DISSERTATION

VECTOR AND VIRUS INTERACTIONS: LA CROSSE ENCEPHALITIS VIRUS AND THE MOSQUITO VECTOR *AEDES (STEGOMYIA) ALBOPICTUS*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY IAN W. SUTHERLAND ENTITLED "VECTOR AND VIRUS INTERACTIONS: LA CROSSE ENCEPHALITIS VIRUS AND THE MOSQUITO VECTOR *AEDES (STEGOMYIA) ALBOPICTUS.*" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

VECTOR AND VIRUS INTERACTIONS: LA CROSSE ENCEPHALITIS VIRUS AND THE MOSQUITO VECTOR *AEDES (STEGOMYIA) ALBOPICTUS.*

La Crosse encephalitis continues to be an important cause of pediatric arboviral encephalitis in the United States. Since 1985, the invasive mosquito vector, *Aedes albopictus*, has spread across the country and into La Crosse virus endemic regions. As an aggressive, daytime feeder, this vector has the potential to change the epidemiology of La Crosse encephalitis. This study investigated 4 components of the La Crosse virus-*Ae. albopictus* system: 1) time course of disseminated and filial infection rates (FIR) among recently colonized field strains, 2) anatomic basis of ovarian infection during the 1st gonotrophic cycle, 3) mitochondrial DNA (mtDNA) variation among geographically dispersed populations in the U.S., and 4) development of transovarially susceptible and refractory strains of *Ae. albopictus.* All geographic strains tested are susceptible to La Crosse virus oral infection and capable of transovarial transmission (TOT). No regional or geographic patterns emerged with respect to dissemination or TOT. 1st gonotrophic cycle vertical transmission was observed at low levels, with an FIR averaging 1%. 2nd gonotrophic cycle FIR averaged approximately 10% and was significantly lower than that of the natural vector, *Ae. triseriatus*. La Crosse virus antigen (Ag) was detected in ovaries by Day 2 after oral infection and prior to detection in head tissues. Ag was not detected in follicles through Day 7, suggesting vertical transmission. Examination of variation in the ND5 mtDNA marker revealed high levels of homogeneity among U.S. *Ae. albopictus* populations. Only 2 haplotypes were observed from 16 geographically dispersed states, including Hawaii. Such broad homogeneity could be due to multiple factors, including founder effects and cytoplasmic incompatibility. *Ae. albopictus* responded poorly to

selection based on FIR for the development of susceptible and refractory strains. This study supports prior literature suggesting *Ae. Albopictus* may be an important secondary vector of La Crosse virus.

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DEDICATION

This work is dedicated to my wife, Genevieve, and my daughter, Imogene. Without them, no endeavor is worth undertaking.

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CHAPTER 1: LITERATURE REVIEW

A. Introduction

Despite early discoveries revealing the basic biology of vector-borne disease and early successes with eradication efforts, vaccination campaigns, and chemical breakthroughs for vector control such as DDT (Dichloro-Diphenyl-Trichloroethane) and DEET (Meta-N,N-diethyl toluamide), arthropod-borne diseases continue to pose a formidable threat to worldwide public health (Gubler 1998). Malaria alone accounts for an estimated 515 million clinical infections a year with 2.2 billion people at risk of infection (Snow et al. 2005). The emergence of West Nile virus in the Western hemisphere illustrates the increasing participation of technological advancement, global scale of trade, and evermore expedient travel between geographic regions, in the potential for spread of infectious diseases (Krajick 2006). Recent outbreaks of La Crosse encephalitis in the southeastern U.S., potentially implicating the imported Asian tiger mosquito, *Aedes albopictus*, underscore the ever-evolving dynamic nature of emerging vector-borne disease and the necessity for ongoing surveillance and research into their prevention (Erwin et al. 2002, Gerhardt et al. 2001).

Arthropod-borne viruses or arboviruses are a great cause of suffering and disease in the world. It is estimated that between 50 -100 million cases of dengue fever, 500,000 cases of dengue hemorrhagic fever and dengue shock syndrome, and more than 20,000 deaths occur each year (Mackenzie et al. 2004, Gubler 2002). Despite a safe and efficacious vaccine, Japanese encephalitis, which has up to a 30% mortality rate, continues to ravage Asia with 30-50,000 cases reported annually (Mackenzie et al. 2004, Solomon and Vaugh 2002).

Nevertheless, from both medical and biological viewpoints, arboviruses are fascinating organisms to study. Although there are over 500 distinct arboviruses, almost all fall within 5 families (Bunyaviridae, Flaviviridae, Reoviridae, Rhabdoviridae, Togaviridae), and they share the ability to infect and replicate within vertebrate and invertebrate host cells (Eldridge et al. 2004). Nearly all have RNA genomes, which may be double stranded, single stranded, positive, or negative sense configuration (Beaty et al. 1988). Some have unsegmented genomes, while others may have multipartite genomes with distinct segments. Segmented RNA arboviruses, such as those in the family Bunyaviridae (3 genomic segments), are potentially capable of rapid genetic evolution both due to the inherently high mutation rate of RNA viruses and due to segment reassortment during viral replication, potentially leading to new viruses (Eigen 1991, Beaty et al. 1988). Many arboviruses are zoonotic in nature, typically cycling within animal hosts but capable of infecting humans, and the most important vectors are mosquitoes (Eldridge et al. 2004). The vector's essential ability to influence the biology of the host and arbovirus makes for a unique biological role. Potentially influencing viral evolution, host immunity, and virus pathogenesis, the vector is more than simply a winged syringe (Tabachnick 1998). Prevention and control of emerging arboviral disease requires a thorough understanding of the complex vector-virus interactions.

B. La Crosse Virus

La Crosse virus (LAC) is named after the site of its 1964 post-mortem isolation from a fatal, 1960 case near La Crosse, Wisconsin (Thompson et al. 1965). It is a member of the largest family of RNA viruses, the Bunyaviridae, which is composed of

five genera: *Hantavirus, Nairovirus, Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* (Schmaljohn and Hooper 2001). The viruses in 4 genera are arthropod-borne; the hantaviruses are not. Notably, the tospoviruses infect plants and the hantaviruses are rodent-borne and transmitted via infected excreta (Lednicky 2003). LAC belongs in the *Orthobunyavirus* genus in the California serogroup (CAL). The serogroup is composed of other viruses including Jamestown Canyon (JC), San Angelo (SA), and Snowshoe Hare (SSH) viruses. These viruses are typically transmitted by mosquitoes, which can exhibit transovarial transmission (TOT), and involve small mammalian hosts (Bishop 1996).

C. LAC Physical Characteristics

LAC has spherical virions that are enveloped by a lipid bilayer containing 2 surface glycoproteins (G1, G2). The genome is negative sense, tripartite, and composed of small (S), medium (M), and large (L) single-stranded RNA species. The S segment is 984 nucleotides in length and codes for the nucleocapsid (N) protein and non-structural protein (NSs). The M segment is 4,326 nucleotides in length and codes for a poly-protein, which is processed to produce the surface glycoproteins (G1, G2) and a non-structural protein (NSm). The L segment is 6,980 nucleotides in length and codes for a RNA-dependent, RNA polymerase (L protein). Each RNA segment complexes with multiple copies of the N protein to form individual S, M, and L helical nucleocapsids with inverted complementarity at the 3' and 5' termini. Virus particles contain three nucleocapsids surrounded by an envelope containing glycoproteins. (Flint et al. 2004, Borucki et al. 2002, Strauss and Strauss 2002).

D. LAC Replication/Coding Strategy

Although not completely understood, initial LAC entry into host cells likely involves a form of receptor-mediated endocytosis following viral surface glycoproteins attaching to host-cell surface receptors. G1 is thought to be important for binding mammalian surface receptors. The G2 protein probably serves a viral attachment function within the mosquito midgut. Following G1 proteolytic processing and cleavage by digestive enzymes, the G2 protein is presumably exposed and can bind to midgut epithelial cells (Ludwig et al. 1991). The early steps in this process have not been well-studied, and the receptors on host cells have yet to be characterized (Schmaljohn and Hooper 2001).

Subsequently, virion entry into and uncoating within the host cell is facilitated by endocytosed coated vesicles. Within this endosomal vesicle, a declining pH induces viral and endosomal membrane fusion and the eventual release of all three viral nucleocapsids into the cellular cytoplasm for transcription and replication (Hewlett and Chiu 1991). During primary transcription, each of the tripartite genome segments transcribes a subgenomic mRNA via the virion-associated polymerase. All three mRNA species (S, M, L) lack poly-A tails, and they contain short primer sequences at their 5' ends that are not virally coded. Instead, they are acquired from cytoplasmic cellular host mRNAs by the endonuclease activity of the L protein in a process termed 'cap scavenging'. Intriguingly, it has been shown that not only is this cap scavenging targeted, but that one putative donor mRNA is from a cellular apoptosis regulatory gene (inhibitor of apoptosis, IAP) (Blitvitch et al. 2002). Cytoplasmic translation by host ribosomes of viral proteins starts after mRNA transcription and produces both structural and non-structural proteins.

S and L segment RNAs are translated by free ribosomes and their gene products (NSs, N, and L proteins) and are not known to be post-translationally modified (Bishop 1996). NSs has been shown to counteract the effects of short interfering RNA (siRNA) in mammalian cells, inhibiting the RNA interference (RNAi) pathway (Soldan et al. 2005). Recently, it has been proposed that the major function of NSs is suppression of the Type I interferon system of the mammalian innate immune response (Blakqori et al. 2007). The M segment mRNA is translated by membrane-bound ribosomes on the endoplasmic reticulum as a polyprotein, which is cleaved co-translationally into the NSm, G1, and G2 proteins (Matsuoka et al. 1991).

After primary transcription and translation of the mRNA species, genome replication begins and genome-complementary RNA accumulates. Although poorly understood, this switching from transcription and translation of vRNAs to genome replication (production of full-length virion complementary vcRNAs, acting as templates for transcription of new genomes), may involve accumulation of viral proteins such as N (Kolakofsky and Hacker 1991). Glycoprotein glycosylation and virion morphogenesis occurs within the golgi apparatus. While G1 and G2 glycoproteins accumulate in the Golgi complex membranes, helical nucleocapsids formed by N protein and RNA genomes interface with glycoproteins on the cytoplasmic side of the Golgi complex (Matsuoka et al. 1991). These nucleocapsids acquire their lipid envelope as they bud into vesicles within the Golgi cisternae. Virion laden vesicles then migrate out of the golgi complex to the cellular surface for fusion with plasma membrane and subsequent exocytosis of mature, infectious virions.

E. Natural History and Epidemiology of LAC

LAC virus is the causative agent of most clinical cases of California group encephalitis, and it has not yet been detected outside of North America (Beaty 2001). The virus is widely distributed throughout the eastern continental U.S. and in states contiguous with the Mississippi river. Although human cases of this viral infection have historically been reported in the upper Midwest, recent outbreaks in the Southeastern U.S. may be indicative of shifting foci of infections (Erwin et al. 2002, Nasci et al. 2000). Reported cases of La Crosse encephalitis average approximately 100 per annum, but it is thought to be grossly underreported (Tsai 1991). A recent epidemiological analysis estimated endemic incidence at a rate higher than that of bacterial meningitis (approximately 25 cases per 100,000 people). LAC is the primary cause of pediatric arboviral encephalitis in the U.S. (McJunkin et al. 2001).

Clinical disease is observed almost exclusively (>90%) in children under the age of 15 years, with twice as many cases reported for boys than girls, possibly indicating a behavioral component of exposure risk (Beaty 2001, Tsai 1991). The incubation period is typically 7 to 14 days, culminating with symptoms ranging from a mild febrile illness (headache, fever, vomiting) to fatal encephalitis (disorientation, seizures, death). About 50% of hospitalized patients present with seizures, with a resultant case fatality rate of 1%. Furthermore, of those patients with seizures, 25% demonstrate recurrent seizures, underscoring the highly epileptogenic nature of LAC. Long-term neurological sequelae such as learning disabilities, cognitive deficiencies, and behavioral disorders are also associated with severe cases of La Crosse encephalitis (Goddard 2000, Rust et al. 1999). A Wisconsin seroepidemiological survey for the presence of antibodies to LAC virus

among the mentally institutionalized revealed the seroprevalence rate for LAC antibody was double that of the general population (Gauld et al. 1979). Taking into account there is no vaccine, treatment is nonspecific, and the high cost of institutionalized or long-term care and convalescence required for some cases, LAC remains a significant threat to public health in the U.S. (Utz et al. 2002).

F. LAC Transmission Cycle

In nature, LAC is maintained in a sylvatic, mosquito-small mammal cycle. The principal vertebrate hosts include the eastern chipmunk (*Tamias striatus*) and the gray squirrel (*Sciurus carolensis*) (Yuill 1983). The main vector is the eastern treehole mosquito, *Aedes (Ochlerotatus) triseriatus* (Say) and research has implicated other species as potential vectors (Gerhardt et al. 2001, Berry et al. 1986, Watts et al. 1972). This mosquito is widely distributed in North America east of the Rocky mountains and from Florida north into parts of Canada (Darsie and Ward 2005). With an extrinsic incubation period (EIP) of approximately 7 days, LAC virus is efficiently transmitted by *Ae. triseriatus*. As the name implies, it is a woodland mosquito often utilizing treeholes as sites of oviposition and larval habitats, although artificial water containers (discarded tires, jars, etc.) may also be used (Ballard et al. 1987, Craig 1983). Transmission of LAC to chipmunks and squirrels usually results in uncomplicated infections with short-lived viremias (average of approximately 2.5 days at an average of 3.0 $\log_{10}$SMICLD$_{50}$/0.025mL) followed by complete recovery (Osorio et al. 1996, Patrican et al. 1985). The acute viremia titers are sufficient to infect *Ae. triseriatus*, but neutralizing antibodies quickly clear infection and mammalian reservoirs have yet to be discovered

(Yuill 1983, Gauld et al. 1974). As with LAC infection of its natural mammalian hosts, the virus does not appear to induce significant pathology in adult mosquitoes (Beaty and Thompson 1975). The infection is lifelong and disseminated, transforming the mosquito into both vector and reservoir. Once orally infected, the adult female promotes virus maintenance in nature through transmission to new immunonaive hosts and by transovarially transmitting the virus to her progeny (Watts et al. 1973). The later is of particular importance, as antibody or seroprevalence rates in reservoir hosts reach up to 100% in endemic areas. TOT bypasses vertebrate herd immunity, allowing the virus to spread to subsequent generations of mosquitoes regardless of the immune status of vertebrate hosts.

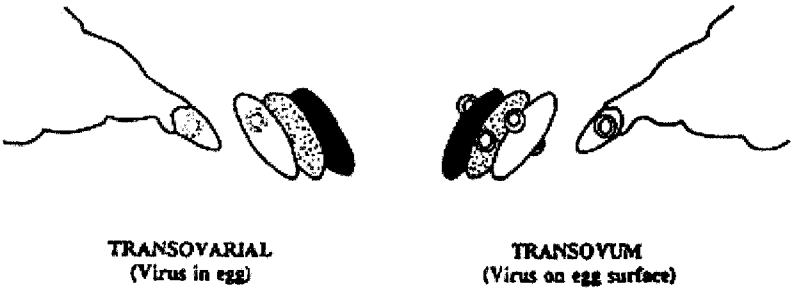
In midwestern and temperate regions of the U.S., winter weather and seasonal cues induce diapause, a form of metabolic hibernation allowing the mosquito to successfully overwinter through egg survival (Shroyer and Craig 1983). With the coming of spring and exposure to the appropriate hatching cues (water, temperature, light cycle, etc.), surviving eggs hatch and infected adults emerge to begin the transmission cycle again. Infected female progeny can transmit the virus orally and transovarially, and infected male progeny provide an additional route of horizontal virus spread through venereal transmission to females during mating (Thompson and Beaty 1977). Studies have demonstrated that although venereal transmission efficiency is low, it can be greatly enhanced (increased from 4% to 50%) if the female is bloodfed prior to mating (Thompson 1979). Insemination rates are also increased (from 45% to 80%) by oral LAC infection in female *Ae. triseriatus* (Gabitzsch et al. 2006). However, the mechanisms that condition these observations remain poorly understood.

In the natural transmission cycle, the eastern treehole mosquito prefers to feed on small mammals in its typical forest range (Nasci 1985). Humans can occasionally become involved in this transmission cycle as dead-end, tangential hosts (Goddard 2000). Most human contact and infections occur in and around the hardwood forest and larval habitats of the mosquito vector. Human infection rates are highest from July through September, with risk factors including outdoor activity, rural residence, and proximity of larval habitats to the residence (Beaty 2001, Nasci et al. 2000).

G. LAC Transovarial Transmission

Transovarial transmission (TOT) is a remarkable viral phenomenon, allowing LAC to overwinter, bypass vertebrate herd immunity, and be amplified in multiple generations of mosquito hosts. Determining that LAC overwinters in the progeny of *Ae. triseriatus* was a landmark discovery. The enigma of how each spring, emerging mosquitoes would feed upon new generations of squirrels and chipmunks, and a new transmission cycle would begin started to unravel with the isolation of LAC from field collected *Ae. triseriatus* larvae and the laboratory demonstration of TOT (Pantuwatana et al. 1974, Watts et al. 1973). This special form of vertical transmission involves the infection of the ovarian follicles. This entails virus entering the egg and is distinguished from other forms of vertical transmission. These include transovum transmission, in which virus is on the egg surface, and infection of the egg through the micropyle during oviposition (Figure 1) (Higgs and Beaty 2005, Rosen et al. 1989).

Figure 1 – Vertical Transmission (from female to progeny): Transovarial and Transovum



from Higgs and Beaty 2005

These experiments were followed by the demonstration of arbovirus overwintering in mosquitoes. This was documented by the observation of LAC infected mosquitoes emerging from eggs overwintering within treeholes of a La Crosse encephalitis endemic area (Beaty and Thompson 1975). *Ae. triseriatus* collected from varied geographic populations have shown differences in TOT efficiencies, which may be a major determinant of disease incidence in an area (Woodring et al. 1998, Miller et al. 1982). Additionally, individual females can display wide variability in their TOT efficiencies, suggesting a genetic component to TOT as well (Graham et al. 1999).

