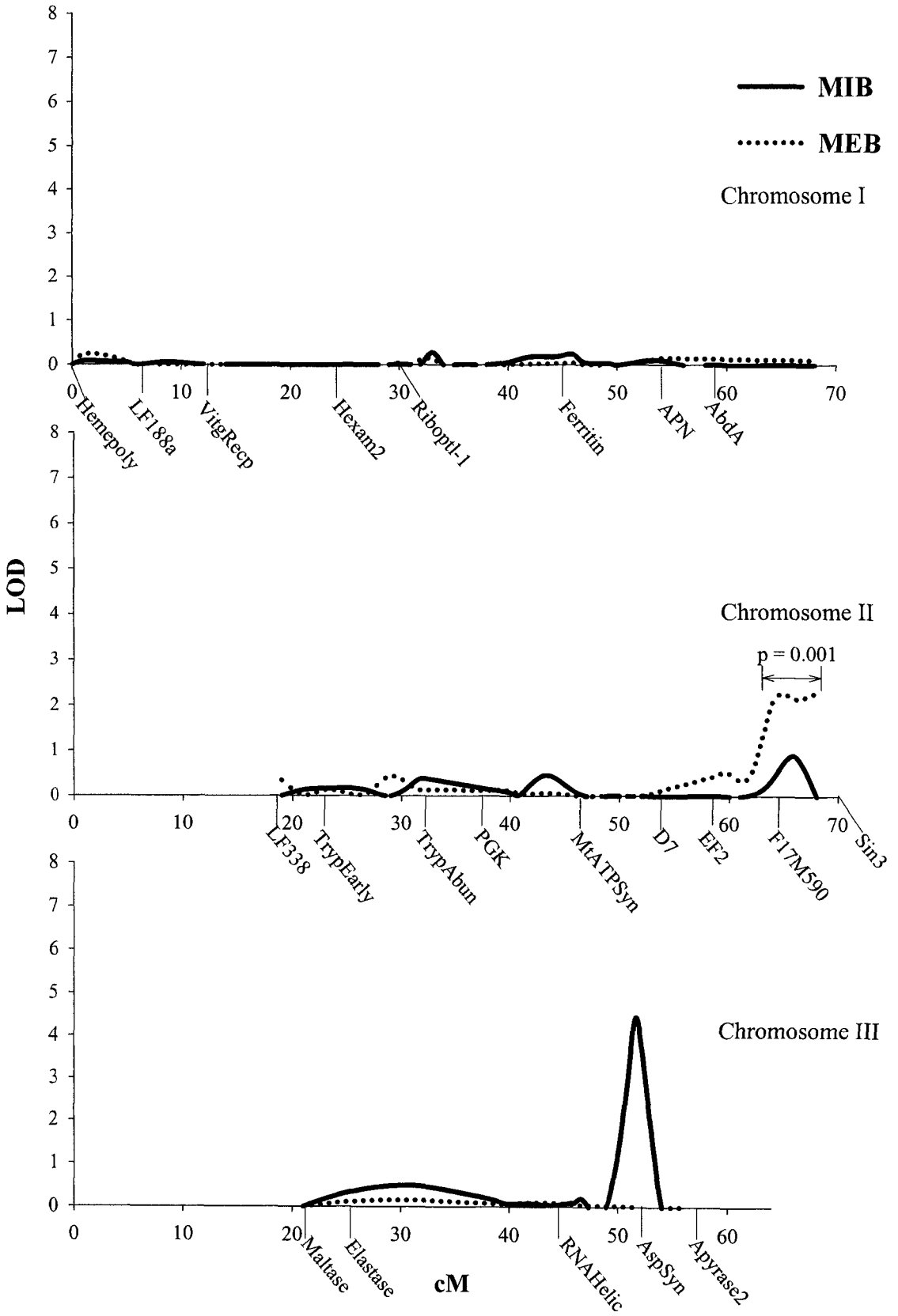


Figure 4.7: Plot of LOD values associated with MIB and MEB along chromosomes *I*, *II* and *III* in the P7.1 F_2 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1B. The LOD estimated for interval mapping (IM) using a heterogeneous residual variance model for binary traits (Xu *et al.* 1998) appears as a solid line for MIB and as a dashed line for MEB. The comparison-wise 95% threshold is a dotted line for MIB and a dot-dot-dash for MEB. The experiment-wise 99% threshold is a dot-dash line across the top of each graph.

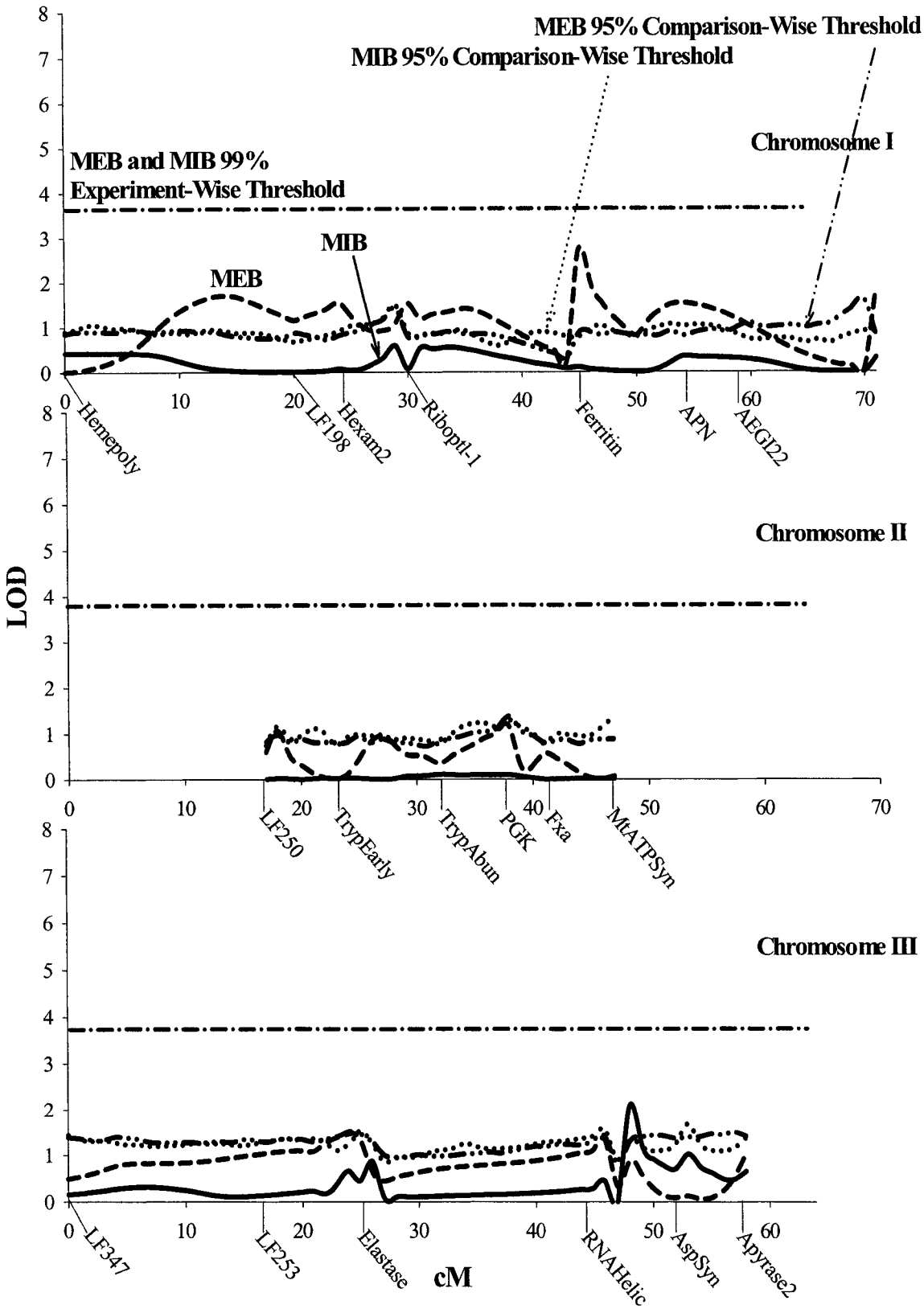
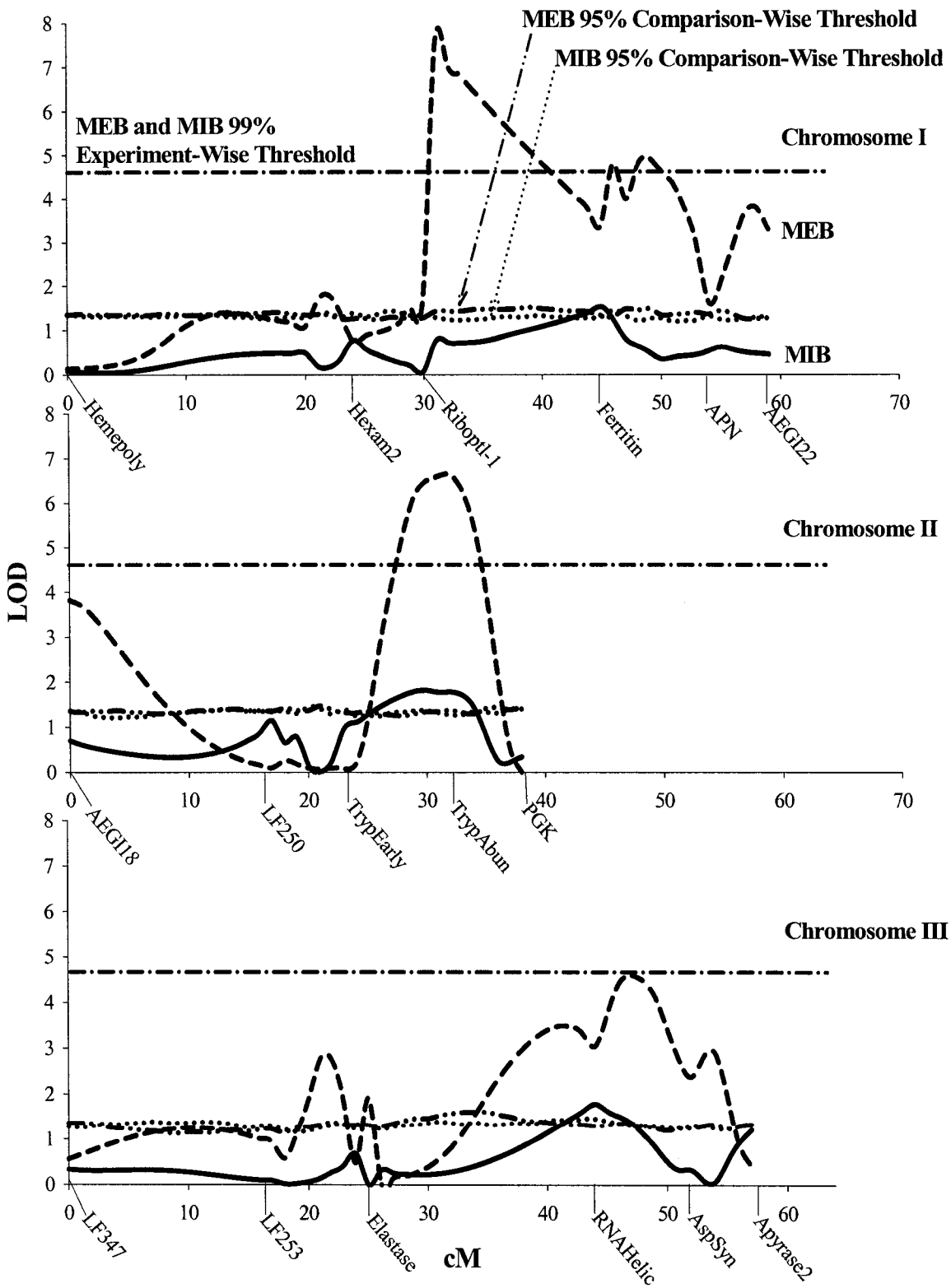


Figure 4.8: Plot of LOD values associated with MIB and MEB along chromosomes *I*, *II* and *III* in the P7.1 F_5 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1C. The LOD estimated for interval mapping (IM) using a heterogeneous residual variance model for binary traits (Xu *et al.* 1998) appears as a solid line for MIB and as a dashed line for MEB. The comparison-wise 95% threshold is a dotted line for MIB and a dot-dot-dash for MEB. The experiment-wise 99% threshold is a dot-dash line across the top of each graph.