In order to understand the anatomic basis of this phenomenon, direct immunofluorescence was used in virogenesis studies of transovarially infected *Ae. triseriatus* (Thompson and Beaty 1978, Thompson and Beaty 1977, Beaty and Thompson 1976). The results from this work elucidated much about viral tropisms during TOT: 1) viral antigen (Ag) increased with each stage of development, from particularly permissive digestive tissues of larvae, culminating in detection in all adult tissues after metamorphosis; 2) newly emerged females had sufficient salivary gland infection to begin oral transmission with their first bloodfeed; 3) transovarially infected

ovarian follicles of the newly emerged female immediately produced infected progeny irrespective of the infection or immune status of host bloodmeals; and 4) newly emerged male reproductive tissues were immediately capable of venereal transmission of LAC to uninfected females, providing another mechanism to amplify the virus in nature.

While it is hard to make direct comparisons between field and laboratory observations, it has nevertheless been shown that TOT can be very efficient. Initial field studies revealed that approximately 1.5% of mosquitoes collected as overwintered eggs were transovarially infected (Beaty and Thompson 1975). In contrast, in laboratory studies a 98% transovarial transmission rate (TOTr) could be attained, and an average 71% filial infection rate (FIR) was maintained through eight generations of *Ae. triseriatus* after only a single LAC infectious bloodmeal (Miller et al. 1977). Additional studies of LAC overwintering in diapause-induced *Ae. triseriatus* eggs that were returned to the field revealed that LAC infection reduced survivability in natural overwintering conditions. Egg mortality was 16% for infected mosquitoes versus 7% for uninfected mosquitoes. This effect on fitness was observed *after* emergence from diapause; suggesting that diapause modulates potential pathology resulting from virus infection (McGaw 1998). Interestingly, transovarially infected eggs broke diapause earlier, possibly allowing earlier establishment of the transmission cycle.

Selection of mosquitoes with high and low TOTr and FIRs can be achieved within three generations(Graham et al. 1999), suggesting: 1) that TOT is conditioned by a single genetic locus, 2) that infection permissiveness is conditioned by a dominant allele, and 3) FIR is also influenced by factors such as viral virulence, titer, vector nutrition, climate, etc..

H. Diapause

A definitive 'resting stage' is a common feature in organisms inhabiting inconstant environments. This dormant state is usually characterized by the temporary restriction of growth, reproduction, metabolism, and enhanced resistance to adverse climatic factors such as cold, heat, and drought. The term diapause was introduced by Wheeler (1893) to describe a stage in the embryogenesis of the grasshopper, *Xiphidium ensiferum*. The complicated pendulum-like movements of the embryo were divided into an ascending stage (anatrepsis) and a descending stage (catatrepsis). The phase intervening between the two, when the embryo was still and poised before resuming movement was called 'the diapause' (from the Greek, διαπαυσς = rest, interruption of work) (Lees 1955). Diapause can be distinguished from quiescence, which is directly induced by environmental cues and exhibits rapid recovery upon cue removal, in that it results from environmental cues occurring before the actual incidence of adverse conditions. Metabolic activity may remain suppressed even if the adverse conditions are removed (Denlinger 2002, Chapman 1998). Photoperiod is the most reliable and consistent indicator of seasons, making it also the most important of the diapause-inducing agents (Chapman 1998). Temperature may also aid in diapause induction and the period of sensitivity to these stimuli varies in different species. In *Ae. triseriatus,* the the embryonated egg is sensitive to photoperiod changes and the primary expression of diapause is in the eggs. In *Ae. albopictus*, the maternal adult is sensitive to photoperiod changes and the primary expression of diapause is also in the eggs (Mitchell 1988, Wang 1966). During diapause, metabolic activity and protein synthesis are greatly reduced, often characterized by a decrease in total RNA levels (Tauber et al. 1986). Egg diapause

can occur in natural oviposition sites independent of the environmental conditions when they were deposited, and it can even be repeatedly induced over multiple winters (Shroyer and Craig 1980, Beaty and Thompson 1976).

Under controlled conditions, *Ae. triseriatus* eggs can be induced into diapause by incubation for 2 weeks at 21°C with a photoperiod of 10 hours of light and 14 hours of dark. Diapause can be terminated when the light cycle is increased to 16 hours of light and 8 hours of dark (McGaw 1998, Shroyer and Craig 1980). In *Ae. triseriatus*, the critical day length for diapause is latitude dependent (30°-46°N), and for every 4.2° increase in latitude, there is 1 hour less of critical photoperiod (the critical photoperiod is the 24 hour light to dark ratio at which half the population switches from one state to another e.g. non-diapausing to diapausing) (Shroyer and Craig 1980). The critical photoperiod is latitude of origin dependent; *Ae. triseriatus* strains from Florida do not diapause at all (Shroyer and Craig 1983).

Cold hardiness is also a component of diapause (Rinehart et al. 2006, Hanson and Craig 1994). Twelve strains of *Ae. albopictus*, 2 of *Ae. aegypti*, and 1 of *Ae. triseriatus*; were tested for their outdoor overwintering ability in Indiana (Hawley et al. 1989). This study documented that increased cold hardiness is conditioned by diapause, and that non-diapausing *Ae. albopictus* eggs had greater survival if conditioned at low temperatures for approximately 2 months (70 days at 7°C). It was also revealed that eggs in diapause had a significantly higher probability of successfully overwintering, and geographic origin correlated with egg survivability. Another study, examining cold hardiness, diapause and cold acclimation in 5 geographically diverse strains of *Ae. albopictus*, *Ae. aegypti*, and *Ae. triseriatus* showed significant differences (Hanson and Craig 1994). Eggs from

temperate region mosquitoes were found to be more cold hardy than subtropical mosquitoes, which in turn, were found to be more cold hardy than eggs from tropical mosquitoes (cold survivorship: temperate > subtropical > tropical). Both cold acclimation and diapause were found to increase temperate region egg survivability. The specific cause of low temperature mortality in *Ae. albopictus* eggs has yet to be determined. Tropical *Ae. albopictus* populations cannot diapause, are much less cold hardy than temperate populations, and even if in diapause and cold acclimated, experience significant mortality (>10%) at temperatures above their supercooling points (Hanson and Craig 1995, Hanson et al. 1993).

Diapause could also function to protect LAC infected eggs when environmental conditions would normally fall within viable temperature ranges for viral replication and host metabolic activity. Thus, mosquitoes capable of "deep" diapause would be the best transovarially transmitting vectors (Borucki et al. 2002). Although TOT rates of temperate and subtropical *Ae. triseriatus* are nearly identical, filial infection rates are significantly higher for northern strains. Temperate strains that are non-diapausing have filial infection rates similar to subtropical, non-diapausing strains (Woodring et al. 1998). Field experiments investigating the overwintering survival of *Ae. triseriatus* eggs showed that LAC infection decreases overall egg survival after emergence from diapause (McGaw et al 1999). This potentially indicates diapause modulates viral virulence. These intriguing results warrant further investigation of the relationship between diapause and La Crosse virus infection. To understand the molecular and anatomic bases of this phenomenon also necessitates an understanding of the general anatomy of the mosquito ovaries and relevant reproductive tissues.

I. Anatomy and Structure of Mosquito Ovaries

Diagram of adult mosquito ovaries, ovarioles, and associated structures are provided below (Figures 2, 3, 4). The paired mosquito ovaries are located dorsolaterally in the posterior region of the abdomen. Each ovary is connected to the common oviduct through a lateral oviduct that runs through the center of the ovary (Figure 2). This conduit is lined with epithelium continuous with the lateral oviduct and is called the calyx (Clements 2000). The functional units of the ovary, the ovarioles, radiate outward from the calyx (Figure 3). Each ovary is connected to the respiratory system by two tracheae, which disperse into a copious array of branches, eventually terminating as tracheoles within the ovarioles (Clements 2000).

Figure 2 – Diagram of Mosquito Ovaries

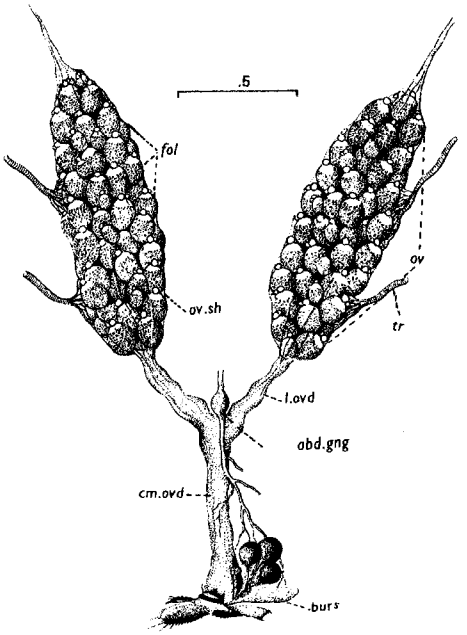


Figure includes: ovaries (ov), including follicles (fol), ovarian sheath (ovsh), tracheae (tr), lateral oviducts (l.ovd), common oviducts (cm.ovd) (Clements 2000).

Each ovary is composed of between 50 and 500 ovarioles. Each ovariole is covered in an ovariolar sheath and is subdivided into the germarium and vitellarium (Figures 3, 4). In the ovariole's anterior portion, the germarium, mitotic division of germ cells occurs, producing follicles. The remaining portion of the ovariole, the vitellarium, is composed of the developing follicles as they mature while approaching the calyx and lateral oviduct. It has been proposed that the follicular stalk (or pedicel) is not a hollow tube opening into the calyx (Clements 2000). Instead, research suggests that the follicular stalk is rod-like and the calyx wall is without perforations until the later stages of the ovarian cycle (Lehane and Laurence 1978). Since follicles within the ovariole are in different stages of development, it is useful to differentiate them as the more mature and closest to the calyx as the proximate or primary (1^{o}) follicle and the younger, furthest from the calyx as penproximate or secondary (2^{o}) follicles (Feinsod and Spielman 1980). With each ovarian cycle, the penproximate follicles from the previous cycle mature and replace the former proximate follicles, and the germarium produces a new penproximate follicle to begin development.

Figure 3 - Cutaway View of Internal Mosquito Ovary Structure (Clements 2000).

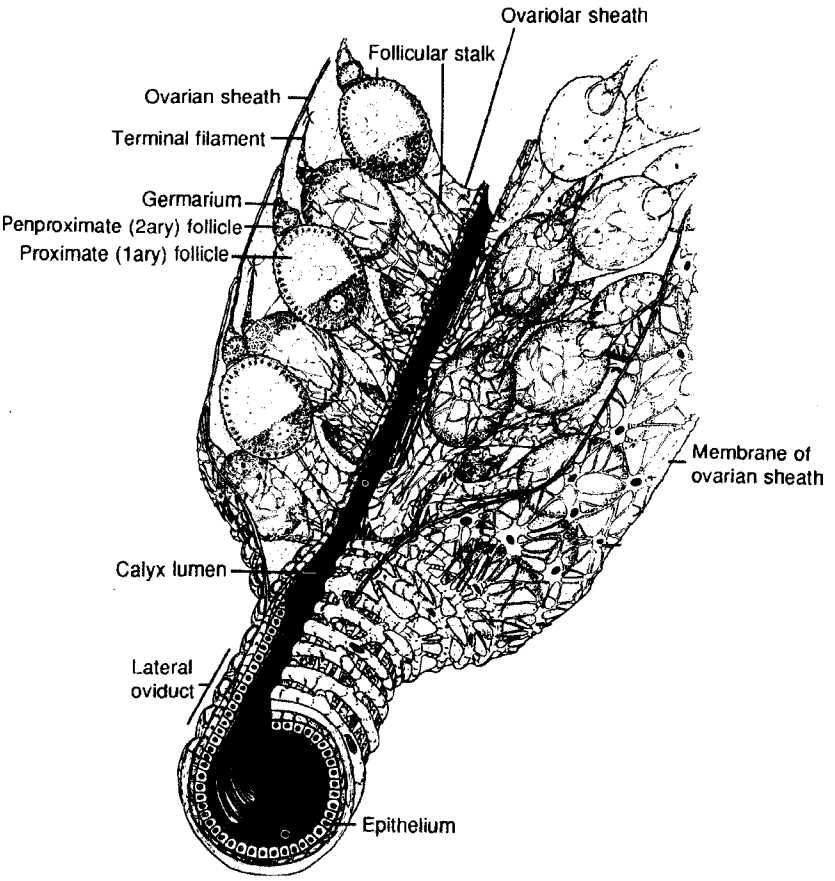


Figure 4 – Lateral Closeup of Mosquito Ovariole Structure.

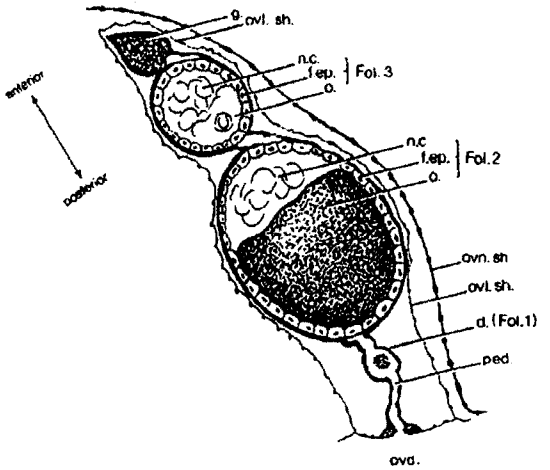


Figure includes: ovarian sheath (ovnsh), ovariolar sheath (ovlsh), follicles (fol), germarium (g), nurse cells (nc), follicular epithelium (fep), and oocytes (o) (Clements 2000).

Oogenesis is characterized by differentiation of oocytes from a terminal germarium. These cells accumulate yolk proteins and pass through to oviducts where they are fertilized as they head to the exterior for oviposition and embryogenesis (Cupp and Jacobs-Lorena 1996). Egg development begins in the adult female upon eclosion: the follicles, containing 8 germ cells surrounded by a follicular epithelium, form into one germ cell and the remaining 7 cells become nurse cells. Initially, the female does not commence mating or bloodfeeding. After 2-3 days of maturation and hormonal development, the female begins to seek mates and bloodmeal hosts (Hagedorn 1996). After the initial phase of differentiation, the ovarian follicles remain in a state of quiescence until metabolically activated by a bloodmeal. Egg production is a cyclical process, alternating between development and quiescence punctuated by bloodmeals and oviposition. Each period of quiescence is terminated by a bloodmeal, which causes a transition to metabolic activation and ovarian development (Clements 2000b,c). Thus, the ovarian cycle comprises the steps from forming a set of sister follicles to forming a set of mature oocytes. Within this cycle, several events occur: 1) the germarium produces new penproximate follicles, 2) former penproximate follicles develop into proximate follicles, and 3) prior proximate follicles and their oocytes mature into eggs.

The principal events of the ovarian cycle are: oocyte differentiation, reductional division of the oocytes' chromosomes, RNA synthesis and deposition by oocytes and nurse cells, yolk synthesis and deposition by the fatbody, and chorion formation (Clements 2000c). Furthermore, the ovarian cycle can be described in 4 sequential stages based on anatomic and physiological differences: 1) Pre-vittelogenic phase, where

follicles separate from the germaria, cell types differentiate, and oocytes become receptive for vitellogenin; 2) Initiation phase, which occurs immediately after a bloodfeed, prompting vitellogenesis and additional follicular growth and maturation; 3) Trophic phase, when vitellogenesis and oocytes growth rapidly ensue, and terminate with a halt in vitellogenesis and production of an endochorionic layer between epithelial cells and oocytes; 4) Post-trophic phase, when the oocytes fully mature and are coated by an unhardened exochorion ready for fertilization and oviposition. (Clements 2000c, Troy et al. 1975).

In addition to bloodfeeding, external factors and environmental cues also affect ovarian development. Larval nutrition may directly influence previttelogenic follicular maturation, in some cases requiring an adult sugar or bloodmeal to supplement inadequate larval nutrition (Feinsod and Spielman 1980b). Cool temperatures and reduced photoperiods may also extend or even inhibit follicular development (Sanburg and Larsen 1973, Service 1968). After successful fertilization through the micropyle, oviposition occurs on or near a given mosquito species' preferred substrate or larval habitat. The soft, white egg then darkens as the chorion sclerotizes and embryonic development starts almost immediately after the eggs have been laid (Becker et al. 2003). Embryogenesis progresses through development of the pharate larva, and pending the absence of diapause and the presence of proper environmental cues (water, temperature, photoperiod, oxygen content, etc.) hatching occurs and a new generation of mosquitoes enters the environment.

J. Characteristics of the Vector *Aedes albopictus*

Aedes albopictus, commonly called the "Asian Tiger" mosquito, was first described from samples collected in Calcutta, India (Skuse 1894). As the name implies, it is a black mosquito with distinctive white markings. Of particular note is the single, conspicuous silvery white line running dorsally along the middle of the thorax and the elegant white bands on the legs. In the last 100 years *Ae. albopictus* has expanded from southeast Asia to all continents of the planet, excluding Antarctica (Ritchie et al. 2006, Derraik 2006, Gratz 2004). This remarkable expansion is in no small part due to increasing transportation and ever-growing trade routes (Tatem et al. 2006, Robertson and Hu 1935).

As an ecological generalist, *Ae. albopictus* can be found throughout urban, suburban, rural, and forested areas. Densely crowded urban areas lacking vegetation and suitable breeding sites are one of the few macrohabitats in which it is rare or absent (Hawley 1988). Eggs of this mosquito are oviposited near the waterline edge of the two most typical larval habitats for this vector: treeholes (including bamboo stumps) and artificial containers such as discarded tires, metal drums, plasticware, glassware, etc. (Hawley 1988). Preferred oviposition substrates are above the waterline, darkly colored, roughly textured, and oriented vertically, with darker colored water being more attractive than clear water (Gubler 1971, Del Rosario 1963). Eggs are desiccation resistant and, particularly among temperate strains, cold resistant for short periods (Hawley et al. 1989). Embryonation may be as long as 3 days. Hatching may be highly dependent on numerous factors such as age, temperature, oxygen tension, photoperiod, and whether or not diapause was induced (Hawley 1988). The duration of the larval stage averages

approximately 7 days. Laboratory studies on *Ae. albopictus* adults have led to several broad conclusions: females live longer than males, females usually live an average of 6 weeks, but may survive up to 3-6 months, and types of nourishment affect longevity, with access to both blood and sugar providing the greatest longevity (Hawley 1988, Gubler 1970). *Ae. albopictus* is well known for being a catholic feeder, with potential hosts ranging from landsnails, and silkmoth caterpillars to lizards, turtles, snakes, frogs, mice, chickens, guinea pigs, and rats (Hawley 1988, Miyagi 1972, Gubler 1970). However, analysis of field collected bloodmeals from *Ae. albopictus* suggest that the mosquito prefers feeding on mammals, particularly humans, but will readily feed on other hosts, such as birds, if the former are not available (Richards et al. 2006, Hawley 1988, Templis et al. 1970). The mosquito is an avid daytime biter, possessing a bimodal diel activity, with peak feeding at early morning and late afternoon (Wang 1962). Average egg batch size is approximately 65 eggs, with a lifetime average fecundity of approximately 322 progeny. However, one female laid 147 eggs after 1 meal and as many as 950 eggs during her lifetime (Hawley 1988, Gubler 1970).