(Figure 4.7) the LOD scores for MIB and MEB never exceeded the 99% experiment-wise threshold. The LOD score for MIB exceeded the 95% comparison-wise threshold on chromosome *III* at 48 cM. This corresponds to the χ^2 goodness of fit test (Figure 4.1B). The LOD score for MEB exceeded the comparison-wise threshold on chromosome *I* on several occasions including: 8-25 cM, 30-40 cM, 45-48 cM, and 51-58 cM. This agreed with results found in the χ^2 goodness of fit tests (Figure 4.1 B).

When the family was expanded to the F_5 generation, the magnitude of QTL increased significantly using IM (Figure 4.8). The LOD for MIB at no time surpassed the 99% experiment-wise threshold. It did, however, exceed the 95% comparison-wise threshold in 3 areas, once on each chromosome. On chromosome *I*, the LOD score for MIB suggested a putative QTL at 45 cM, on chromosome *II,* from 25 to 33 cM, and on chromosome *III*, from 42 to 47 cM. This corresponded well to results of the χ^2 goodness of fit test (Figure 4.1C). The LOD scores for MEB was very large, and exceeded the 99% experiment-wise threshold in 4 areas on chromosomes *I* and *II*. On chromosome *I*, the LOD score for MEB reached 7.9 at 32 cM, much higher than the 99% experiment-wise threshold of 4.6. This QTL spanned an area on chromosome *I* from 31 to 41 cM. It then surpassed the 99% experiment-wise threshold again on chromosome *I* at 45 and 47-50 cM. On chromosome *II*, the LOD score of 6.5 for MEB exceeded the 99% experiment-wise threshold from 28-37 cM. The LOD score for MEB exceeded the 95% comparison-wise threshold additionally in other locations. On chromosome *I*, there was a putative QTL at 21-23 cM, and the entire lower half of the chromosome, below 31 cM exceeded this threshold. On chromosome *II*, LOD scores for MEB at 0-9 cM and 26-38 cM were larger than the 95% comparison-wise threshold. On chromosome *III*, the LOD for MEB

surpassed the 95% comparison-wise threshold at 19-23, 25, and 34-56 cM. This corresponded well with results of the χ^2 goodness of fit test (Figure 4.1C). LOD scores that surpassed the 99% experiment-wise threshold were QTL of major effect and LOD scores that exceeded the 95% comparison-wise threshold indicated QTL of minor effect.

CIM was performed in both the P7.1 F_2 individuals (Figure 4.9) and F_5 individuals (Figure 4.10). In the F_2 generation a single LOD score for MIB was significant on chromosome *III* at 47-48 cM ($p = 0.04$). LOD scores for MEB, although small, were found to be significant at several locations. A putative QTL for MEB was found on chromosome *I*, from 22 - 28 cM ($p = 0.002$). On chromosome *III*, several putative QTL were found for MEB: 3-11 cM ($p = 0.001$), 16-21 cM ($p = 0.002$), 29-44 cM ($p = 0.007$) and 47-48 cM ($p = 0.03$). This corresponded less well to the χ^2 goodness of fit test results (Figure 4.1C) than did the IM results.

Expanding the family through the F_5 generation greatly increased the magnitude of these QTL when using CIM (Basten *et al.* 2002). Interestingly, a single QTL was found for MIB, but not in the same location as in the F_2 generation. The QTL was found on chromosome *II*, 22-25 cM, which correlated to the marker *TrypAbun*, also found to be significantly associated to MIB by the χ^2 goodness of fit tests. QTL for MEB were found on all three chromosomes. On chromosome *I*, they were located: 8-14 ($p = 0.03$), 30-33 ($p < 0.05$), and 52-59 cM ($p = 0.003$). On chromosome *II*, a QTL with a very large LOD score of 6.3 spanned 21-29 cM ($p < 0.0001$). Finally, on chromosome *III*, there were putative QTL at 16-17 cM ($p < 0.05$) and 26-50 cM ($p < 0.007$) and within this region, 30-48 cM ($p < 0.0001$). These results agree with results of the χ^2 goodness of fit tests (Figure 4.1C).

Figure 4.9: Plot of LOD values associated with MIB (solid line) and MEB (dotted line) along chromosomes *I*, *II* and *III* for the P7.1 F_2 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1B. The LOD score was estimated using composite interval mapping (CIM) (Zeng 1994, Basten *et al.* 1994, 2002). Intervals at which the CIM LOD estimated in ZmapQTL (QTL Cartographer 1.16) is significant are bracketed and labeled with the *p*-value..

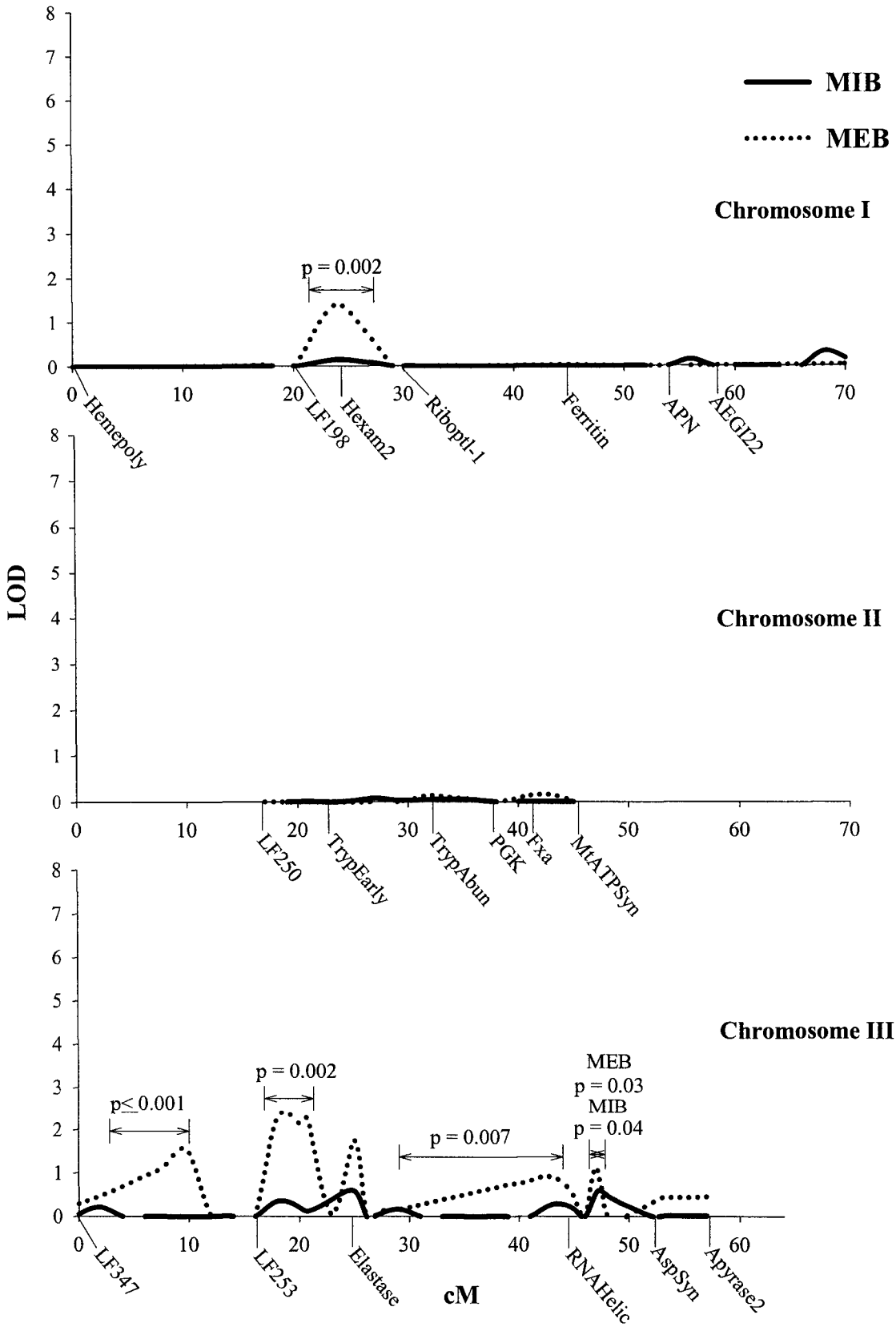
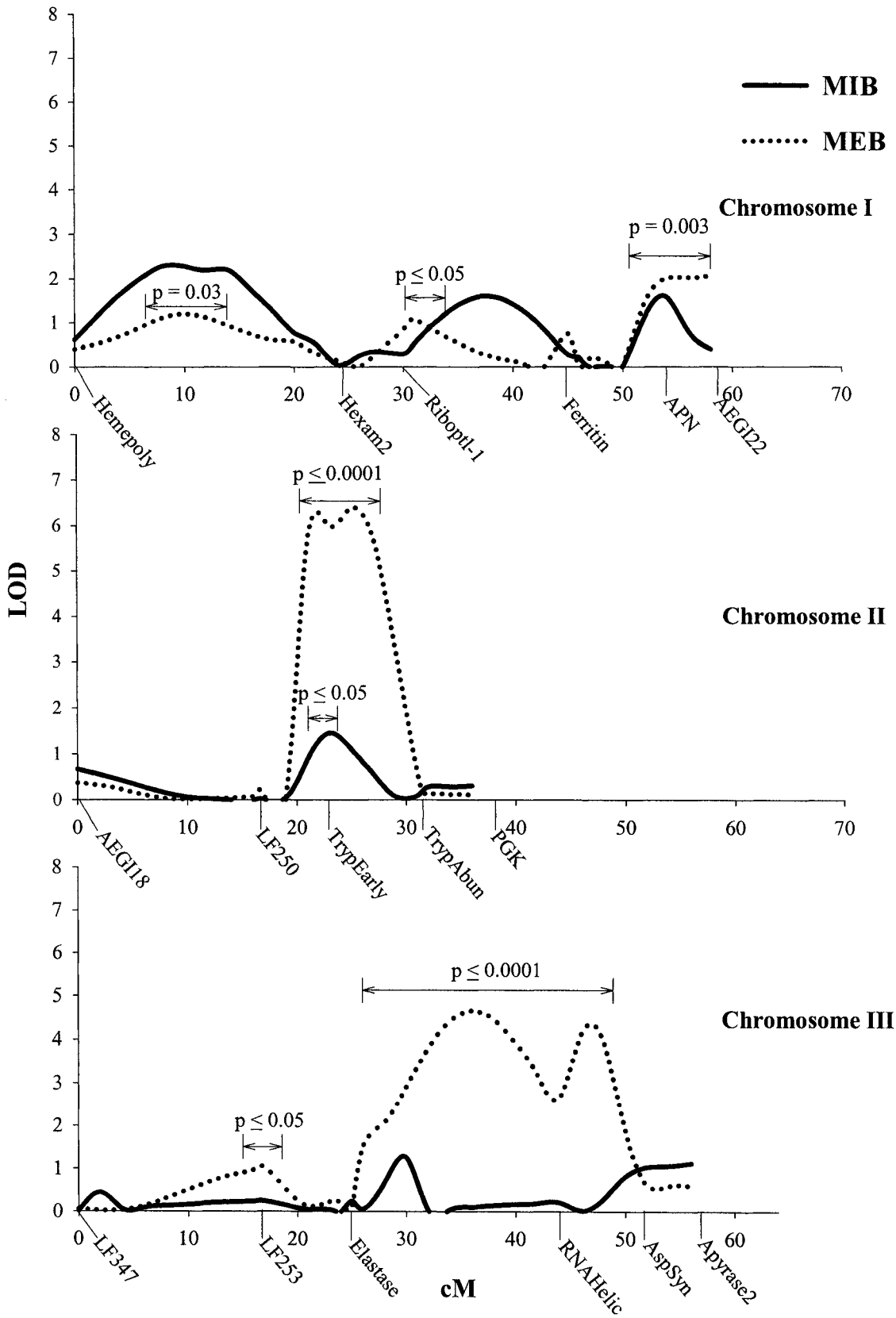


Figure 4.10: Plot of LOD values associated with MIB (solid line) and MEB (dotted line) along chromosomes *I*, *II* and *III* for the P7.1 F_5 generation. Marker names are listed along the x-axis to orient QTL positions relative to the linkage maps shown in Figure 4.1C. The LOD score was estimated using composite interval mapping (CIM) (Zeng 1994, Basten *et al.* 1994, 2002). Intervals at which the CIM LOD estimated in ZmapQTL (QTL Cartographer 1.16) is significant are bracketed and labeled with the *p*-value.



The results of IM (Xu *et al.* 1998) and CIM (Basten *et al.* 2002) tended agree on QTL locations in both families. CIM gave a somewhat more conservative estimate of LOD scores. In results for the P7.1 F+ generation, most of the QTL peaks were shifted slightly to the left (higher on the chromosome) when using CIM for all QTL detected. One example of this includes the major QTL on chromosome *II*, which spanned 28-37 cM according to IM and 21-29 cM according to CIM. Results of the χ^2 goodness of fit test tended to have better agreement with IM than with CIM.