In temperate climes, *Ae. albopictus* overwinters as an egg, with adults being temperature and photoperiod sensitive and diapause expressed in the egg (Mori et al. 1981, Wang 1966). *Ae. albopictus* from tropical or subtropical areas do not appear sensitive to photoperiodicity and survive poorly in harsh, winter conditions (Hanson and Craig 1994, Hawley et al. 1989, Hawley et al. 1987).

Ae. albopictus is unquestionably a mosquito of public health significance. The tiger mosquito is a competent vector for at least 22 arboviruses (Table 1), including all 4 serotypes of dengue, yellow fever, Chikungunya, Japanese encephalitis, eastern equine

encephalitis, and Ross River viruses (Gratz 2004). *Ae. albopictus* is also a vector of filariae, such as dog heartworm, *Dirofilaria immitis* and *Dirofilaria repens* (Cancrini et al. 2003a,b).

Table 1 - Arboviruses Transmitted by *Aedes albopictus*

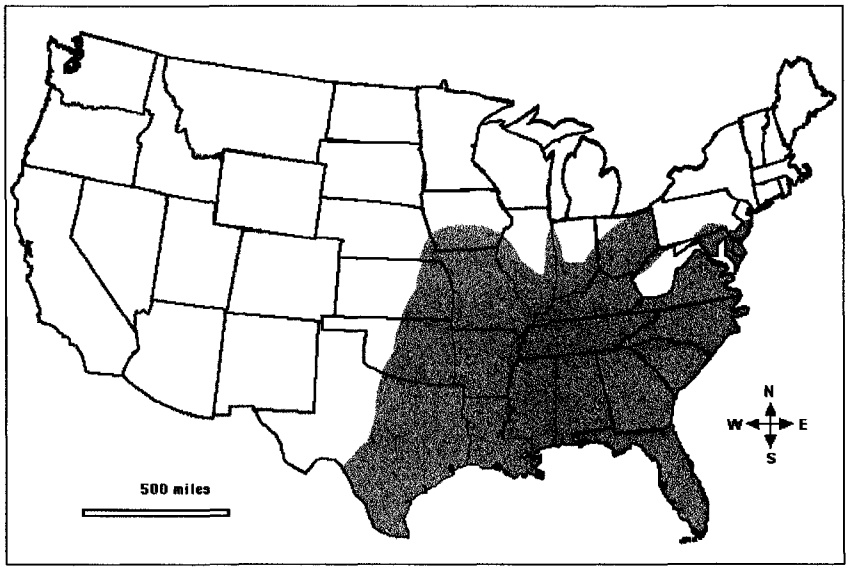
Arboviruses capable of being transmitted by *Aedes albopictus*		
Cache Valley	La Crosse Encephalitis	Ross River
Chikungunya	Mayaro	San Angelo
Dengue (1-4)	Nodamura	Saint Louis Encephalitis
Eastern Equine Encephalitis	Oropouche	Trivittatus
Jamestown Canyon	Orungo	West Nile
Japanese Encephalitis	Potosi	Western Equine Encephalitis
Keystone	Rift Valley	Venezuelan Equine Encephalitis
		Yellow Fever

adapted from Gratz 2004

Consequently, the U.S. public health community was greatly concerned when the first breeding populations of this mosquito were discovered in the continental U.S. in Harris County, Texas, in August 1985 (Sprenger and Wuithiranyagool 1986). Adults had been detected in gravid traps on the U.S. mainland before (Memphis, TN) and larvae had been detected in the cargo holds of ships on the Pacific coast (Los Angeles, CA) (Reiter and Darsie 1984, Pratt et al. 1946). Therefore, it is surprising that breeding populations from Hawaii (present since the 19th century) didn't establish a mainland foothold sooner (Usinger 1944). The mosquito most likely entered the U.S. from Northern Asia within shipments of used tires (Craven et al. 1988, Hawley et al. 1987). Since being introduced, *Ae. albopictus* has been detected in well over 1105 counties and parishes and more than 28 states. (Figure 5) (Moore CG, personal communication, Gerhardt et al. 2001, Moore 1999, Moore and Mitchell 1997). In 2001 the U.S. was again faced with an introduction

within shipments of *Dracaena spp.* ("lucky bamboo") plants from China, and potential establishment in California (Linthicum et al. 2003, Madon et al. 2003).

Figure 5 – Approximate U.S. distribution of *Ae. albopictus* ca2005

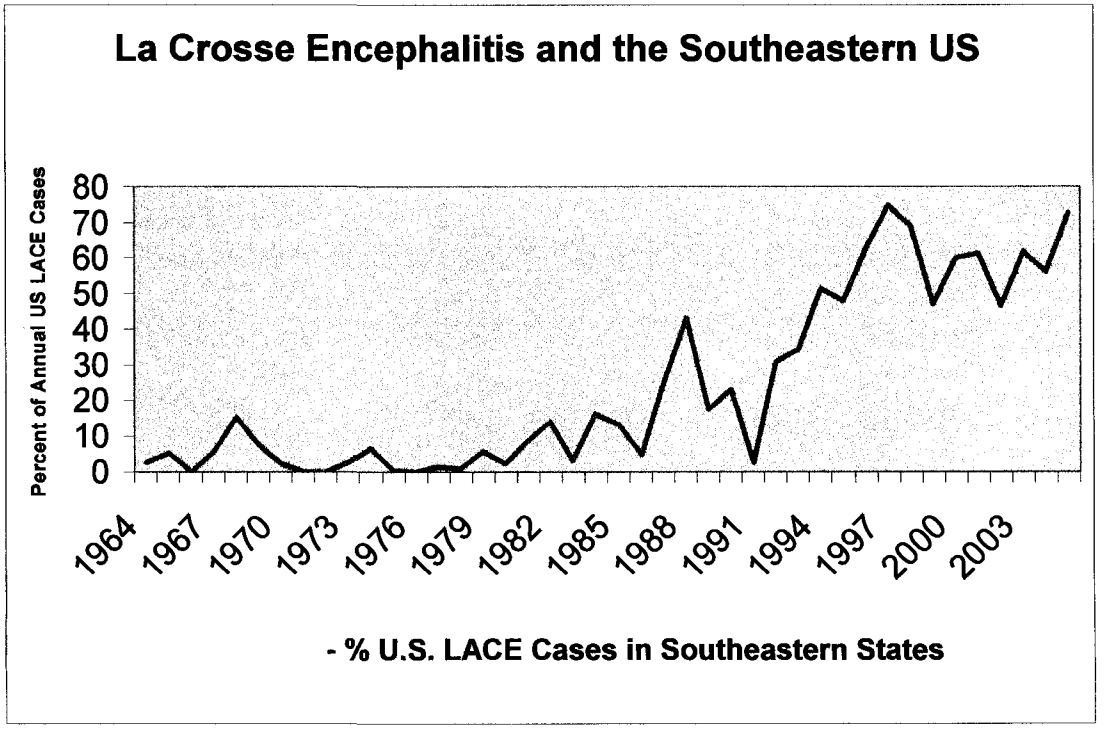


Adapted from Darsie and Ward 2005

With the introduction of this aggressive, old world vector into North America, there was understandable concern about its ability to transmit pathogens indigenous to this biome. Indeed, in the U.S., *Dirofilaria* and 8 arboviruses (Cache Valley, eastern equine encephalitis, Jamestown Canyon, Keystone, La Crosse, Potosi, Tensaw, West Nile) have been isolated from this introduced species (Gratz 2004). Of particular concern has been the possibility that *Ae. albopictus* may alter or enhance the transmission dynamics of LAC. In fact, there is evidence La Crosse encephalitis epidemiology is changing, with many more cases occurring outside of the traditional, upper Midwest "La Crosse belt" range and an emergence in the southeastern U.S. (Erwin et al. 2002, Jones et al. 2000, Nasci et al. 2000, Jones et al. 1999, Szumlas et al. 1996).

The CDC nationwide dataset on confirmed and probable California serogroup (mainly LAC) encephalitis cases in the U.S. shows that prior to the introduction of *Ae. albopictus* in 1985, the southeastern U.S. accounted for an annual average of 5% of all cases. After 1985, the southeastern region accounts for an annual average of 45% of all cases (CDC dataset 2006) (the southeastern U.S. was defined as 14 states: AL, AR, FL, GA, KY, LA, MS, MO, NC, SC, TN, TX, VA, WV) (Figure 6). These results could be due to numerous factors, including surveillance artifacts, increased disease reporting, and diagnostic efforts. In at least one La Crosse encephalitis outbreak in the south, the presence of *Ae. albopictus* represented a 3 times greater risk of infection (Erwin et al. 2002).

Figure 6 – Occurence of La Crosse Encephalitis (LACE) in the Southeastern U.S. (1964-2005).



Southeastern U.S. (1964-2005). The southeastern U.S. was defined as 14 states: AL, AR, FL, GA, KY, LA, MS, MO, NC, SC, TN, TX, VA, WV. Prior to 1985, the Southeastern US accounted for an annual average of 5% of all cases. Following 1985, the Southeastern US accounts for an annual average of 45% of all cases.

The medical importance of a suspected vector species is often determined using laboratory colonized populations to determine its ability to become infected and transmit a given pathogen. Since large numbers of mosquitoes are usually required to assess these vector competence parameters, the use of laboratory colonies is both convenient and practical. Although it is possible to acquire field samples of vector populations, this is frequently and logistically restricted to seasonally permissive times of the year, making the capture process inconsistent and unreliable. Additionally, wild-caught specimens of interest must be separated from other species, which is time-consuming and labor intensive. Manipulating vectors for identification of species, sex, etc. can cause mortality, potentially imposing an additional source of selection pressure. In addition, the infection status for a host of natural pathogens in wild caught specimens must be determined in order to assess potential study confounders. Thus, laboratory colonized populations are a convenient surrogate for natural populations.

Unfortunately, a potential concern with the use of long colonized vector strains is that selection pressures during colonization may produce strains that does not well-represent the original, wild populations. During the process of colonization, sample specimens are taken from a complex environment and colonized within a relatively austere but stabilized insectary environment with photoperiod, humidity, temperature, food source, etc. regularly monitored and maintained to constant conditions. Specimens that do not readily adapt to such environs have reduced fitness and decreased reproduction, resulting in bottlenecking from the founding population. Two major issues are of concern in the use of colonized strains to determine vector competence

phenotypes: 1) is the colonized population representative of field strains, and 2) are the vector competence phenotypes stable from generation to generation?

Studies have indicated that when mosquitoes are removed from field conditions and reared in the laboratory, they undergo drastic selection and subsequent rapid gene pool alterations (Craig 1964). Stress factors, including overcrowding, underfeeding, and bottlenecking have been reported to be among the major reasons why vector populations kept in the laboratory for any length of time may undergo rapid evolutionary change (Sonleitner 1964). Also, the founder effect may account for observed differences between natural and laboratory populations of a species (Powell and Richmond 1974). Laboratory colonization has been reported to have a diverse and unpredictable effect on the infection and transmission of La Crosse virus by *Ae. triseriatus* (Grimstad et al. 1977). During colonization, transmission rates ranged from 27% to 90% in different regional strains of *Ae. triseriatus*. Colonization has also been reported to alter the photoperiodic response of *Ae. triseriatus* for induction of egg diapause (Shroyer and Craig 1983). Laboratory colonization significantly changed the infection and transmission rates of *Culex spp.* for Rift Valley fever virus (Gargan et al. 1983). Susceptibility to infection with yellow fever virus declined dramatically during colonization (from 55% at F_0 to 12% in F_{10}) (Lorenz 1981).

To date, only 5 laboratory studies have examined the vector potential of *Ae. albopictus* for LAC. Of those, 2 dealt primarily with oral transmission to small mammals (Cully et al. 1992, Grimstad et al. 1989). *Ae. albopictus* strains from Indiana, Missouri, and Texas were colonized for at least 2-3 generations. The mosquito strains had similar transmission potential as the native vector, *Ae. triseriatus*. In Hawaii (Tesh and Gubler

1975), the interactions between LAC and *Ae. albopictus* were studied a full decade before the vector was introduced to the mainland. Three studies have dealt primarily with TOT of LAC (Hughes et al. 2006, Streit and Grimstad 1990, Tesh and Gubler 1975); one of which investigated TOT potential in a North American strain (LA) having been in continuous laboratory culture for over 20 years (Nasci RS, personal communication). All 3 studies demonstrated that *Ae. albopictus* is able to transovarially transmit LAC. However, no single study can definitively encompass all aspects of the complex vector-virus interaction. Due to the known issues related to laboratory colonization, the effects of inbreeding, the continuing dispersal of *Ae. albopictus* throughout North and South America, and the genuine public health threat of this disease, field-relevant studies into this arbovirus-vector system are warranted (Gargan et al. 1983, Lorenz 1981, Grimstad et al. 1977).

K. Summary and Research Objectives

LAC emerged in the 1960s and remains an important threat to public health (McJunkin et al. 2001, Thompson et al. 1965). Although the virus is historically transmitted in the midwest by the primary vector, *Ae. triseriatus*, recent isolations of the virus from *Ae. albopictus*, and the potential for transmission in the southern U.S. is cause for concern (Erwin et al. 2002, Gerhardt et al. 2001). Previous laboratory studies have demonstrated the ability of *Ae. albopictus* to orally, venereally, and transovarially transmit the virus (Grimstad et al. 1989, Tesh and Gubler 1975). However, as this mosquito continues to spread throughout the U.S. and overlap with LAC endemic regions, it is important to characterize this vector-virus relationship using freshly-

colonized North American strains that are geographically representative of this non-native mosquito.

The first objective of this research was to investigate the vector competence of *Ae. albopictus* for LAC virus. This included the study of TOT and filial infection rates of recently colonized, geographically diverse strains of *Ae. albopictus*. Results of experiments characterizing the vector competence, oral infection, and the detection of viral antigen (Ag) in head tissue, and horizontal and TOT transmission are described in Chapter 2. It was hypothesized that regional strains of *Ae. albopictus* express significant variability in both disseminated infection and filial infection rate (FIR). The identification of field strains with widely divergent horizontal and TOT transmission potential could both be of public health significance and provide insight into the epidemiology of this emerging disease.

The second objective of this research was to characterize the basic infection kinetics and tropisms of LAC in ovaries of orally exposed adults. It was hypothesized that virus enters the oocytes through the follicular epithelium of surrounding structures such as the oviducts, calyx, and ovariolar sheath. Elucidating the manner in which LAC gains entry into the developing egg would provide insight into the basic mechanisms underlying vertical transmission in orally exposed adults.

The third objective of this research was to broadly determine the phylogeography of field collected populations of *Ae. albopictus*, using a mitochondrial DNA (mtDNA) marker. The collection, extraction, amplification, and analysis of DNA samples from field populations are described in Chapter 3. The hypothesis was that populations of greatest geographical or physical separation would be more genetically distinct than

populations in close proximity to one another (i.e. Tobler's first law of geography) (Sui 2004, Tobler 1970). The identification of genetically distinct field populations could provide insight into the genetics underlying the potential for emerging patterns in LAC infections.

The final objective of this project was to investigate the genetics of TOT by selecting refractory and susceptible strains of *Ae. albopictus* for TOT of LAC. The experiments and selection scheme are described in Chapter 4. The hypothesis was that there would be a genetic basis for TOT of LAC in *Ae. albopictus*. A strong and consistent response to selection is an indication that the trait has a significant genetic basis (Graham et al. 1999, Falconer and MacKay 1996). Determining the degree to which TOT is genetically conditioned in the tiger mosquito would provide a foundation for future research revealing the mechanisms underlying this phenomenon.

CHAPTER 2: VECTOR COMPETENCE OF GEOGRAPHIC STRAINS OF *AE. ALBOPICTUS* FOR TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS

A. Introduction

Since the initial detection of *Ae. albopictus* breeding populations in a single Texas county, this vector has spread into over 1105 counties and parishes and over 28 states of the mainland U.S. (Moore CG, personal communication, Gerhardt et al. 2001, Moore 1999, Moore and Mitchell 1997). Unfortunately, this vector has become permanently established in North America. In over 2 decades the vector has become established in and colonized this country, with a broad ecological range of distribution from New Jersey south to Florida to as far west as California (Darsie and Ward 2005, Madon et al. 2003). With the adult dispersal range of approximately 200m, *Ae. albopictus* from different geographic regions in the U.S. may possess distinct genetic traits (Hawley 1988). Indeed, studies have shown strains of this mosquito encompass different vector competencies for an array of arboviruses; including Flaviviruses, Alphaviruses, and members of the Bunyaviridae (Mitchell 1991, Boromisa et al. 1987, Gubler and Rosen 1976). Previous studies have demonstrated that *Ae. albopictus* is a competent vector for La Crosse virus (LAC) and that transovarial transmission (TOT) does occur (Hughes et al. 2006, Tesh and Gubler 1975). Such studies are typically done on long colonized strains from a single geographic location. It is important to assess the field-relevant transmission dynamics of different populations. Most prior studies used a limited number of strains or strains of long-colonized laboratory populations.

The studies described here were conducted to test the hypothesis that geographic strains of *Ae. albopictus* differ significantly in vector competence for LAC virus.

Recently colonized (<F2) strains were orally exposed to LAC and then assayed for susceptibility to infection, disseminated infection rates, and filial infection rates (FIR). After oral exposure to virus, the kinetics and rates of dissemination were determined by direct immunofluorescence assay in 21 strains of *Ae. albopictus* and 1 strain of *Ae. triseriatus*. FIR and TOT potential were determined for both 1st and 2nd gonotrophic cycles. The results obtained from these studies provide insight into the potential role of local geographic strains of *Ae. albopictus* in the epidemiology of LAC.