The results obtained for the P7.1 F_5 individuals had a much greater significance than results seen in either of the F_2 groups. Because of this, the remainder of the analysis focuses on the F_5 generation. Once data have been obtained for the F_5 generation of the Y2.2 family, the same procedures will be followed.

In summary, there were 3 putative QTL for MIB, located as follows: on chromosome *I* at 45 cM, on chromosome *II* at 25-33 cM and on chromosome *III* at 42-47 cM (Figure 4.8). These will be called MIB1, MIB2 and MIB3 for the remainder of this chapter. In total there were 8 putative QTL for MEB. Seven of these were identified in the P7.1 F_5 individuals. They were located on chromosome *I* at 21-23 cM (MEB1), 31-41 cM (MEB2), and at 45-50 cM (MEB3), on chromosome *II* at 0-9 cM (MEB4) and at 28-37 cM (MEB5), and on chromosome *III* at 19-25 cM (MEB6) and 34-56 cM (MEB7) (Figure 4.8). An 8^{th} QTL for MEB (MEB8) was identified in the Y2.2 F_2 individuals, on chromosome *II*, at 64-70 cM (Figure 4.6).

Variance Components: Pearson's correlation coefficients estimated between marker genotypes and MIB and MEB were significant ($p < 0.05$) at the same loci that were statistically associated with MIB and MEB in the χ^2 goodness of fit tests. For MIB,

the largest correlation coefficients were found for *TrypAbun* at 32 cM on chromosome *II*, and *RNAHelic* at 44 cM on chromosome *III*. For MEB, the largest correlation coefficients were found for *Riboptl-1* and *Aamy2* at 30 and 45 cM on chromosome *I*, and at *AEGI18*, and *TrypAbun* at 0 and 32 cM on chromosome *II*. MIR, DIR, or VC phenotypes were regressed on *Riboptl-1*, *Aamy2*, *TrypAbun*, *Elastase*, and *RNAHelic* genotypes (Table 4.3). Genotypes at the *Aamy2*, *TrypAbun*, and *RNAHelic* loci counted for 13.7, 53.7 and 32.6% of the σ^2_g respectively and cumulatively accounted for 5% of the σ^2_p in MIB. Genotypes at the *Riboptl-1*, *AEGI18*, *TrypAbun*, *Elastase* and *RNAHelic* loci account for 51.2, 3.7, 34.0, 2.7, and 8.4% respectively of the σ^2_g and cumulatively accounted for 22% of the σ^2_p in MEB. Genotypes at the *Riboptl-1, Aamy2*, *AEGI18*, *TrypAbun*, *Elastase* and *RNAHelic* loci account for 40.9, 0.3, 4.5, 49.1, 2.1, and 3.1% respectively of the σ^2_g and cumulatively account for 21% of the in σ^2_p overall VC.

Discussion

The results of these QTL mapping experiments suggest that there are at least 3 loci that condition MIB, and at least 8 loci that condition MEB for DEN-2 in *Ae. aegypti*. A single QTL for MEB was identified in the Y2.2 F_2 family and the remaining QTL were identified in the P7.1 F_5 family. This latter group includes putative QTL found initially in the P7.1 F_2 individuals, and subsequently confirmed and reinforced by QTL mapping in the F_5 generation. The dramatic increase in significance levels of the QTL between the F_2 and F_5 generations indicates the importance of sample size when testing for correlations between phenotype and genotype. The 76 individuals used for mapping in the Y2.2 F_2 individuals was probably adequate to detect QTL of large effect, but not optimal for detection of QTL of smaller effect (Zeng 1994, Zeng 1993, Lander and Botstein 1989,

Table 4.3: Phenotypic variance accounted for by genotype markers at QTL for MIB, MEB, and VC in the family P7.1 F_5 generation.

Phenotype	Genetic Marker	ΔR^2 (% total variance)	Partial F[a]
Midgut Infection Barrier			
	Intercept	—	1292.64***
	Aamy2	0.006882(13.7)	1.10
	TrypAbun	0.026981(53.7)	6.87**
	RNAHelic	0.016377(32.6)	4.85*
	Total	0.050240(100)	
Midgut Escape Barrier			
	Intercept	—	344.242***
	Riboptl-1	0.11222(51.2)	22.386***
	Aamy2	0.00004(0)	0.028
	AEGI18	0.00808(3.7)	1.522
	TrypAbun	0.07437(34.0)	16.423***
	Elastase	0.00592(2.7)	0.973
	RNAHelic	0.01842(8.4)	4.150*
	Total	0.21905(100)	
Vector Competence			
	Intercept		148.249***
	Riboptl-1	0.08644(40.9)	15.584***
	Aamy2	0.00054(0.3)	0.149
	AEGI18	0.00964(4.5)	2.290
	TrypAbun	0.10373(49.1)	26.618***
	Elastase	0.00438(2.1)	0.661
	RNAHelic	0.00663(3.1)	1.706
	Total	0.21136(100)	
MEB as a Function of Individual Loci			
	Riboptl-1	$R^2 = 0.6866$	455.59***
	Aamy2	$R^2 = 0.6510$	388.04***
	AEGI18	$R^2 = 0.2205$	58.84***
	TrypAbun	$R^2 = 0.0751$	16.88***
	Elastase	$R^2 = 0.0721$	16.16***
	RNAHelic	$R^2 = 0.2114$	9.07***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

[a] The *F*-Statistics and *P* values represent a partial *F*-statistic for the general linear model where $F = [(SSE_R - SSE_F)/d.f._R - d.f._F)]/(SSE_F/d.f._F)$. SSE, error sum of squares; d.f., error degrees of freedom; *F*, model with all genotypes; *R*, model with one genotype. H_0 is rejected when $F_c > F(\alpha = 0.05, d.f._R - d.f._F)$.

Maliepaard *et al.* 1997). The 41 individuals used in the P7.1 F_2 generation was too small to detect most QTL, and the 296 individuals used for QTL mapping in the P7.1 F_5 generation was probably closer to an ideal sample size.