These studies also investigated the anatomic basis of vertical transmission of LACV by *Ae. albopictus*. The broadly accepted paradigm for LAC TOT is that the 1st infectious bloodmeal will produce a disseminated infection, but the 1st gonotrophic cycle progeny produced from this meal are not infected (Miller et al. 1979). Therefore, the subsequent bloodmeals are considered critically important for TOT. However, previous reports suggest that some mosquito progeny resulting from the 1st gonotrophic cycle may be infected with LAC (Cully et al. 1992, Tesh and Gubler 1975, Watts et al. 1973). Additionally, research with *Ae. triseriatus* has indicated that ovarian infection can occur prior to dissemination from the midgut (Chandler et al. 1998, Chandler et al. 1996). Despite previous work on the pathogenesis and tropisms of California group viruses in mosquitoes, the mechanisms for viral entry into the developing follicle or egg are unknown. Viruses may directly infect developing follicles from the surrounding ovarian tissues, but it remains unclear why some follicles within an infected female become infected and others do not (Tesh and Beaty 1983). Early investigations of San Angelo virus (California group, Orthobunyavirus) tropisms demonstrated that the virus was widely disseminated in the ovaries (including follicles, follicular epithelia, nurse cells,

and oocyte). Similar tropisms have been demonstrated for LAC and *Ae. triseriatus* (Kempf et al. 2006, Paulson and Grimstad 1989, Beaty and Thompson 1978).

Because of the lack of information on the anatomic basis of LAC infection of *Ae. albopictus*, as well as the observation of 1st gonotrophic cycle transmission, studies were conducted to characterize the basic, early infection kinetics and tropisms of LAC in ovaries of adults infected *per os*. Elucidating the time and manner by which LAC infects the developing egg may provide insight into the basic mechanisms underlying TOT in orally exposed adults.

B. Materials and Methods

Cells

BHK-21 Cells. Baby hamster kidney epithelial (BHK) cells were grown in Dulbecco's modified Eagle medium (DMEM), including 10% fetal calf serum and 1500 units penicillin per mL, 1500 units of streptomycin per mL, and 1.5 mg of gentamicin per mL in 75-cm^2 flasks in 5% CO_2 at 37°C. The cells were diluted 1:20 and passed every 4 days until virus inoculation.

Vero Cells. African green monkey kidney epithelial (Vero) cells were maintained in Liebovitz medium (L-15), containing 10% fetal calf serum, and 1500 units penicillin per mL, 1500 units of streptomycin per mL, and 1.5 mg of gentamicin per mL in 75-cm^2 flasks (no CO_2) at 37°C. The cells were diluted 1:20 and passed every 7 days until virus inoculation.

Virus. LAC (Human/78, also called H78) was originally isolated from the brain of a patient with a fatal case of La Crosse encephalitis in 1978 and maintained at the

Arthropod-borne and Infectious Diseases Laboratory (AIDL), Colorado State University, Fort Collins, CO. The virus had been passed 4 times in BHK-21 cell culture. A working stock of La Crosse virus was prepared by infecting confluent BHK-21 cultures in 150-cm^2 flasks at a multiplicity of infection (MOI) of 0.001. After approximately 72 hours, when cytopathic effects (CPE) reached 100%, virus titers were quantified by 50% tissue culture infectious dose ($TCID_{50}$) endpoint titration on Vero cells. Titers were determined in replicates of 4 per dilution by the Karber method (Karber 1931). Aliquots (25μL) of the stock virus preparations were stored at -70°C and used in all infection experiments. The stock La Crosse virus had a titer of 3.16×10^7 $TCID_{50}$/mL.

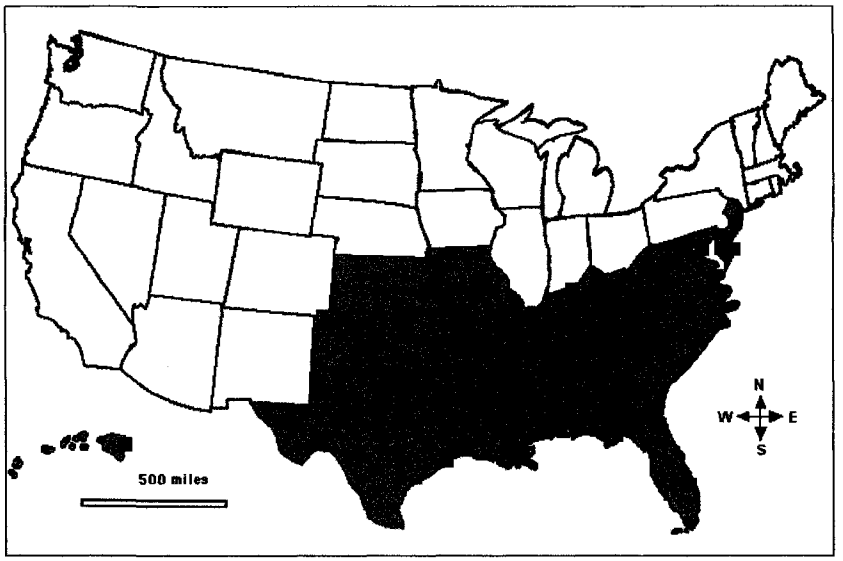
Mosquito Acquisition and Maintenance. All strains were reared under standard insectary conditions, ≥80% RH, 14:10 light-dark cycle and 28°C (Gerberg et al. 1994). Adult mosquitoes were provided sugar cubes and water *ad libitum.* Forty-eight hours before artificial membrane feeding, sugar cubes were removed, and mosquitoes were provided only water. The laboratory strain of *Ae. albopictus* (LACLA) originated from field material collected near Lake Charles, Louisiana in 1987. It was originally colonized at the Centers for Disease Control and Prevention (CDC) Coordinating Center for Infectious Diseases (CCID), National Center for Zoonotic, Vector-Borne, and Enteric Diseases (NCZVED), Fort Collins, CO, and subsequently continuously colonized at the AIDL. The laboratory strain of *Ae. triseriatus* (LACWI) originated from field material collected near La Crosse, Wisconsin in 1981 and colonized continuously at the AIDL. Between 2004 and 2006, the remaining 20 other *Ae. albopictus* strains were acquired from the field as either eggs or larvae (Table 2, Figure 7).

Table 2 – Source of Field Collected *Aedes albopictus* Strains.

State	City	County/Parish	LAT	LONG	Strain ID	Source
AL	Mobile	Mobile	N 30 41 38	W 88 02 34	MOBAL	Kelly Micher
AR	Farmington	Washington	N 36 02 30	W 94 14 49	FARAR	Dr. Max Meisch
						Bryce Blackman
DE	Georgetown	Sussex	N 38 41 23	W 75 23 09	GEODE	Dr. Gulnihal Ozbay
FL	Vero Beach	Indian River	N 27 38 18	W 80 23 50	VERFL	Dr. Roxanne Rutledge
GA	Macon	Bibb	N 32 50 26	W 83 37 56	MACGA	Dr. Mike Womack
HI	Hilo	Hawaii	N 19 43 47	W 155 05 23	HILHI	Dr. Sheldon C. Furutani
KS	Lawrence	Douglas	N 38 58 18	W 95 14 06	LAWKS	Jason Botz
KY	Ft. Knox	Hardin	N 36 45 52	W 88 17 43	FTKKY	Ben Pagac
LA	New Orleans	Orleans	N 29 57 16	W 90 04 29	NORLA	Bethany Peel
MD	Ft. Meade	Anne Arundel	N 39 06 18	W 76 44 29	FTMMD	Ben Pagac
MO	Columbia	Boone	N 38 57 06	W 92 20 01	COLMO	Dr. Brenda Beernstein
MS	Jackson	Hinds	N 32 17 54	W 90 11 05	JACMS	Dr. Gerome Goddard
						Wendy Varnado
NC	Raleigh	Wake	N 35 46 19	W 78 38 19	RALNC	Dr. Charles Apperson
NJ	New Brunswick	Gloucester	N 40 29 09	W 74 27 08	NBRNJ	Dr. Lena Brattsten
						Debin Sun
OK	Stillwater	Payne	N 36 06 55	W 97 03 28	STIOK	Lisa Coburn
SC	Columbia	Richland	N 34 00 02	W 8102 06	COLSC	Chris Evans
TN	Knoxville	Knox	N 35 57 38	W 83 55 14	KNOTN	Dr. Reid Gerhardt
TX	Galveston	Galveston	N 29 17 56	W 94 47 38	GALTX	Dr. Stephen Higgs
						Kate McElroy
VA	Williamsburg	Williamsburg-James City	N 37 16 14	W 76 42 28	WILVA	Maj. Jason Richardson, USA
WV	Richwood	Nicholas	N 38 13 29	W 80 31 59	RICWV	Humbert "Zap" Zappia

P1 samples were collected as either eggs or larva, and reared to adults. Only *Ae. albopictus* were retained for experiments and strain propagation (P1 = n≥65/strain)

Figure 7 – Distribution of *Aedes albopictus* Field-Collection Sites



Eggs were hatched and larvae reared to pupae. Upon ecdysis, only adult *Ae. albopictus* were keyed to species, retained for experimentation, and designated generation P1 ($n \geq 65$/strain) (Darsie and Ward 2005). In order to produce an F1 generation for phenotyping, P1 adults were offered 2 artificial bloodmeals at 1 week intervals of mechanically defibrinated sheep blood (Colorado Serum Company, Denver, CO) for a minimum of 2 hours each. Eggs were collected from both gonotrophic cycles, designated generation F1, and partially dried until ready for hatching and experimentation. All P1 adults were then screened for disseminated LACV infection by headsquash and a direct immunofluorescence assay (IFA). The samples were assayed using a 1:100 dilution of a fluorescein-isothiocyanate conjugated, polyclonal anti-LAC antibody created from the sera of hyperimmune mice (F92) (Beaty and Thompson 1978, Beaty and Thompson 1976, Beaty and Thompson 1975). No P1 samples had detectable LAC antigen (Ag). After LAC infection assay, the headless P1 bodies were then stored at -70°C for DNA extraction and phylogeography studies (Chapter 3).

Infection of Mosquitoes by Artificial Bloodmeal. Three days prior to oral exposure, 15μL of stock virus were added to 15mL of DMEM and used to replace the medium of a confluent 75cm^2 flask of BHK-21 cells. The infected flask was then placed into a plastic, sealed secondary container with an infectious material label. After 3 days incubation, 15mL of newly propagated (unfrozen) infected BHK-21 cell culture supernatant of LAC was mixed with an equal volume of mechanically defibrinated sheep blood. This infectious bloodmeal solution was then transferred to a 37°C water-jacketed membrane feeder for oral infection of mosquitoes (Rutledge et al. 1978). Mosquitoes that had been deprived of sugar for forty-eight hours were allowed to feed for a maximum of 2 hours.

After feeding, females were visually inspected and fully engorged females were retained for study. Partially fed and non-fed females were discarded.

Virus Titration Protocol. Virus titer in infected bloodmeals was determined by serial 10-fold dilution in sterile 96-well flat bottom plates with lids. First, 180µL of L-15 was placed in each well. The infectious bloodmeal was then centrifuged (3500Xg for 10 minutes) to deposit red blood cells (RBCs). Next, 20µL of the infectious supernatant plasma was pipetted into each well of the first column. Then, 20µL was transferred in serial dilutions from column 1 through column 12, moving from one column to the next and pipetting to mix. Filtered tips were changed between each column to prevent carry over contamination. A $75cm^2$ flask of Vero cells was then trypsinized with 5mL of trypsin/EDTA for 3-4 minutes at 37°C in an incubator. Trypsin activity was halted by adding 5mL of L-15 medium (containing 10% fetal bovine serum, FBS) and pipetting to mix. Next, 100µL of Vero cell suspension was then added to each well of the plate, starting from the highest dilution (lowest titer) to lowest dilution (highest titer). The infected plate was then placed into a plastic, sealed secondary container with an infectious material label. After 7 days incubation, the wells of the plate were microscopically examined for CPE and cell death. Titers were calculated in replicates of 4 dilutions by the Karber method (Karber 1931). Virus titers averaged $4.89x10^6$ $TCID_{50}$/mL (Standard deviation: 0.210, Variance: 0.044, Range $1.58x10^6$ - $1.00x10^7$ $TCID_{50}$/mL). The equation is as follows:

Log_{10} of $TCID_{50}$ = X –d (P-0.5)

X = log_{10} of the highest concentration (lowest dilution) used

d = log_{10} of dilution factor

$$P = \frac{\text{sum of percent infection at each dilution}}{100}$$

Oral infection and dissemination of LACV in *Ae. albopictus* strains. Studies were conducted to determine the relative susceptibility of *Ae. albopictus* strains to LACV oral infection and dissemination. The kinetics and rate of disseminated infection (DI) were determined for each strain of mosquito by sampling on days 0, 7, 10, and 14 after oral exposure to virus (n=50/timepoint). To determine if mosquitoes had disseminated infections, the head was removed from each mosquito, squashed onto a microscope slide, and fixed in cold acetone for 15 minutes. Slides were then air dried and small circles were made around each sample of tissue using a yellow, fine-tip Texpen latex paint marker and allowed to air dry for 20 minutes. Approximately 65µL of F92, a polyclonal antibody for LAC Ag described above (page 35), was then added onto each circle to cover the entire area of tissue. The slides were then placed for 30 minutes into an environmental chamber set to 37°C and ≥80% RH. Slides were then put into a metal slide holder and immersed twice in a 1X PBS wash for 10 minutes. Slides were then placed for 2 seconds into dH_2O, before mounting. A single drop of mounting medium, consisting of a 3:1 solution of glycerol and PBS, was deposited onto each circle of tissue and a coverslip was then placed over the slide. Stained slides were visualized using an

Olympus BH2 fluorescent microscope (at 100-200X magnification). A mosquito was considered to have a disseminated infection if virus Ag was present in the head.

Determination of Vertical Transmission Potential. To determine the 1st and 2nd filial infection rate (FIR) for each strain, the corresponding eggs were collected, hatched, and reared to pupation. Within 12-24 hours of ecdysis, the head was removed from each mosquito, squashed onto a microscope slide, and assayed for LAC Ag as described above. One hundred progeny were assayed per gonotrophic cycle per strain. The DI for each timepoint was determined as the percentage of mosquitoes with detectable viral Ag in head tissues within the total number of females tested. The FIR was determined as the percentage of progeny for a given gonotrophic cycle (1st or 2nd) with detectable viral Ag in the head tissue within the total number of progeny tested.

Oviposition and Larval Rearing. Bloodfed mosquitoes were maintained in cartons containing oviposition (OP) cups containing distilled H_2O (dH_2O) and paper strips to enhance egg laying. Ten days after the 1st infectious bloodmeal, OP liners were collected, partially dried, and stored until ready for hatching. To induce egg hatching, the OP liners were placed in ~1L of water containing 0.1% brain heart infusion broth. After larval hatching, progeny mosquitoes were given adequate food (Purina Mouse Chow and Tetramin fish food) and raised under conditions identical to that of the parents.

Second Bloodmeal. Ten days after the 1st infectious bloodmeal, adults were sugar deprived in preparation for a 2nd non-infectious bloodmeal. Mechanically defibrinated sheep blood was transferred to a 37°C water-jacketed membrane feeder (Rutledge et al. 1978). Mosquitoes that had been deprived of sugar for forty-eight hours were allowed to feed for a minimum of 2 hours. After feeding, females were visually inspected and fully

engorged females were retained for study. Partial or non-fed females were discarded. Fresh OP cups were placed into the cartons for oviposition of 2nd gonotrophic cycle eggs. Ten days after the 2nd non-infectious bloodmeal, OP liners were collected, partially dried, and stored until ready for hatching. To induce egg hatching, the OP liners were placed in ~1L of water containing 0.1% brain heart infusion broth. After larval hatching, progeny mosquitoes were given adequate food and raised under conditions identical to that of the parents.

Sample Processing. After screening for full engorgement status, mosquitoes were collected in triplicate at 8 timepoints post oral exposure (Day 0, 1, 2, 3, 4, 5, 6, 7) (n=10 mosquitoes per timepoint; combining all replicates results in a total of n=30 mosquitoes per timepoint). Mosquitoes were briefly chilled at -20°C to immobilize for immediate decapitation and ovarian dissections. Determination of disseminated head infections were as previously described (page 38).

Concurrent with head assay, individual mosquitoes also had both ovaries removed for determination of infection status. For each collected mosquito, 1 ovary was dissected *in toto* and 1 ovary was briefly disrupted with clean minutens pins (Monarch, size 0) in order to expose individual follicles for examination and viral detection. Ovarian tissues were fixed in a 4% paraformaldehyde solution for 2 hours at room temperature while gently rocking in clear, 500µL eppendorf tubes. The paraformaldehyde solution was carefully removed by pipette and 200µL of a 1× PBS, 1% BSA, 0.2% Triton X-100 solution (PBT-2) was added. The PBT-2 solution was removed by pipette and an additional 200µL of PBT-2 was added. Following gentle rocking at room temperature for 1 hour, the PBT-2 was again carefully pipetted out and 100µL of F92 was added to

each tube and gently rocked overnight at 4°C. The conjugate was removed and 200µL of a 1× PBS, 1% BSA, 0.1% Triton X-100 solution (PBT-1) was added. Tissue was mounted onto yellow, 6 well microscope slides (Erie Scientific, 30-618). Excess PBT-1 was removed by pipette and 2 drops of Vectashield mounting medium (Vector Labs, H-1400) were added. A coverslip was applied and slides were stored vertically within a slide box overnight at 4°C. Clear nail polish was used to seal the edges between the coverslip and slide prior to imaging. Stained tissues were visualized as above. For details on reagent preparation, see Appendix A (page 138).

Data analysis. Data were compiled using Microsoft Excel and then analyzed by ANOVA, Chi squared test for independence, and the Grubb's test for outliers using Microsoft Excel and Graphpad Instat 3 programs (Barnett and Lewis 1998, Iglewicz and Hoaglin 1993).

C. Results

Disseminated Infections. Twenty-one strains of *Ae. albopictus* and one strain of *Ae. triseriatus* (20 F1 *Ae. albopictus*, 1 long laboratory colonized *Ae. albopictus*, and 1 long laboratory colonized *Ae. triseriatus*) were examined for a total of 8,800 individual mosquitoes. The resulting kinetics and rates of disseminated infection of LAC are provided in Table 3, Table 4, and Figure 8. Percent infection by timepoint was calculated by dividing the total number of mosquitoes with disseminated infection by the total number of mosquitoes tested per timepoint by strain.