Use of the *D2MEB* line allowed for detection of MEB QTL that in the past were not detected, or were only weakly supported (Bosio *et al.* 2000). An increased sample size increased statistical power in detection of QTL. Bosio, in his dissertation (2000) identified 3 putative QTL for MEB using CIM, but did not have enough statistical power to support them. Interestingly, the QTL he detected were located on chromosomes *I*, *II*, and *III* (59-63, 28-34, and 46-50 cM respectively) and roughly correspond to putative QTL for MEB identified in this study (MEB3, MEB5 and MEB7), particularly those found on chromosomes *II* and *III*. One of the QTL detected for MIB by Bosio (2000) (chromosome *II,* 34-45 cM) was also identified in this study (MIB2 on chromosome *II,* 25-33 cM). The use of selected lines helped refine the search for QTL and increased the statistical power for mapping.

Alleles at individual loci significantly associated with MIB (Figure 4.2) or MEB (Figure 4.3) by χ^2 analysis acted additively in some cases and with dominance in others. In cases where the alleles acted dominantly, the dominance was not consistent in its direction. In some cases, alleles from the susceptible parent were dominant (as with markers *Aamy2, APN, AEGI18*, and *Elastase*), and in other cases, they were refractory (as with markers *TrypAbun, RNA Helic* and *AspSyn*) (Figure 4.3).

Regardless of the variation in additivity and dominance at individual loci, the overall effect of susceptibility alleles can be seen in Figure 4.4, where it is clear that having more alleles inherited from the susceptible parent leads to a higher infection rate.

The exception to this is an apparent decrease in infection rates from 28% in individuals with 1 copy of a susceptibility allele to 0% in individuals with 2 or 3 copies of susceptibility alleles. This is likely an artifact of the samples sizes found in these groups (n = 4, 2, 5 respectively), and not an accurate depiction of what one would see with a larger sample size.

Because so many components determine susceptibility to infection with DEN-2 in *Ae. aegypti*, the overall effect of alleles (Figure 4.4) is probably a better depiction of the effects of individual loci. Both MIB and MEB can be considered threshold traits conditioned by several loci throughout the genome. A certain threshold must be overcome in order for mosquitoes to become midgut infected, and a subsequent threshold must be overcome in order for them to exhibit a DI. Thus, carrying a single susceptible allele at a given locus is not likely to cause a mosquito to be a good vector, and susceptibility alleles at all loci must be considered concurrently. Additionally, as Bosio showed in his work (1998, 2000), environmental and/or stochastic effects can play a significant role in mosquito phenotype. Thus, mosquitoes expected to be highly susceptible (having many susceptible alleles) may remain uninfected after ingesting a viremic bloodmeal. In theory, the more susceptibility alleles an individual mosquito has, the less likely it is that this will happen. The presence of susceptibility alleles can be considered as a risk factor for dengue transmission, and should not be considered as an absolute guarantee of infection.

There was not strong support for an MIB QTL in the Y2.2 F_2 family using either IM or CIM, although IM revealed several putative QTL that barely surpassed the 95% comparison-wise threshold LOD score. These results were difficult to interpret given the

fact that the family size was fairly small (76 phenotyped females). Whether these regions actually contain QTL will be determined once analysis of the F_5 generation is completed. A single well supported QTL for MEB (MEB8) was found in this family using both IM and CIM (Figures 4.5 and 4.6). Several other putative QTL for MEB were also found in the Y2.2 F_2 individuals using IM (Figure 4.5), but were not recovered using CIM. Because of the relatively small sample size, the magnitude of these QTL may increase and be better supported once the expanded F_5 generation is analyzed. The fact that the majority of the putative QTL for MEB were located on chromosome *I,* and corresponded with locations of QTL in the P7.1 F_5 family, makes this a likely scenario. More than one thousand F_5 individuals of the Y2.2 family have been phenotyped for susceptibility to DEN-2, and are currently being genotyped in preparation for QTL analysis.

The fact that the χ^2 goodness of fit tests (Figure 4.1) associated markers within the regions covered by most of the putative QTL shows its utility as a fairly accurate, yet procedurally simple and rapid indicator of potential QTL locations. Because the process of using IM and CIM is so time consuming, the χ^2 goodness of fit test is a valuable tool when scanning for families that might be useful in QTL analysis. In the future, it might be more efficient to scan smaller families using this test, and then expand those that give promising results through several generations to get a more statistically powerful sample size before attempting the more labor-intensive IM and CIM procedures. The χ^2 test shows linkage between a marker locus and a QTL while IM and CIM use maximum likelihood estimates to find the most probable location of a QTL. For this reason, the χ^2 test is not sufficient when looking for QTL, as the specific loci that are associated with

the QTL may or may not actually be involved in conditioning vector competence. They may simply be linked to the markers that actually are responsible.

The majority of the QTL in this study were identified in the P7.1 family. Putative QTL found using the F_2 generation are generally confirmed at a much greater magnitude in the F_5 generation. As such, the remainder of this discussion will focus on the results obtained for the F_5 individuals. The ability to detect a greater number of QTL with greater statistical power lends credence to the idea that QTL mapping can be refined and optimized by increasing sample sizes.

The experiment-wise threshold is likely to underestimate the number of QTL, while the comparison-wise threshold may overestimate the QTL number, or may detect QTL of small effect. With CIM, only one of the QTL for MIB detected in IM was supported, and was given a most probable location slightly left of that given by IM, at 25-33 cM (Figure 4.10). This shift was likely due to the detection of interactions among intervals using CIM. However, the QTL area of the genome contains, or nearly contains, the *TrypEarly* marker for both mapping programs. This is interesting, given results of quantitative trait nucleotide mapping (QTN) which have indicated that there may be individual nucleotides within the *early trypsin* gene that contribute to MIB in *Ae. aegypti* (Gorrochotegui, personal communication). Another marker within this QTL region is *TrypAbun*, a different trypsin gene currently under QTN analysis as a candidate gene that may in part condition a dengue MEB.

A likely contributor to the low LOD scores seen for the MIB QTL is the small proportion of individuals negative for midgut infection. Because these crosses were designed to look for MEB, even a faint detection of QTL for MIB is surprising. The

location of MIB2 loosely corresponds and lends further support to the QTL for MIB identified by Bosio *et al.* (2000). Despite the fact that CIM only corroborates one of the QTL identified by IM, this chapter will acknowledge all 3 QTL identified by IM. This is with the understanding that this family was not designed for mapping MIB, and that MIB is not likely an important contributor to overall vector competence in these mosquitoes.

The additional QTL for MEB found in the Y2.2 F_2 individuals could not be mapped in the P7.1 family, as all markers tested thus far on the bottom of chromosome *II* have been monomorphic. It will be interesting to see if this area of the genome remains significantly associated with MEB in the Y2.2 F_5 generation. Looking for polymorphic markers for this region in the P7.1 family is not likely to be a productive use of time and resources. Numerous markers have already been looked at to no avail (Table 4.1).

The remaining 7 QTL for MEB were found in the P7.1 family (Figures 4.8 - 4.10). For the most part, IM and CIM were in agreement with the placement of the QTL, though CIM tended to shift the QTL to the left (higher) 3-10 cM on the chromosome *II* and *III* QTL. The QTL with the largest LOD score using CIM was MEB5 on chromosome *II*. This corresponded to an area of the chromosome that contains the *TrypAbun* marker in IM and the *TrypEarly* marker in CIM. MEB2 had the largest LOD score when using IM. This area corresponded to a region on chromosome *I* that contained the *Riboptl-1* marker. This area of the genome was also associated with MEB using CIM, but at a lower significance level. This was likely due to the mapping strategy implemented by CIM, which incorporated the potential for interactions among intervals. Because there were several QTL for MEB identified on chromosome *I*, CIM likely found interactions among them, and the overall effect of each individual QTL was decreased.

Ideally, Multiple Interval Mapping (MIM) should be included as an analysis for data of this sort. MIM maps multiple intervals at the same time rather than one at a time, allowing for the detection of potential epistatic effects between QTL. Because MIM is computer intensive, it has not yet been completed for this data set, but will be once results of the Y2.2 F_5 generation are obtained.

The 2 QTL with the largest LOD scores (MEB2 and MEB5) make up a total of 85.2% (51.2 and 34% respectively) of the total V_G associated with MEB when looking at midgut positive individuals (Table 4.3). This increases to a total of 90% (40.9 and 49.1% respectively) of the total V_G associated with VC for individuals. That leaves only 14.8% of the total genetic variance to be distributed among the remaining 4 QTL. Thus, for MEB, there are 2 QTL of major effect, and 5 of minor effect found in the P7.1 F_5 individuals. MEB1 was not included in this analysis, as its low LOD score indicated that it was of extremely small effect. The 8th QTL could not be analyzed along with these, as it was identified in the Y2.2 F_2 individuals, and was not part of the same family. Despite accounting for up to 90% of the total V_G in VC, the fact that they account for only 21% of the total V_P in VC suggests that environmental factors are also extremely important in DEN-2 susceptibility in *Ae. aegypti*, and corroborates findings of Bosio *et al.* (1998, 2000).

Bennett *et al.* (in preparation) found that lines selected for susceptibility phenotype using one DEN serotype did not exhibit similar infection phenotypes for other DEN serotypes. This suggests that the QTL identified here may not be involved in susceptibility to other DEN serotypes. To identify QTL associated with VC for other serotypes, QTL mapping must be done using the specific DEN serotypes. Alternatively, it

is possible that QTL common among the different serotypes will be identified. These may contribute in different amounts to susceptibility phenotype, and a QTL of major effect for one serotype may be a QTL of minor effect for another. This may in part explain why selection for susceptibility to one serotype does not confer a similar susceptibility to a different serotype. Additionally, subtle differences in viral requirements such as proteolytic processing or receptor binding might be factors contributing to differences in susceptibility. Certain virus serotypes might require more or less proteolytic processing in the mosquito midgut, or have different affinities for the receptors present in the mosquito midgut.

The fact that some of the QTL identified by Bosio *et al.* (2000) were identified in this study suggests that there may be some continuity in loci affecting VC for DEN-2 among *Ae. aegypti* populations. However, it is also possible, and even quite likely that there is some degree of polymorphism in QTL among populations, and there are likely QTL unique to given populations.

Candidate genes are being looked at within some of the QTL regions identified here. These are being studied using QTN analysis (Gorrochotegui, personal communication), which should be able to identify risk factor for dengue susceptibility down to the nucleotide level. QTN mapping is an excellent tool, but is only practical once a candidate gene has been identified. QTL mapping helps in this regard, by limiting the number of genes to choose from. It is likely that QTL mapping will still be a useful technique in the foreseeable future.

CHAPTER 5: CONCLUSIONS

The results of this dissertation confirm (albeit with important caveats) what has been known for a long time; there is a wide range of variation among natural *Aedes aegypti* populations in their ability to transmit dengue viruses, and that this variation is, at least in part, conditioned by genetic factors. However, in addition to corroborating earlier studies, this dissertation has revealed several new things about vector competence of *Ae. aegypti* for DEN viruses.

The first chapter focused on detecting variation in DEN susceptibility in *Ae. aegypti* populations in a relatively small geographic area. It is interesting that the variation seen in the southern USA and throughout Mexico rivals that observed on a global scale. It is amazing that a population of mosquitoes highly susceptible to infection with DEN-2 can live within a few hundred miles of another population that is largely resistant to DEN-2 infection and yet both retain distinct susceptibility phenotypes.

The amount of variation in MIB and MEB exhibited by these natural populations supports the concept that DEN susceptibility must be conditioned by multiple genes, or many alleles at a single, highly polymorphic locus to give the range of DEN-2 susceptibility found among local populations. The QTL mapping done in Chapter 3 confirmed that there are several genes conditioning these characteristics. Gorrochotegui-Escalante *et al.* (1998, 2000) has shown that there is a great amount of overall genetic variation in these populations. Whether or not this genetic variation is related to DEN susceptibility remains to be seen. It also remains to be seen whether or not there is

polymorphism in the actual QTL that condition vector competence for DEN-2 in *Ae. aegypti*. It is possible that there are different QTL responsible for conditioning vector competence in each population. Because QTL mapping requires the crossing of only 2 mosquito lines, it greatly underestimates the amount of genetic variation associated with a phenotype. A better understanding of whether or not the QTL conditioning DEN-2 susceptibility are constant across mosquito populations could be gained through multiple crosses using different parental lines. The amount of work that this would entail, however, does not make this a very practical option.

The observation in Chapter 3, that lines of *Ae. aegypti* selected for specific phenotypes using DEN-2 do not fully retain this phenotype for other DEN serotypes suggests that susceptibility to DEN may not be completely conditioned by general genetic factors. However, the fact that the *D2S3* line is consistently more susceptible than the *D2MEB* line, suggests that susceptibility to the different DEN serotypes may be at least partially determined by common genetic factors. It will be important to determine what genetic factors do condition susceptibility of *Ae. aegypti* to other DEN serotypes. It may be that there are common QTL, but that the contribution of each QTL varies from serotype to serotype.