Dissemination infections were determined for 22 mosquito strains on Day 0, Day 7, Day 10, and Day 14 for a total of 4400 samples (Table 3). No populations were

completely refractory to LAC infection. On Day 0, no detectable LAC Ag was found in head tissues of mosquitoes in any experimental population. In contrast, by Day 7, virus dissemination had occurred in all mosquito strains, with DI rates ranging from 48 to 100%. Among field strains, all contained detectable LAC Ag by Day 7, with variability among strains decreasing at each subsequent timepoint. Field strains possessed an average DI rate on Day 7, 85.4% (SD 13.38), on Day 10, 95.8% (SD 6.12), and on Day, 14 98.1% (SD 2.72) (Figure 8). The DIs differed significantly over time (Day 7 χ^2=136.38, P<0.001; Day 10 χ^2=86.12, P<0.001; Day 14 χ^2=37.50, P<0.01) (Table 4). On Day 7 after oral exposure, the greatest variability in DI occurred and steadily decreased through each subsequent timepoint (DI variability on Day 7: 0.0179, Day 10: 0.0037, Day 14: 0.001) (Figure 9). The overall average DI was lowest at Day 7, and steadily increased through Day 14 (DI on Day 7: 85.4%, Day 10: 95.8%, Day 14: 98.1%). Multiple strains exhibited statistically significant differences with respect to the kinetics of DIs (Table 4). Both MOBAL and NBRNJ expressed significantly lower DI rates from Day 7 through Day 14 (P<0.05, P<0.01, respectively). DI for GEODE was significantly lower only on Day 7 (P<0.005). While the DIs for LAWKS and FTMMD were significantly lower only on Day 14 (P<0.05 each). Three strains were particularly susceptible to early infection; HILHI, STIOK, and COLSC possessed significantly higher DIs on Day 7 (P<0.01 each). The DI rate of the *Ae. triseriatus* lab strain (LACWI) was significantly lower than the *Ae. albopictus* field strains, with a DI rate on Day 7 of 52% (P<0.001) and on Day 10 84% (P<0.05).

Table 3 – Relative Susceptibility of Geographic Strains *Aedes albopictus* and *Aedes triseriatus* to LACV Infection.

STRAINS	Day 0		Day 7		Day 10		Day 14	
FIELD	Nº Infected	Percent	Nº Infected	Percent	Nº Infected	Percent	Nº Infected	Percent
MOBAL	0/50	0%	37/50	74%	41/50	82%	47/50	94%
FARAR	0/50	0%	39/50	78%	50/50	100%	50/50	100%
GEODE	0/50	0%	29/50	58%	50/50	100%	50/50	100%
VERFL	0/50	0%	45/50	90%	46/50	92%	48/50	96%
MACGA	0/50	0%	46/50	92%	47/50	94%	48/50	96%
HILHI	0/50	0%	50/50	100%	50/50	100%	50/50	100%
LAWKS	0/50	0%	41/50	82%	46/50	92%	47/50	94%
FTKKY	0/50	0%	44/50	88%	50/50	100%	50/50	100%
NORLA	0/50	0%	44/50	88%	50/50	100%	50/50	100%
FTMMD	0/50	0%	45/50	90%	50/50	100%	47/50	94%
COLMO	0/50	0%	43/50	86%	49/50	98%	49/50	98%
JACMS	0/50	0%	48/50	96%	50/50	100%	50/50	100%
RALNC	0/50	0%	44/50	88%	48/50	96%	50/50	100%
NBRNJ	0/50	0%	24/50	48%	39/50	78%	46/50	92%
STIOK	0/50	0%	50/50	100%	50/50	100%	50/50	100%
COLSC	0/50	0%	50/50	100%	50/50	100%	50/50	100%
KNOTN	0/50	0%	40/50	80%	47/50	94%	49/50	98%
GALTX	0/50	0%	41/50	82%	49/50	98%	50/50	100%
WILVA	0/50	0%	48/50	96%	48/50	96%	50/50	100%
RICWV	0/50	0%	46/50	92%	48/50	96%	50/50	100%
LAB								
LACLA	0/50	0%	40/50	80%	48/50	96%	48/50	96%
LACWI	0/50	0%	26/50	52%	42/50	84%	48/50	96%

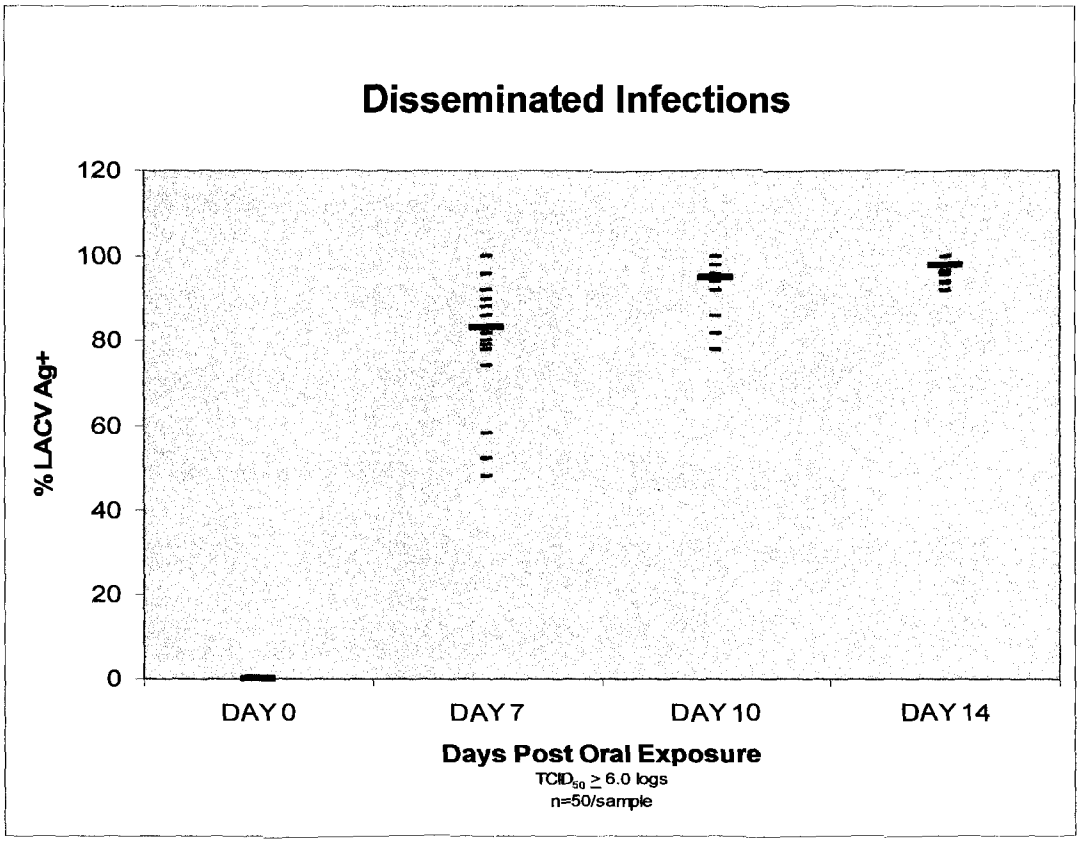
Number and percentage testing LACV Ag+ in head tissues of orally exposed adult mosquitoes. Lab strains include long colonized *Ae. albopictus* (LACLA) and *Ae. triseriatus* (LACWI).

Table 4 – Comparison of Relative Susceptibility to Disseminated Infections in *Ae. albopictus* strains.

		χ^2		
STRAINS	Day 0	Day 7	Day 10	Day 14
FIELD				
MOBAL	0	**4.450**	**21.087**	**4.424**
FARAR	0	1.875	2.196	0.950
GEODE	0	**4.395**	2.196	0.950
VERFL	0	0.725	1.483	1.161
MACGA	0	1.248	0.295	1.161
HILHI	0	**7.300**	2.196	0.950
LAWKS	0	0.396	1.483	**4.424**
FTKKY	0	0.232	2.196	0.950
NORLA	0	0.232	2.196	0.950
FTMMD	0	0.725	2.196	**4.424**
COLMO	0	0.012	0.651	0.003
JACMS	0	0.658	2.196	0.950
RALNC	0	0.232	0.017	0.950
NBRNJ	0	**8.189**	**35.305**	**9.792**
STIOK	0	**7.300**	2.196	0.950
COLSC	0	**7.300**	2.196	0.950
KNOTN	0	0.999	0.295	0.003
GALTX	0	0.396	0.651	0.950
WILVA	0	0.658	0.017	0.950
RICWV	0	1.492	1.290	0.950
Degrees of freedom	19	19	19	19
χ^2	0	**136.377**	**86.118**	**37.502**
P	p=1	p<		p<0.01
LAB				
LACWI				
Degrees of freedom	1	1	1	1
χ^2	0	**13.511**	**4.000**	0.344
P	p=1	p<0.001	p<0.05	p<1
LACLA				
Degrees of freedom	1	1	1	1
χ^2	0	0.638	0	0.344
P	p=1	p<1	p=1	p<1

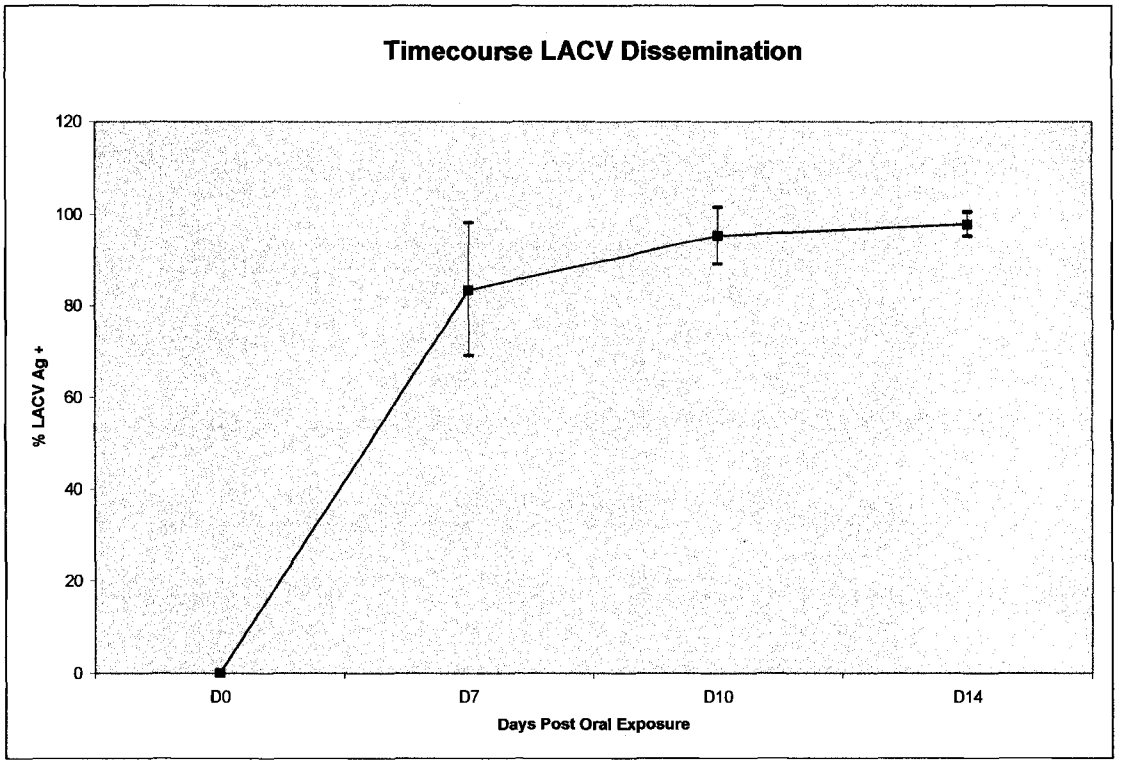
Chi Square values for head tissue infection rates of various strains of *Aedes albopictus* and *Ae. triseriatus*. Lab strains include long colonized *Ae. albopictus* (LACLA) and *Ae. triseriatus* (LACWI). Numbers in bold represent statistically significant values. At a significance level of 0.05, the F_{crit} for 1 degree of freedom is 3.84.

Figure 8 - Disseminated Infections of Geographic Strains of *Aedes albopictus*



Disseminated infections were calculated as the number of adults with LACV Ag present in head tissues divided by the total samples tested. Figure shows the general distribution of infection among strains. Small dashes represent the percentage of infected head tissues for an individual strain. Large dashes represent the average percentage of infected head tissues for all strains. Bloodmeal titer of $TCID_{50} \geq 6.0$ logs. Number tested is n=50/sample.

Figure 9 – Distribution of Disseminated Infection (DI) and Standard Deviation Post Oral Infection



Average DI and standard deviation per timepoint. Results were combined for all strains to display the average disseminated infection and standard deviation at each timepoint. Disseminated infections were calculated as the number of adults with LAC Ag present in head tissues divided by the total samples tested. Results are provided to show the general distribution of disseminated infection and standard deviation versus time.

Filial Infection Rates. The results for 1st and 2nd gonotrophic cycle filial infection rates of LAC by individual mosquito per strain and cycle are given in Figure 10, Table 5, and Table 6. Percent infection by cycle was calculated by dividing the total number of mosquito progeny with LAC infection by the total number of progeny tested per cycle by strain.

1st and 2nd gonotrophic cycle samples were collected for 22 mosquito strains for a total of 4400 samples (Figure 10, Table 5). Six strains produced infected 1st gonotrophic cycle progeny (GEODE, HILHI, FTMMD, GALTX, RICWV, LACLA). The overall 1st cycle FIRs did not differ significantly (χ^2=20.73, P<1) (Table 6). Infection of 1st gonotrophic cycle progeny was infrequent and low, averaging 1.17% (SD 0.41). All strains were susceptible to 2nd gonotrophic cycle infection (Table 5). Females from all field strains were permissive for TOT, with an average 2nd FIR of 10.1% (SD 6.48). The FIRs did differ significantly (χ^2=87.86, P<0.001) (Table 6). Two strains, MOBAL and STIOK were found to have significantly higher FIRs than other *Ae. albopictus* strains (31% and 20%, respectively, P<0.005 each). One strain, GALTX, was found to have a significantly lower FIR of 3% (P<0.05). Between field and lab strains, only LACWI, was found to have a significantly higher FIR in the 2nd gonotrophic cycle (49%, P<0.001).

Table 5 – 1st and 2nd Gonotrophic Cycle Filial Infection Rates

Number and percentage of progeny testing LACV Ag+ in head tissue

STRAINS	1ST Gonotrophic Cycle		2ND Gonotrophic Cycle	
FIELD	Nº Infected	Percent	Nº Infected	Percent
MOBAL	0/100	0%	31/100	31%
FARAR	0/100	0%	4/100	4%
GEODE	1/100	1%	6/100	6%
VERFL	0/100	0%	5/100	5%
MACGA	0/100	0%	5/100	5%
HILHI	1/100	1%	15/100	15%
LAWKS	0/100	0%	6/100	6%
FTKKY	0/100	0%	12/100	12%
NORLA	0/100	0%	9/100	9%
FTMMD	2/100	2%	8/100	8%
COLMO	0/100	0%	7/100	7%
JACMS	0/100	0%	5/100	5%
RALNC	0/100	0%	12/100	12%
NBRNJ	0/100	0%	11/100	11%
STIOK	0/100	0%	20/100	20%
COLSC	0/100	0%	10/100	10%
KNOTN	0/100	0%	12/100	12%
GALTX	1/100	1%	3/100	3%
WILVA	0/100	0%	13/100	13%
RICWV	1/100	1%	8/100	8%
LAB				
LACLA	1/100	1%	7/100	7%
LACWI	0/100	0%	49/100	49%

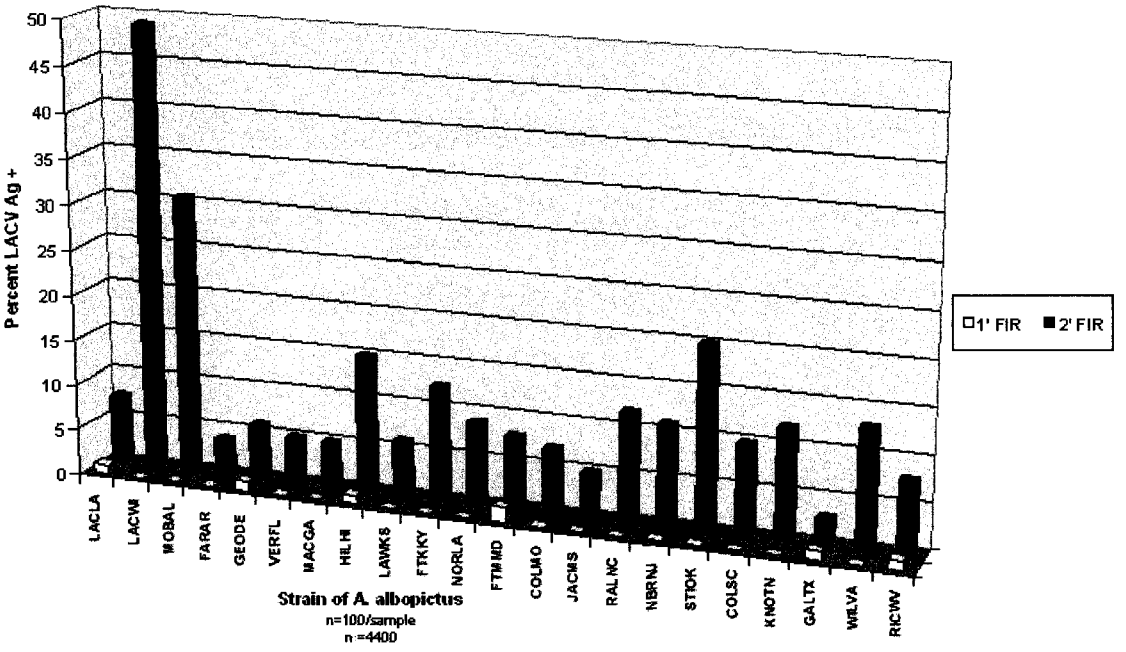
Filial infections were calculated as the number of progeny expressing LACV Ag in head tissues divided by the total number of progeny tested. Lab strains include long colonized *Ae. albopictus* (LACLA) and *Ae. triseriatus* (LACWI).