If QTL contribute differently to vector competence to each serotype, or if there are polymorphisms in QTL among mosquito populations, it is unlikely that using this information to create refractory mosquitoes for release in disease control strategies will be a viable option. The more genes involved, the more complicated such a tactic becomes. Taken in conjunction with the large amount of phenotypic variance explained by environmental effects, it is unlikely that such a strategy will be effective. Additionally,

as more QTL are found to condition DEN-2 susceptibility in selected lines of *Ae. aegypti*, this goal becomes more and more unrealistic. This has implications for other strategies of vector manipulation including the creation of transgenic mosquitoes in that they too will have to consider the genetic complexity of vector competence in *Ae. aegypti*. Despite this, selected or transgenic lines remain important as tools for better understanding what factors are involved in pathogen transmission, and what factors might be good targets for control.

The results of Chapter 4 expanded upon the findings of Bosio in his dissertation (1999). This study supports the presence of a QTL for MIB on chromosome *II* (Bosio *et al.* 2000). Additional QTL for MIB were identified on chromosome *I* and *III*, and 8 QTL for MEB were identified across all three chromosomes. The ability to use selected lines for QTL mapping enhanced the ability to detect QTL for MEB, and increasing the sample size clearly gave additional power to the statistical analysis.

This work further supports Bosio's conclusion (1998, 2000) that there are multiple major controlling loci and several loci of small effect that condition vector competence for DEN-2 in *Ae. aegypti*. This is contrary to other QTL mapping studies of vector competence for *Plasmodium* (Severson *et al.* 1994, 1995b) and filarial parasites (Zheng *et al.* 1997) in mosquitoes, where a single QTL of major effect has been found to be responsible for the majority of the genetic variance in the susceptibility phenotype examined. Based on the results of this study, it would be interesting to see if these other QTL mapping studies would identify a greater number of QTL if they also increased their sample sizes and examined recently colonized, unselected mosquito lines.

The observation that there is a loose correlation between MIB and MEB in Chapter 2, is interesting when taken in conjunction with the fact that QTL for MIB and MEB detected in the crossing experiments of Chapter 4 largely overlap. It is possible that there are genes within these QTL regions that are responsible for both MIB and MEB. Alternatively, there may be genes near each other that independently condition MIB and MEB, but which are linked due to their proximity in the genome. It will be interesting to see if QTL for MIB and MEB still overlap once mapping techniques are able to localize them more precisely. This is especially important in *Ae. aegypti,* given that the genome has a relatively low rate of recombination (165 cM total) and that each cM still corresponds to such a large physical distance.

A large effort has focused on increasing the number of markers used for linkage mapping in *Ae. aegypti* (Severson *et al.* 2002, Fulton *et al.* 2001) in an attempt to increase the resolution of the map, and the ability to more precisely delineate QTL regions. Although current linkage maps for *Aedes aegypti* have hundreds of markers, each cM still translates to several megabase pairs (Mbp). The *p*-arm of chromosome *I* has an estimated 1.7 Mbp/cM. The *p*-arm of chromosome *II* is resolved to 3.0 Mbp/cM while the *q*-arm is resolved to 2.2 Mbp/cM. For chromosome *III*, the *p*-arm is resolved to 2.8 Mbp/cM and the *q*-arm to 1.4 Mbp/cM (Brown *et al.* 2001). Based on these estimates, there remains a vast amount of genetic material within each linkage interval, as most QTL regions span several cM of the linkage map. This leaves a lot of DNA to sift through once QTL analysis is complete, and even with sophisticated techniques such as map based positional cloning, distinguishing genes responsible for vector competence within these regions is a daunting task.

However, the potential for use of candidate genes that fall within the region of a QTL is being examined. Gorrochotegui Escalante (personal communication) has been looking at both the *Early trypsin* and *Abundant trypsin* genes for quantitative trait nucleotides (QTN) associated with MIB and MEB, with promising results. These genes were chosen for further examination based on their location within a QTL, and because both are involved in blood meal digestion at a point when virus may be infecting the midgut epithelial cells. It is hypothesized that they may be involved in proteolytically processing the virions to enhance their ability to infect the midgut.

Evidence of a relationship between the presence of trypsin and the ability of dengue virions to infect *Ae. aegypti* also appeared in studies done using soybean trypsin inhibitor (STI) to knock out the activity of trypsin. When STI was added to an infectious blood meal, the disseminated infection rate of the virus was significantly reduced and expression of virus proteins in the midgut was inhibited (Gupta and Bennett, unpublished). Use of the Sindbis virus expression system to knock out the expression of early trypsin gave a similar result (Sanchez-Vargas, personal communication). Unfortunately, biologically relevant genes that might serve as good candidate genes were not always so evident within each QTL region. As both treatments have an inhibitory effect on blood meal digestion, the decrease in infectivity or dissemination may be due to the limited availability of amino acids, reducing the overall translation of viral proteins. Further experiments have been designed to determine whether this is the case. These include pre-digestion of the blood meal, and pre-proteolytic processing of the virus before *per os* infection of mosquitoes.

Completion of the human genome project (The Genome International Sequencing Consortium) as well as genome projects for *Anopheles gambiae* (Holt *et al.* 2002) and the malaria parasite (Gardner *et al.* 2002a and 2002b, Hall *et al.* 2002), give hope for rapid advances in identification of key vector-pathogen and pathogen-host interactions (Aultman *et al.* 2002). Sequencing of the *Aedes aegypti* genome will similarly help delineate vector interactions with dengue. Despite the fact that this sequencing will certainly provide a lot of information that can be used in vector research, there is still much to be learned about what each genomic segment contributes to the overall organism. Even in sequenced genomes there are still large expanses of DNA for which there is no known function. Any or all of these could be the genetic factors underlying vector competence for DEN. One relatively quick and effective way to test the effect of each candidate gene within a QTL might be through RNA silencing using in vivo injection of double stranded RNA.

To date, there remains much to learn about the factors that condition vector competence for dengue in *Ae. aegypti*. It is hoped that these studies have in some way contributed to a growing body of knowledge that will someday culminate in a complete understanding of the myriad factors that govern vector competence of not just *Ae. aegypti*, but all vectors for their respective pathogens. Understanding and control of disease transmission are, however, two different matters, and despite growing understanding, it is difficult to imagine that humankind will ever be completely rid of vector borne disease.

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