Table 6 – Comparison of 1st and 2nd Gonotrophic Cycle Filial Infection rates (FIR) in Geographic Strains of *Ae. albopictus*

	χ^2	
STRAINS	1st Cycle FIR	2nd Cycle FIR
FIELD		
MOBAL	0.001	**43.249**
FARAR	0.001	3.684
GEODE	0.005	1.664
VERFL	0.001	2.575
MACGA	0.001	2.575
HILHI	0.005	2.377
LAWKS	0.001	1.664
FTKKY	0.001	0.357
NORLA	0.001	0.120
FTMMD	0.029	0.437
COLMO	0.001	0.951
JACMS	0.001	2.575
RALNC	0.001	0.357
NBRNJ	0.001	0.080
STIOK	0.001	**9.704**
COLSC	0.001	0.001
KNOTN	0.001	0.357
GALTX	0.005	**4.991**
WILVA	0.001	0.833
RICWV	0.005	0.437
Degrees of freedom	19	19
χ^2	20.729	**87.864**
p	p<1	p<0.001
LAB		
LACWI		
Degrees of freedom	1	1
χ^2	0.001	**43.703**
p	p<1	p<0.001
LACLA		
Degrees of freedom	1	1
χ^2	0.005	0.951
p	p<1	p<1

Chi Square Values for FIRs of Various Strains of *Aedes albopictus* and *Ae. triseriatus*. Numbers in bold represent statistically significant values. At a significance level of 0.05, the F_{crit} for 1 degree of freedom is 3.84. Lab strains include long colonized *Ae. albopictus* (LACLA) and *Ae. triseriatus* (LACWI).

Figure 10 – Filial Infection Rates of Geographic Strains of *Aedes albopictus*



1st and 2nd gonotrophic cycle FIR. FIR was determined for a strain by dividing the number of progeny expressing LACV Ag in head tissues by the total of progeny tested. One hundred progeny were tested for each strain for both the 1st and 2nd gonotrophic cycles. Lab strains include long colonized *Ae. albopictus* (LACLA) and *Ae. triseriatus* (LACWI).

Table 14 - Haplotype Analysis of Field Collected *Ae. albopictus*

ND5 Polymorphism and Haplotype Analysis for *Aedes albopictus* field collections	
Number of Sequences	34
Number of Sequences Used	34
Selected region	1-405
Number of Sites	405
Total Number of Sites	366
Number of variable sites(S)	1
Total Number of Mutations (Eta)	1
G-C Content	
at noncoding positions	0
at coding positions	0.219
total	0.219
Haplotype/Nucleotide Diversity	
Number of Haplotypes	2
Haplotype(gene) diversity (Hd)	0.114
Variance of Hd	0.00509
STDDEV of Hd	0.071
Nucleotide Diversity per site (π)	0.00031
Sample variance of π	0
STDDEV of π	0.00019
Average number of nucleotide differences (k)	0.11408
Theta (per sequence) from S, θ-W	0.24457
Theta (per site) from S, θ-W	0.00067
Neutrality Tests	
Tajima's D	-0.79933
not significant (p>0.10)	
Fu and Li's D test statistic	0.5804
not significant (p>0.10)	
Fu and Li's F test statistic	0.22484
not significant (p>0.10)	
Strobeck's S statistic	0.929
Haplotype Distribution	
Number of Haplotypes (h)	2
Haplotype diversity (Hd)	0.1141
h 1	2 (1-2)
h 2	32 (3-34)

with a rate of 93% for infection of head tissues (Table 9). LAC Ag was detectable in *Ae. albopictus* heads by Day 3 (17%) post oral exposure, with an increasing rate of infection until Day 6, decreasing at Day 7 (87%). LAC Ag was detectable in mosquito calyx tissue as early as Day 2 (23%), also with an increasing rate of infection until Day 7 (87%). Infection of the calyx tissues occurred before detection of Ag in the head and at significantly higher rates: Day 2 calyx infection rate of 23% vs. head infection rate of 0% ($p<0.01$, $\chi^2 = 7.9$), Day 3 calyx infection rate of 47% vs. head infection rate of 17% ($p<0.025$, $\chi^2 = 6.2$). Although by Day 4, infection rate of the calyx was greater than in head tissue (47% vs. 30%), the difference was no longer statistically significant ($p<0.2$). Regardless of head or calyx infection status, LAC Ag was not detected in the follicles at any time through Day 7 (0%).

Table 8 –Detection of La Crosse Virus Antigen in *Aedes albopictus* Tissues

Replicate Group 1

	Head		Calyx		Follicle	
Day	LACV Ag+	Percent	LACV Ag+	Percent	LACV Ag+	Percent
0	0/10	0%	0/10	0%	0/10	0%
1	0/10	0%	0/10	0%	0/10	0%
2	0/10	0%	2/10	20%	0/10	0%
3	1/10	10%	4/10	40%	0/10	0%
4	3/10	30%	3/10	30%	0/10	0%
5	8/10	80%	6/10	60%	0/10	0%
6	10/10	100%	7/10	70%	0/10	0%
7	7/10	70%	7/10	70%	0/10	0%

Replicate Group 2

	Head		Calyx		Follicle	
Day	LACV Ag+	Percent	LACV Ag+	Percent	LACV Ag+	Percent
0	0/10	0%	0/10	0%	0/10	0%
1	0/10	0%	0/10	0%	0/10	0%
2	0/10	0%	3/10	30%	0/10	0%
3	2/10	20%	4/10	40%	0/10	0%
4	3/10	30%	6/10	60%	0/10	0%
5	8/10	80%	8/10	80%	0/10	0%
6	9/10	90%	8/10	80%	0/10	0%
7	10/10	100%	10/10	100%	0/10	0%

Replicate Group 3

	Head		Calyx		Follicle	
Day	LACV Ag+	Percent	LACV Ag+	Percent	LACV Ag+	Percent
0	0/10	0%	0/10	0%	0/10	0%
1	0/10	0%	0/10	0%	0/10	0%
2	0/10	0%	2/10	20%	0/10	0%
3	2/10	20%	6/10	60%	0/10	0%
4	3/10	30%	5/10	50%	0/10	0%
5	9/10	90%	8/10	80%	0/10	0%
6	9/10	90%	8/10	80%	0/10	0%
7	9/10	90%	9/10	90%	0/10	0%

Three groups of *Ae. albopictus* mosquitoes were orally exposed to a La Crosse virus infected bloodmeal. Each day post exposure (Day 0 – Day 7), 10 mosquitoes per group were individually dissected and assayed for presence of La Crosse virus antigen in head, calyx, and follicle tissues.

Table 9 – Detection of La Crosse Virus Antigen in *Aedes albopictus* Tissues (Combined results)

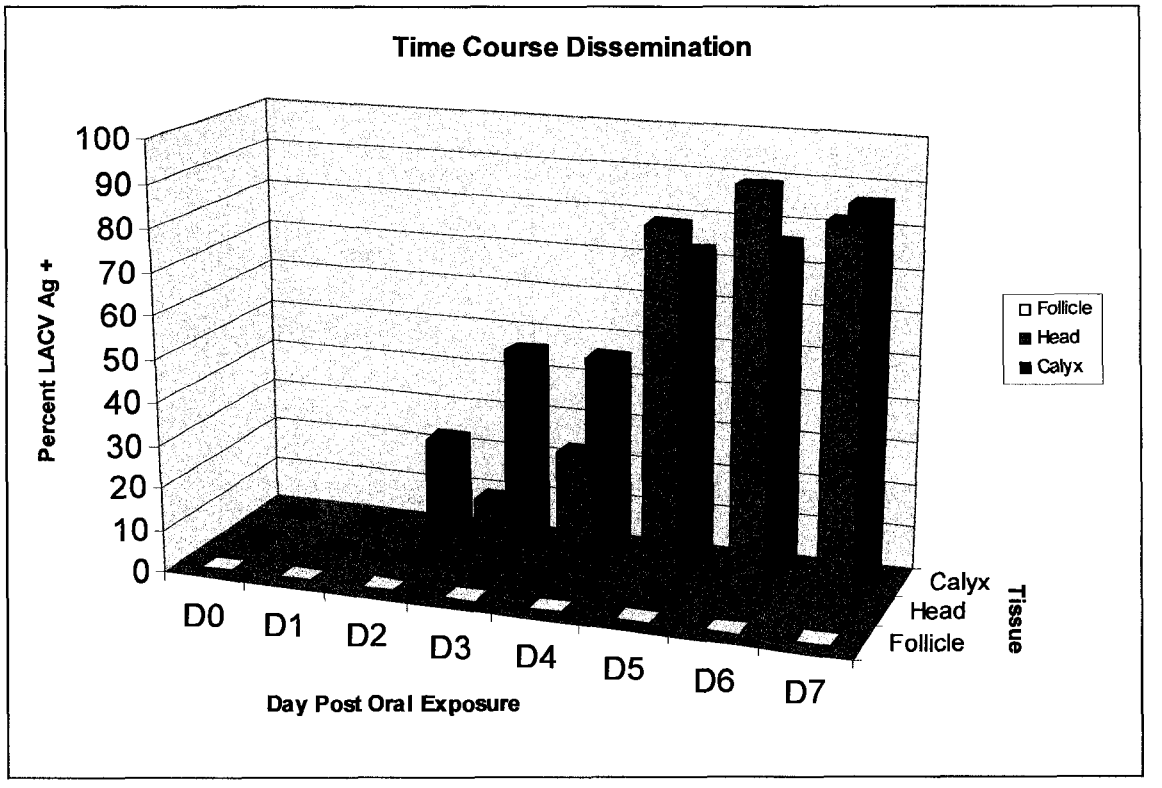
	Head		Calyx		Follicle	
	LACV Ag+	Percent	LACV Ag+	Percent	LACV Ag+	Percent
Day 0	0/30	0%	0/30	0%	0/30	0%
Day 1	0/30	0%	0/30	0%	0/30	0%
Day 2	0/30	0%	7/30	23%	0/30	0%
Day 3	5/30	17%	14/30	47%	0/30	0%
Day 4	9/30	30%	14/30	47%	0/30	0%
Day 5	25/30	83%	22/30	73%	0/30	0%
Day 6	28/30	93%	23/30	77%	0/30	0%
Day 7	26/30	87%	26/30	87%	0/30	0%

Comparison of LAC Ag Detection by Tissue

		Head	Calyx	Follicle
Day 0	SUM	0	0	0
	AVE	0	0	0
	PER	0	0	0
	χ^2		p<1	
Day 1	SUM	0	0	0
	AVE	0	0	0
	PER	0	0	0
	χ^2		p<1	
Day 2	SUM	0	7	0
	AVE	0	2.33	0
	PER	0	23.33%	0
	χ^2		**p<0.01 (7.9245)**	
Day 3	SUM	5	14	0
	AVE	1.66	4.66	0
	PER	16.66%	46.66%	0
	χ^2		**p<0.025 (6.2388)**	
Day 4	SUM	9	14	0
	AVE	3	4.66	0
	PER	30%	46.66%	0
	χ^2		p<0.2	
Day 5	SUM	25	22	0
	AVE	8.33	7.33	0
	PER	83.33%	73.33%	0
	χ^2		p<1	
Day 6	SUM	28	23	0
	AVE	9.33	7.66	0
	PER	93.33%	76.66%	0
	χ^2		p<0.1	
Day 7	SUM	26	26	0
	AVE	8.66	8.66	0
	PER	86%	86.66%	0
	χ^2		p<0.1	

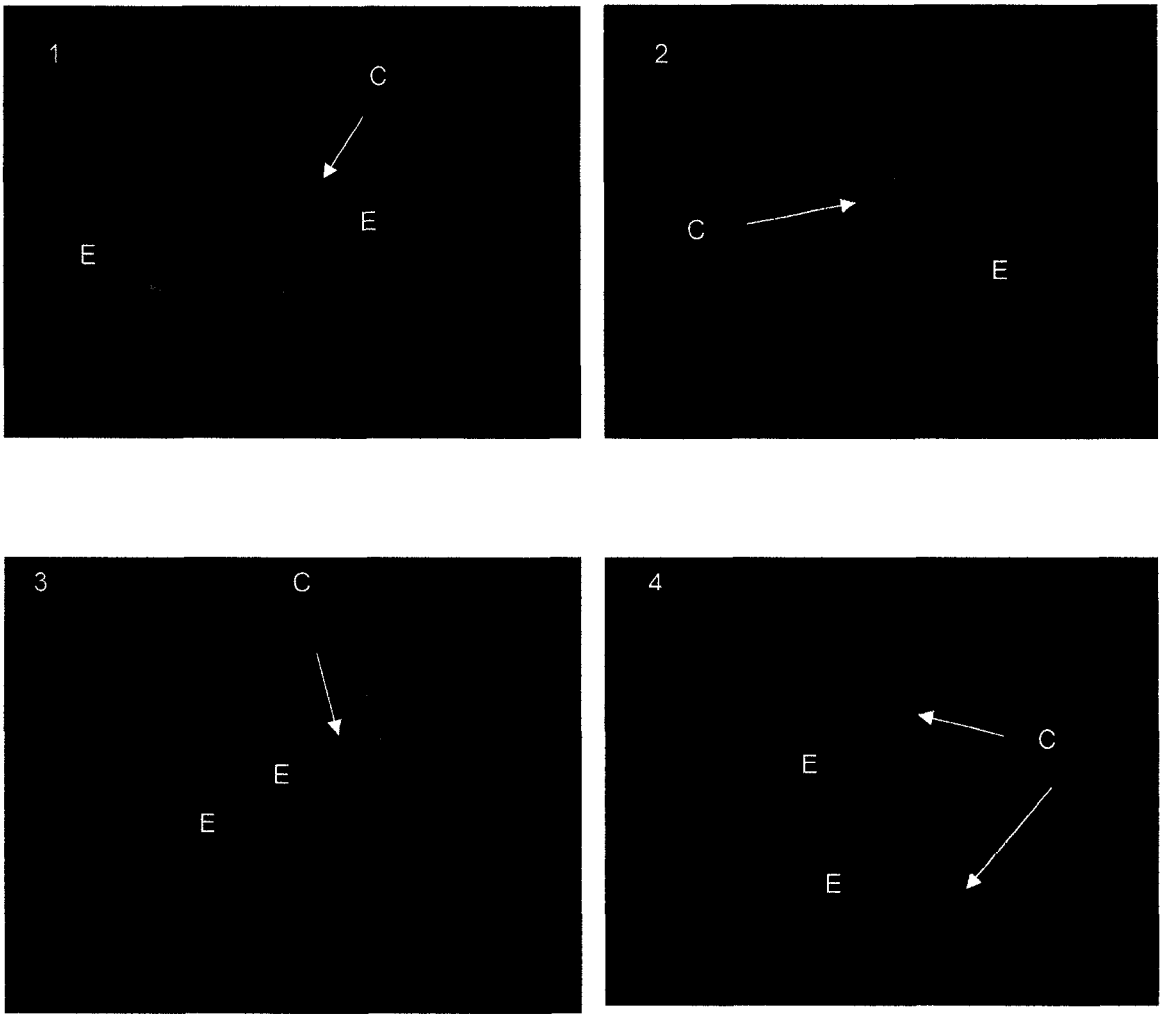
χ^2 analysis of distribution of La Crosse Ag by tissue. Combined results from three groups of *Ae. albopictus* mosquitoes orally infected with La Crosse virus. Each day post exposure (Day 0-Day 7), 30 mosquitoes were individually dissected and assayed for presence of viral antigen in head, calyx, and follicle tissues. Chi-square analysis of the distribution of antigen in tissues are given by timepoint. Bold values indicate statistical significance.

Figure 11 – **Time Course Results For Detection of La Crosse Virus Antigen in Various Mosquitoes Tissues**



n = 30 samples per timepoint

Figure 12 – Detection of LAC Antigen in *Aedes albopictus* Ovaries



Fluorescent microscopy (100X) of *Ae. albopictus* ovaries containing both developing egg (E) and calyx (C) tissues. 1: Day 4 post oral exposure to La Crosse virus, 2: Day 4 negative control, 3: Day 6 post oral exposure to LAC, 4: Day 6 negative control.

D. Discussion

All *Ae. albopictus* geographic strains tested were susceptible to LAC infection following oral exposure. This remarkable susceptibility to infection confirms previous LAC transmission studies with *Ae. albopictus* (Grimstad et al. 1989). All of the geographic strains of *Ae. albopictus* were susceptible to LAC infection and appear to be competent vectors. There were, however, some differences in the replication and dissemination kinetics of LAC. All strains displayed evidence of viral infection by Day 7. Some geographic variability was seen in DIs, with MOBAL and NBRNJ less susceptible to early dissemination through the time course in relation to other strains. However, the Day 14 DIs of 94% and 92% respectively, indicate that both populations are quite susceptible to infection. Similarly, the DIs of LAWKS and FTMMD were statistically lower with respect to other field strains, yet it would be hard to argue that populations with a 97% DI were not highly susceptible to LAC infection. Of particular interest are the three strains susceptible to early infection (HILHI, STIOK, and COLSC), with 100% DI by Day 7 and maintained at 100% through Day 14. This is both statistically and biologically relevant. A significant reduction in the extrinsic incubation period (EIP) may translate into a higher and earlier transmission potential.

Between field and lab strains, only the lab strain of *Ae. triseriatus* (LACWI) exhibited statistically lower DI from Day 7 to Day 10. However, as *Ae. triseriatus* is the natural vector of LAC and an entirely different species of mosquito than *Ae. albopictus*, this temporarily delayed dissemination could simply be due to general genetic and biological differences between the two species. Midgut barriers are not uncommon in *Ae.*

triseriatus. Wide variability in DI among strains does occur, with disseminated LAC infections controlled primarily by a midgut escape barrier (Paulson et al. 1989).

For both dissemination and vertical transmission, here doesn't appear to be any strong evidence for regional patterns of difference with respect to North-South or East-West gradations. All geographic strains were remarkably susceptible to LAC and are presumably very competent horizontal transmission vectors. As *Ae. albopictus* continues to spread throughout the U.S., overlapping with *Ae. triseriatus* in La Crosse encephalitis endemic areas, it will likely be involved in virus transmission among small mammal populations (Erwin et al. 2002). After over 2 decades of colonization, both in and outside of the laboratory, *Ae. albopictus* appears remarkably permissive to productive infection by LAC.

Since LAC can be efficiently transovarially transmitted and overwinter in diapausing eggs of its natural vector, *Ae. triseriatus*, the ability of local mosquito strains to transovarially transmit virus may be a major determinant in the prevalence of the disease for a given locale (Monath 1979). Variation in FIRs of LAC has been reported for local populations of *Ae. triseriatus* in Wisconsin (Miller et al. 1979). Geographic strains of *Ae. triseriatus* also differ in their vector competence and FIRs (Miller et al. 1982, Grimstad et al. 1977). While TOT of LAC in *Ae. albopictus* has previously been demonstrated, no one has yet investigated both the 1st and 2nd cycle FIRs of many recently colonized and widely separated geographic strains of this species.

The distribution of FIRs for the 21 geographic strains of *Ae. albopictus* in this study differed significantly from each other ($P<0.001$)(average 2nd cycle FIR = 10.1%, range = 3-31%) (Table 6). Specifically, two strains, MOBAL and STIOK, had

significantly higher FIRs of 31% and 20%, respectively ($P<0.005$ each) than the other strains. One strain, GALTX, was found to have a significantly lower FIR of 3% ($P<0.05$). The average 2nd FIR of the *Ae. albopictus* populations studied here falls almost exactly between the 1st and 3rd cycle FIRs reported in previous studies (Hughes et al. 2006, Tesh and Gubler 1975). The 2nd gonotrophic cycle FIR range of 3-31% and statistically significant differences of FIRs ($P<0.001$), are suggestive of genetic differences in the ability of strains of *Ae. albopictus* to transovarially transmit LAC.

Genetic determination of TOT of California group viruses by *Ae. albopictus* is not unprecedented. San Angelo virus FIRs range from 3-23% with variation among individual females for a given strain (Tesh and Shroyer 1980). A genetic basis for TOT was strengthened by selection of *Ae. albopictus* lines with FIRs of up to 75% over several generations. Similarly, a strain of *Ae. triseriatus* with a single oral exposure to LAC had a mean FIR of 71% for 8 consecutive generations (Miller et al. 1977). Since TOT is the natural maintenance and overwintering mechanism of this virus, the importance of FIR variability to the epidemiology of LAC is of obvious significance. Although the average 2nd gonotrophic cycle FIRs of *Ae. albopictus* strains are significantly lower than *Ae. triseriatus*, 10.1% vs. 49% ($P<0.001$) respectively, the range varies widely from 3-31%. Since *Ae. triseriatus* is the natural vector for LAC, it is not surprising that the FIR is nearly 5 times that of a recently introduced vector (Hughes et al. 2006). Lower FIRs in *Ae. albopictus* might be the result of genetic differences in the ability of geographic strains to TOT LAC. These FIR differences are likely independent of the mosquitoes' ability to become orally infected and horizontally transmit LAC. Indeed, it has already been demonstrated that *Ae. albopictus* mosquitoes are capable of transmitting LAC to

vertebrates as efficiently as *Ae. triseriatus* mosquitoes (Cully et al. 1992, Grimstad et al. 1989).

Based on these observed FIRs, there are differences among geographic strains of *Ae. albopictus* to transovarially transmit LAC. It remains to be established whether these differences have epidemiologic significance in relation to LAC endemicity within the range of the geographic distribution of *Ae. albopictus*. By using recently colonized strains to investigate oral susceptibility, kinetics of disseminated infection, and variability with respect to FIR, this study has contributed to the understanding of the emerging role of *Ae. albopictus* in La Crosse encephalitis. As an aggressive, daytime feeder, *Ae. albopictus* transmission could dramatically change the epidemiology of LAC encephalitis. Laboratory studies have demonstrated potential TOT, virus has been isolated from field collected larvae, and epidemiological studies have associated *Ae. albopictus* presence with risk for LAC infections (Erwin et al. 2002, Reid et al. 2001, Tesh and Gubler 1975). This study has demonstrated that field populations are readily susceptible to oral infection and that geographic differences exist with respect to TOT. However, in order to make any strong conclusions about any state or regional differences in TOT, confirmation and additional studies must be conducted with more strains and samples to investigate if regional genetic differences exist. The recent detection in the western U.S. of additional new strains of *Ae. albopictus* from mainland China underscores the necessity of continued investigation of the interaction with California group viruses (Linthicum et al. 2003, Madon et al. 2003, Madon et al. 2002).

La Crosse virus is not typically transmitted to *Ae. triseriatus* progeny in the 1st ovarian cycle following an infectious bloodmeal (Miller et al. 1979). This is due to the

need for virus to first replicate in and then escape from the midgut and then to infect the ovaries. Classically, *Ae. triseriatus* females become infected following an initial infectious bloodmeal, but virus does not disseminate and infect ovarian follicles before resulting in an initial virus-free batch of eggs. Following the second bloodmeal, the resulting progeny from the 2nd ovarian cycle may be infected transovarially. However, in the initial laboratory studies demonstrating TOT of LAC, virus was isolated from pools of *Ae. triseriatus* larvae from the 1st ovarian cycle (Watts et al. 1973). Subsequent studies suggested that this could occur infrequently, and virus could be detected in *Ae. triseriatus* ovaries as early as day 2 after an infectious bloodfeed (Chandler et al. 1998, Chandler et al. 1996). In another study, *Ae. albopictus* mosquitoes infected during the 1st ovarian cycle transmitted La Crosse virus to chipmunks (Cully et al. 1992). This present study supports these prior findings, and demonstrates that La Crosse virus may be transmitted to 1st ovarian cycle progeny. The low filial infection rate (1.17%) of 1st gonotrophic cycle progeny suggests this transmission is very inefficient. Similar rates of approximately 1% have been observed with vertical transmission of Flaviviruses and may suggest a similar mechanism of infection (Rosen 1989). Although poorly characterized, several possible mechanisms have been hypothesized to account for early ovarian infection. These include: 1) passive diffusion – early infection of tissues such as the calyx (Clements 2000) may result in the simple diffusion of virus into the ovaries (Chandler et al. 1998, Rosen et al. 1989); 2) leaky midgut – rapid infection results from the increased permeability of ovarian tissues after a bloodmeal in preparation for vitellogenesis and nutrient uptake for oocyte development (Koller et al. 1989); 3) tracheoles – permeating both the midgut and ovaries, they may serve as a viral transport

conduit, as observed for Lepidopteran Baculoviruses, Alphaviruses in *Ae. albopictus,* and dengue virus in *Ae. aegypti* (Salazar et al. 2007, Bowers et al. 1995, Kirkpatrick et al. 1994). The 'Conduit Model' of arbovirus dissemination may indicate involvement of tracheae in early viral egress from the midgut as well as the possible participation of midgut visceral muscle fibers (Romoser et al. 2004). Further work confirming and characterizing this phenomenon is warranted.

This study examined the early infection kinetics of LAC in ovaries of orally exposed *Ae. albopictus*. The first laboratory investigation of LAC TOT by *Ae. albopictus* demonstrated 1st gonotrophic cycle transmission (Tesh and Gubler 1975). Virus was isolated from pools of adult progeny produced from an intrathoracic (IT) injection and a subsequent, non-infectious bloodmeal. A follow-up to this highly artificial system also demonstrated 1st gonotrophic cycle transmission of LAC by *Ae. albopictus* (Cully et al. 1992). Pools of 1st cycle progeny produced after an infectious bloodmeal were allowed to feed upon suckling mice, which subsequently died. LAC was then isolated from these mice. It has been demonstrated that 1st gonotrophic cycle TOT to *Ae. albopictus* progeny can occur (Table 5, page 49). Although previous studies with *Ae. triseriatus* suggest LAC is not typically transmitted to 1st ovarian cycle progeny, it has been reported to occur (Miller et al. 1979, Watts et al. 1973). This may be similar to the mechanism observed with Flaviviruses (Rosen et al. 1989). Furthermore, infection of the ovaries can be detected as early as 1 day post oral exposure and prior to disseminated infection of head tissues (Kempf et al. 2006, Chandler et al. 1998). In this study, LAC Ag was detected in ovarian tissue prior to midgut dissemination, as determined by Ag detection in head tissue. Both whole and disrupted ovarian dissections indicated this early infection

did not infect the follicles. Indeed, images of early infected ovaries reveal heavily infected calyx tissues enveloping the developing eggs during movement to the oviducts (Figure 12). These results support the previous work with *Ae. triseriatus* suggesting the ovaries and eggs can become infected by mechanisms other than dissemination from midgut infection (Chandler et al. 1998).

The "leaky midgut" phenomenon could account for rapid infection of ovaries in lieu of broad dissemination and egress from the midgut (Hardy 1988). Shortly after a mosquito bloodfeeds, the permeability of her ovaries rapidly increases (Clements 2000c). In order to facilitate the uptake of important developmental nutrients such as vitellogenin into the oocyte, intercellular spaces between the ovarian sheath and follicular epithelium expand (Koller et al. 1989, Anderson and Spielman 1971). With such a high transport receptivity within the ovaries, any escaped virus could quickly invade the ovaries.

Tracheal involvement after oral infection presents an attractive explanation. The conduit model of dissemination from the midgut proposes that tracheoles may be involved in the rapid escape of virus into tissues such as the ovaries (Romoser et al. 2004). Members of the Baculoviridae (Baculoviruses), Flaviviridae (dengue virus) and Togaviridae (Sindbis virus) have been shown to rapidly replicate in tracheole cells after oral infection (Salazar et al. 2007, Barrett et al. 1998, Bowers et al. 1995, Kirkpatrick et al. 1994). Studies of LAC ovarian infection before dissemination from the midgut, have also suggested that tracheal cells could be used as a viral transport route (Kempf et al. 2006, Chandler et al. 1998). Ultrastructure studies of *Culex pipiens* using electron microscopy have examined the association between midgut epithelium, tracheae, and visceral muscles (Romoser et al. 2004). Examination of these tissues revealed near-

complete penetration of the midgut epithelium and basal lamina by tracheal cells, as well as that at points of close contact between visceral muscle fibers and midgut epithelium, the basal lamina appears irregularly modified or porous. The authors proposed that tracheae could either penetrate the basal lamina itself or simply terminate near areas of this muscular modified basal lamina. Virus from an infected bloodmeal could then have multiple fates after infecting a cell of the midgut epithelium: infection of additional adjacent midgut epithelia, infection of tracheal cells and subsequent bypass of the basal lamina, or infection of the tracheole-muscular complex of the modified basal lamina and again bypass the basal lamina. Viral dissemination from the midgut would therefore be related to the distance between the site of first midgut cell infection and a tracheal cell or area or modified basal lamina. Thus, bloodmeals with high viral titer may increase the likelihood of infecting midgut epithelia near tracheal cells or modified basal lamina and the observation of rapid dissemination or even early ovarian infection.

The mechanisms underlying the early infection of *Ae. albopictus* follicles have yet to be elucidated. The absence of early follicle infection and the rapid infection of the calyx suggests that LAC could infect some of the 1st gonotrophic cycle eggs during oviposition. During these critical developmental stages and early oviposition, the chorion has not yet sclerotized and the micropyle remains open (Clements 2000d). Virus could then enter and infect the eggs. Flaviviruses can be vertically transmitted in this manner (Rosen et al. 1989, Beaty et al. 1980, Aitken et al. 1979).

This study supports previous investigations observing early ovarian infection after oral exposure to LAC (Kempf et al. 2006, Chandler et al. 1998). More studies will be essential to determining the significance and early route of LAC ovarian infection. While

viral Ag was detected in ovarian tissues using immunofluorescence, real-time quantitative-polymerase chain reaction (Q-PCR) for LAC has been found to be more sensitive than either IFA or reverse transcription polymerase chain reaction (RT-PCR) (Kempf et al. 2006). Further research characterizing the events surrounding early ovarian infection and TOT of La Crosse virus in *Ae. albopictus* may be aided by investigation of the potential for tracheal involvement, temporal dynamics of follicle infection, and the role of multiple bloodfeedings at varying viral doses.

As the invasive mosquito vector *Ae. albopictus* continues to spread across the United States and North America, more work will be needed to characterize its developing relationship with this important, native arbovirus.

CHAPTER 3: GENETIC VARIATION IN U.S. POPULATIONS OF *AE. ALBOPICTUS*

A. Introduction

Since its permanent introduction into the mainland U.S., an assortment of genetic markers (including allozymes, rDNA cistron variation, etc.) have been used to study the population genetics of *Ae. albopictus* (Kambhampati et al. 1990, Black et al. 1989, Black et al. 1988). One important early accomplishment was confirmation of the theory that the founding U.S. population of *Ae. albopictus* geographically originated in northern Asia. Research employing comparative photoperiodic sensitivity and cold-hardiness characteristics determined that *Ae. albopictus* strains derived from North America and northern Asia exhibit a clear photoperiodic response and diapause, which was absent in mosquitoes tested from both subtropical and tropical regions (Hawley et al. 1987). The geographic origin was subsequently corroborated with experiments using genetic analysis of allozyme variation in 57 populations of *Ae. albopictus* to trace the geographic source of U.S. populations (Kambhampati et al. 1991). Within a 98% probability, North American populations of *Ae. albopictus* were discovered to be closest in terms of genetic distance to Japanese populations. Of the 28 US strains tested, 57% were classified into the Japan group, 25% were classified into the China group, and 18% were classified into the Malaysia group. This was of public health interest for this introduced species because the ability to transmit dengue virus to a host has been shown to be a function of the geographical origin of *Ae. albopictus* (Boromisa et al. 1987, Gubler and Rosen 1976).

Mitochondrial DNA (mtDNA) is commonly used as a molecular marker for genetic studies of insect populations (Kambhampati and Smith 1995, Avise 1994).

Polymorphisms in mtDNA act as a sensitive tool for investigating population genetics of a given species (lineages, evolutionary history, gene flow, effective population, etc.). The mitochondrial genome is circular and usually contains 13-20kb of DNA. It is maternally inherited and lacks recombination events between maternal clones, making it useful for a wide variety of genetic studies, ranging from phylogeny reconstruction to determination of population structure (Rai 1991). Because mtDNA has a smaller effective population size than does nuclear DNA, it is more sensitive to genetic drift, resulting in greater genetic differentiation between populations (Avise 1994). Thus, mtDNA has been successfully utilized in the population genetics of important disease vectors such as *Anopheles gambiae s.l.* and *Ae. aegypti* (Gorrochotegui-Escalante et al. 2002, Gorrochotegui-Escalante et al. 2000, Besansky et al. 1997).

Overall, *Ae. albopictus* has demonstrated remarkably low genetic variation between populations. Restriction enzyme analysis of mtDNA has been used to survey polymorphism in 17 U.S. and global populations (Kambhampati and Rai 1991). Of those examined, only 3 populations (Hong Kong, Mauritius, Singapore) showed the presence of novel mtDNA haplotypes. Furthermore, an analysis of 9 populations from the U.S. and Japan for within-population variation among individual mosquitoes revealed only a single mosquito from the U.S. expressing a restriction site variation. Thus it was concluded that the level of polymorphism was low, with over 99% of the fragments being shared in common among the 17 populations. Although this early study revealed low variation in mitochondrial restriction sites in *Ae. albopictus*, more information could be obtained through direct sequencing. DNA sequence data represent the highest level of genetic

resolution and allow the development of more powerful statistical approaches in the analysis of collection samples (Li 1997).

Sequence analysis of mtDNA has also suggested a low degree of variability between 16 global populations of *Ae. albopictus* (Birungi and Munstermann 2002). Out of 8 U.S. populations surveyed from 5 states, samples from 4 of the states possessed the exact same haplotype. In addition to a single, widespread haplotype, only 1 state, Florida, yielded multiple unique haplotypes, but their frequency averaged 20% or less among sampled populations. Similarly, a recent phylogeographic study of *Ae. aegypti* and *Ae. albopictus* based on mtDNA sequence variations using 3 markers also demonstrated little genetic variation for *Ae. albopictus* (Mousson et al. 2005). However, during the course of the global study, only 13 individual mosquitoes were used, including 2 specimens from the U.S.; and of those, only 1 came from the mainland. Although genetic variation of *Ae. albopictus* populations has been surveyed globally, little work using sequenced mtDNA markers to characterize the genetic structure of this species within the United States. Indeed, only *Ae. albopictus* populations from 4 mainland U.S. states have been characterized. Clearly a more intensive study, sampling a large number of populations and individuals from throughout the U.S. and analyzing for mtDNA sequence polymorphisms would be of great value. This study was undertaken to elucidate basic aspects of the population genetic variation of strains of *Ae. albopictus* in the U.S..

The studies described here were conducted to test the hypothesis that regional strains of U.S. *Ae. albopictus* express significant genetic variability in their mtDNA. Specifically, we analyzed the sequence diversity of the mitochondrial gene encoding the

NADH dehydrogenase subunit 5 (ND5), one of the most variable protein-coding genes (Besansky et al. 1997). After mosquito collection, DNA extraction, PCR amplification, haplotype screening by single strand confirmation polymorphism (SSCP), and automated sequencing, mtDNA variation was determined for 16 strains of *Ae. albopictus* and compared to 1 outgroup strain of *Ae. aegypti*. The results obtained from these studies provide insight into the general population structure and genetic diversity of local populations of *Ae. albopictus* within the U.S..

B. Materials and Methods

Acquisition and Maintenance of Mosquito Populations. *Ae. albopictus* were collected from 16 different locations, representing 14 U.S. mainland states as well as Hawaii, and a long-colonized lab strain was also used in the analysis (Table 10). An outgroup control of long-colonized *Ae. aegypti* were also used. All strains were reared under standard insectary conditions, ≥80% RH, 14:10 light-dark cycle and 28°C (Gerberg et al. 1994). Adult mosquitoes were provided sugar cubes and water *ad libitum.* Forty-eight hours before artificial membrane feeding, sugar cubes were removed, and mosquitoes were provided only water.

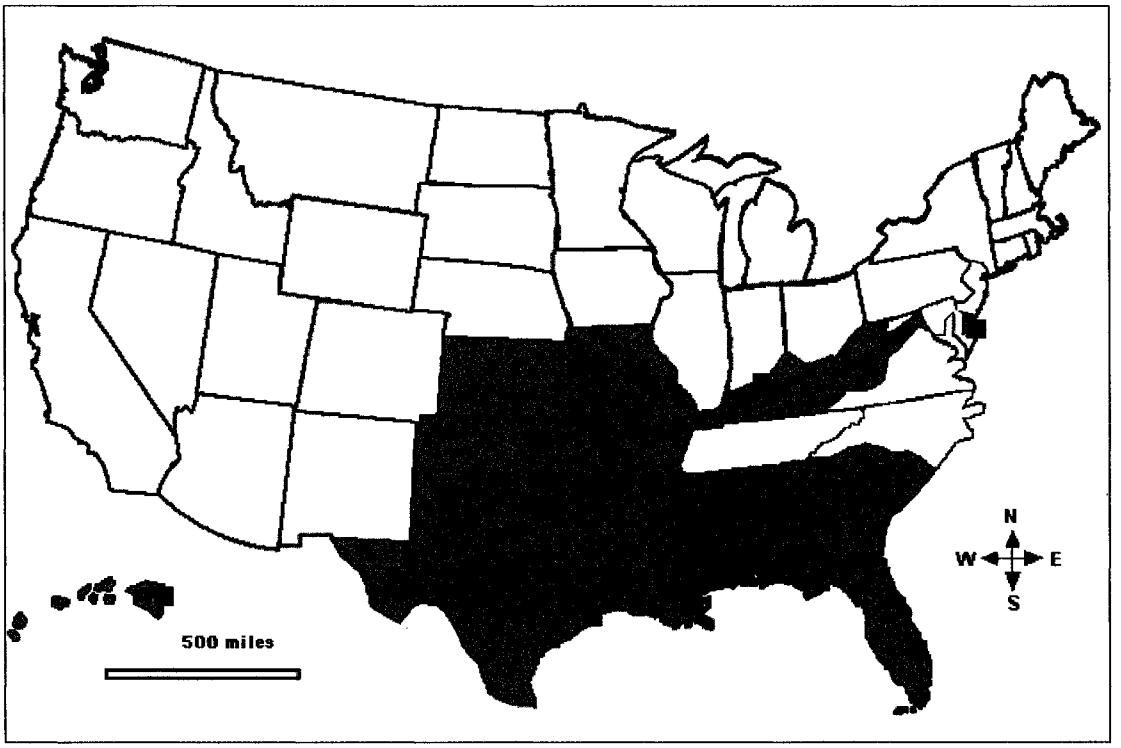
The laboratory strain of *Ae. albopictus* (LC) originated from field material collected near Lake Charles, Louisiana in 1987. It was originally colonized at the Centers for Disease Control and Prevention (CDC) Coordinating Center for Infectious Diseases (CCID), National Center for Zoonotic, Vector-Borne, and Enteric Diseases (NCZVED), Fort Collins, CO, and subsequently continuously maintained at the AIDL. The laboratory strain of *Ae. aegypti* (RXD) originated from field material collected near Rexville, San Juan, Puerto Rico in 1991. It was originally colonized at the CDC, CCID,

NCZVED, in Fort Collins, CO; and continuously maintained at the AIDL (Miller and Mitchell 1991). Between 2004 and 2006, all *Ae. albopictus* field strains were acquired as either eggs or larvae. The source locations of *Ae. albopictus* collections are listed in Table 10 and the geographic locations of all sampling sites appear in Figure 13. Eggs were hatched and larvae reared to pupae. Upon ecdysis, only *Ae. albopictus* adults (designated generation P1, n≥65/strain) were keyed to species and retained for experimentation (Darsie and Ward 2005). In order to produce an F1 generation for vector competence studies (Chapter 2), P1 adults were offered 2 artificial bloodmeals at 1 week intervals of mechanically defibrinated sheep blood (Colorado Serum Company, Denver, CO) for a minimum of 2 hours each. Eggs were collected from both gonotrophic cycles and partially dried until ready for hatching and experimentation.

Table 10 – Origin of *Aedes albopictus* Field DNA Samples

State/Strain ID	City	County/Parish	LAT	LONG	Source
AL	Mobile	Mobile	N 30 41 38	W 88 02 34	Kelly Micher
AR	Farmington	Washington	N 36 02 30	W 94 14 49	Dr. Max Meisch
					Bryce Blackman
DE	Georgetown	Sussex	N 38 41 23	W 75 23 09	Dr. Gulnihal Ozbay
FL	Vero Beach	Indian River	N 27 38 18	W 80 23 50	Dr. Roxanne Rutledge
GA	Macon	Bibb	N 32 50 26	W 83 37 56	Dr. Mike Womack
HI	Hilo	Hawaii	N 19 43 47	W 155 05 23	Dr. Sheldon C. Furutani
KS	Lawrence	Douglas	N 38 58 18	W 95 14 06	Jason Botz
KY	Ft. Knox	Hardin	N 36 45 52	W 88 17 43	Ben Pagac
LA	New Orleans	Orleans	N 29 57 16	W 90 04 29	Bethany Peel
MO	Columbia	Boone	N 38 57 06	W 92 20 01	Dr. Brenda Beernstein
MS	Jackson	Hinds	N 32 17 54	W 90 11 05	Dr. Gerome Goddard
					Wendy Varnado
OK	Stillwater	Payne	N 36 06 55	W 97 03 28	Lisa Coburn
SC	Columbia	Richland	N 34 00 02	W 8102 06	Chris Evans
TX	Galveston	Galveston	N 29 17 56	W 94 47 38	Dr. Stephen Higgs
					Kate McElroy
WV	Richwood	Nicholas	N 38 13 29	W 80 31 59	Humbert "Zap" Zappia

Figure 13 - Map of North American Origin of *Aedes albopictus* Field DNA



Each black square represents the collection site within a given state.

DNA Extraction. DNA was extracted from individual mosquitoes based on previously described methods (Bender et al. 1983). Fifty specimens from each location were triturated in a 1.5mL Eppendorf tube containing 25µL of lysis buffer (see Appendix B, page 139) using a Kontes pestle. After triturating, pestles were rinsed with an additional 25µL of buffer to remove trace amounts of the sample. All tubes were centrifuged for 3-

5 seconds at 14,000 rpm (Hermle Z231 M benchtop centrifuge) to remove remaining tissue from tube walls into the lysis buffer at the tube's bottom. Samples were incubated at 65°C in a dry bath for 30 minutes. Potassium acetate was then added to make a final 1M concentration (i.e. 7μL of 8M KAc per 50μL of sample). Tubes were gently tapped to mix. Samples were incubated on ice for 30 minutes then centrifuged for 15 minutes at 14,000 rpm. Supernatant was transferred to fresh 1.5mL tubes, and 100μL of absolute ethanol was added. After incubation for 5 minutes at room temperature and then an overnight at -20°C, tubes were centrifuged for 15 minutes at 14,000 rpm. Supernatant was carefully removed and the remaining pellet was washed with 100μL of cold 70% ethanol, and the tube was gently tapped to resuspend. Tubes were again centrifuged for 5 minutes at 14,000 rpm. Supernatant was carefully removed and the pellet was washed with 100μL of cold absolute ethanol. Tubes were centrifuged for 5 minutes at 14,000 rpm. Supernatant was carefully removed and the pellet was dried in a Savant SC100 Speed Vac for 5 minutes. DNA pellets were resuspended in 300μL TE for 10 minutes at 65°C and subsequently stored in 50μL aliquots at -70°C.

DNA Amplification. DNA samples were used as templates for the amplification of a 450bp fragment of ND5 mtDNA. The following set of primers were used: ND5FOR (5'-TCCTTAGAATAAAATCCCGC-3') and ND5REV (5'-GTTTCTGCTTTAGTTCATTCTTC-3') (Birungi and Munstermann 2002). PCR amplifications were performed in an MJ Research PTC-200 Peltier thermal cycler in a final volume of 25μL per reaction. The PCR mixture contained 2μL of template, 10μM of each primer, and 12.5μL of Promega GoTaq Green master mix (Promega, Madison, WI) which was then brought to 25μL with nuclease-free water. The amplification profile

was initial denaturation at 98°C for 2 minutes, followed by five cycles of 95°C for 30 seconds, 45°C for 30 seconds, and 72°C for 45seconds. Then 25 cycles of 95°C for 30 seconds, 46°C for 45 seconds and 72°C for 45 seconds, and a final extension step at 72°C for 5 minutes. Negative controls lacking template DNA were included in all reactions. Amplification of product was confirmed by subjecting aliquots to electrophoresis on a 1.5% agarose gel, which was then stained with ethidium bromide (Westermeier 2005).

SSCP and Sequencing. Based on earlier experiments with mosquitoes, SSCP has proven to be a sensitive, rapid, and low-cost technique to screen for haplotype variation among amplified mitochondrial genes from individual samples (Birungi and Munstermann 2002, Gorrochotegui-Escalante et al. 2000). The detailed SSCP protocol and reagents are provided in Appendix C (page 140). Briefly, the SSCP procedure was as follows: the PCR product was added (1:1) to a tube containing denaturing loading buffer. This solution was denatured at 98°C for 5 minutes then quickly cooled on ice. Products were then loaded onto a 5% acrylamide gel with 5% glycerol and 2% cross linker. Electrophoresis was performed at room temperature at 15mA for 15-18 hours using 1X TBE buffer, pH 8.0 in both chambers. Gels were silver-stained immediately (Black and DuTeau 1997). After fixing and drying, gels were scored for haplotype, digitally scanned, and catalogued using a Microsoft Excel spreadsheet. Prior to sequencing, PCR products of detected haplotypes were purified with the Qiagen MinElute PCR purification Kit (Qiagen, Valencia, CA) according to manufacturer's specifications. The nucleotide sequences of observed haplotypes were determined by automated sequencing on an ABI 3130 Genetic Analyzer at Macromolecular Resources at Colorado State University. Two representatives of each observed haplotype were sequenced.

Genetic Analysis. DNA sequences were aligned using ClustalW (Thompson et al. 1994). After recording the nucleotide sequence and the frequency of each haplotype for a collection, the haplotype distribution between populations, number of haplotypes, and number of unique haplotypes were calculated using DNAsp version 4.10.9 (Rozas et al. 2003). Variation in haplotype frequencies within and among collection sites was examined using the Analysis of Molecular Variance (AMOVA) Arlequin version 2.000 (Schneider et al. 2000, Excoffier et al. 1992). Phylogenetic relationships among haplotypes were estimated with PAUP4.0B10 using maximum likelihood, maximum parsimony, and distance/neighbor joining analyses (Tamura and Nei 1993, Saitou and Nei 1987, Hasegawa et al. 1985). The consistency with which the dataset support each branch in the resolved phylogeny was estimated using a bootstrap analysis with 1,000 replications. The average number of pairwise nucleotide differences (nucleotide diversity, π) was calculated using the program DNAsp 4.10.9 (Rozas et al. 2003). Where indicated below, the GenBank listed sequences of *Ae. albopictus* from previously published studies were included for comparison with this study's results (Table 11).

Table 11 - Origin of *Aedes albopictus* ND5 mtDNA Sequences from Prior Studies

STRAIN ID	CITY	COUNTRY	AUTHOR	ACCESSION NUMBER
3DS11b	Jacksonville	USA	Birungi and Munsterman 2002	AY049972
3DS6b	Jacksonville	USA	Birungi and Munsterman 2002	AY049971
AAS13b	Jacksonville	USA	Birungi and Munsterman 2002	AY049974
BRZLm	Represa do Cigano	Brazil	Mousson et al. 2005	AJ971016
CAMBm	Seam Reap	Cambodia	Mousson et al. 2005	AJ971019
FRANm	Naintre	France	Mousson et al. 2005	AJ971022
JXFLm	Jacksonville	USA	Mousson et al. 2005	AJ971018
MADGm	Diego Suarez	Madagascar	Mousson et al. 2005	AJ971020
NO1b	New Orleans	USA	Birungi and Munsterman 2002	AY049970
OAHI	Oahu	USA	Mousson et al. 2005	AJ971024
OM7b	Jacksonville	USA	Birungi and Munsterman 2002	AY049973
REUNm	La Possession	Reunion	Mousson et al. 2005	AJ971025
THAIm	Chiang Mai	Thailand	Mousson et al. 2005	AJ971028
VIETm	Hanoi	Vietnam	Mousson et al. 2005	AJ971017

C. Results

Seventeen strains were examined over the course of this study for a total of 802 individual mosquitoes. These strains included *Ae. albopictus* from 16 different locations, representing 14 mainland U.S. states, as well as Hawaii, and a long-colonized lab strain from Louisiana. An outgroup control of long-colonized *Ae. aegypti* was also used (RxD). The results for SSCP haplotype screening by strain are given in Table 12. A single haplotype for ND5 overwhelmingly dominated the mtDNA sequences among all U.S. populations. Fifteen collections or 93.75% of our sample groups possessed identical sequences. One collection or 6.25% of our sample groups, representing a single mainland U.S. state, KY, possessed multiple haplotypes, 1 of which was unique. Two haplotypes were observed, with KY composed of both haplotype A (66%) and haplotype B (34%).

Sequencing results for two representatives of each observed haplotype are given in Table 13. Of the 405 nucleotide sites, 1 was variable at the 268bp position. This consisted of a single transition from adenosine to guanine (A→G) for both samples KY02B and KY04B (Table 13, page 79).

The ClustalW neighbor joining tree results per strain based on this study are given in Figure 14. All strains resolved into 3 clades representing the outgroup (RxD), KY, and all other U.S. strains. A ClustalW neighbor joining tree, combining sequence information from this study and previously published *Ae. albopictus* ND5 sequences, is given in Figure 15. Strains resolved predictably, with the outgroup forming a separate clade (RxD) and the majority of remaining strains resolving within 4 highly homogeneous clades. These clades indicate populations with high rates of genetic similarity.

Table 12 – Frequency of ND5 Haplotypes (Hap) for Collections of *Ae. albopictus*

	Collection															
	AL	AR	DE	FL	GA	HI	KS	KY	LA	MO	MS	OK	SC	TX	WV	LC
Sample ID																
01	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
02	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
03	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
04	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
05	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
06	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
07	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
08	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
09	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
10	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
11	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
12	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
13	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
14	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
15	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
16	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
17	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
18	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
19	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
20	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
21	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
22	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
23	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
24	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
25	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
26	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
27	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
28	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
29	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
30	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
31	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
32	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
33	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
34	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
35	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
36	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
37	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
38	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
39	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
40	A	A	A	A	A	A	A	B	A	A	A	A	A	A	A	A
41	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
42	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
43	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
44	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
45	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
46	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
47	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
48	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
49	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
50	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
Percent Hap A	100	100	100	100	100	100	100	66	100	100	100	100	100	100	100	100
Percent Hap B	0	0	0	0	0	0	0	34	0	0	0	0	0	0	0	0

Table 13 – Sequences of Observed ND5 Haplotypes for *Aedes albopictus*

(1 of 6)

ID	(bp)	SEQUENCE
AL01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
AL02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
AR01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
AR02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
DE01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
DE02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
FL01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT

ID	(bp)	SEQUENCE
FL02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
GA01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
GA02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
HI015A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
HI016A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
KS07A	405	AATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTCCTATAAAACGAATATCTTGAGCATTT TTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAAGCATGTGTTAATAAATGAAAAAAAGC TAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAATGTAGATAAAGCAATAATCTTTTTTA AATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATATTAATAATAAAAATTGACCTAATGTA GAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACTAAAGTAGAAGAATGAACTAAGCAGAA ACACA
KS08A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT

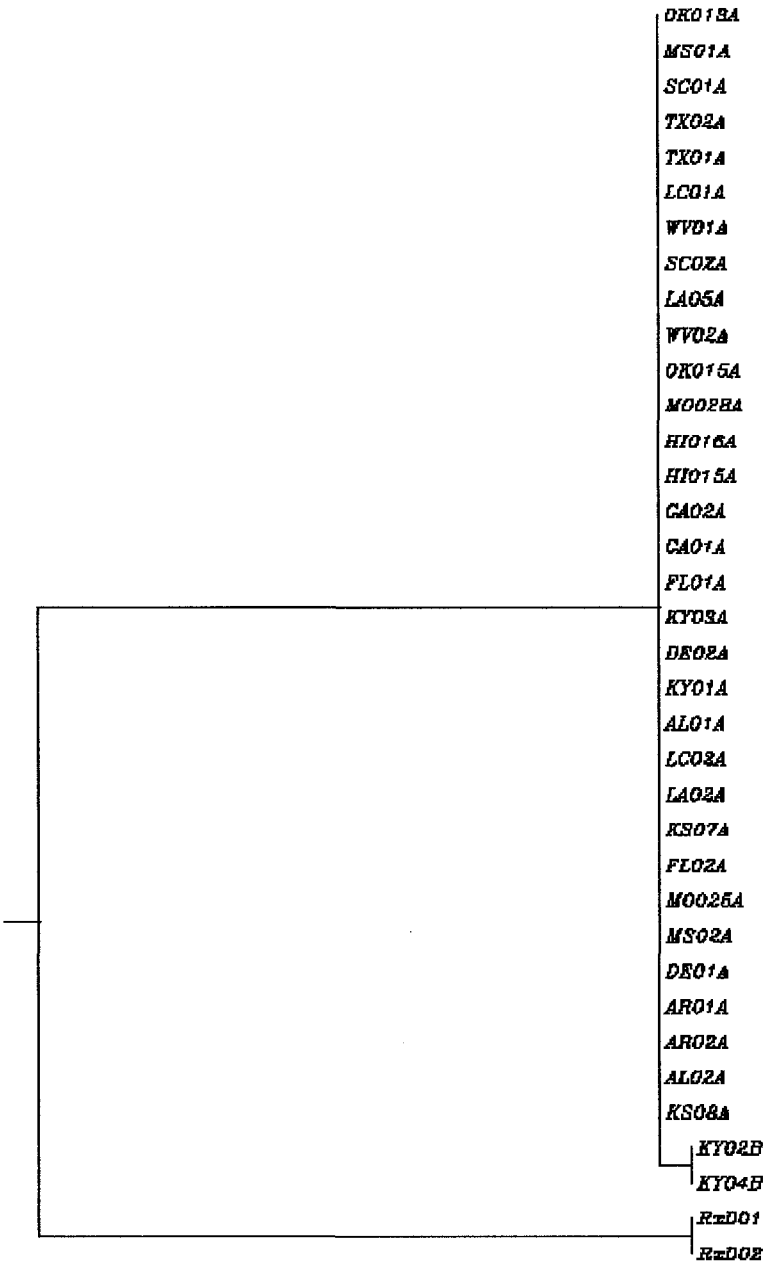
ID	(bp)	SEQUENCE
KY01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
KY03A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
KY02B	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAA**G**TCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
KY04B	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAA**G**TCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
LA02A	392	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTG
LA05A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT

ID	(bp)	SEQUENCE
MO025A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
MO028A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
MS01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
MS02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
OK013A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
OK015A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT

ID	(bp)	SEQUENCE
SC01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
SC02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
TX01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
TX02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
WV01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
WV02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT

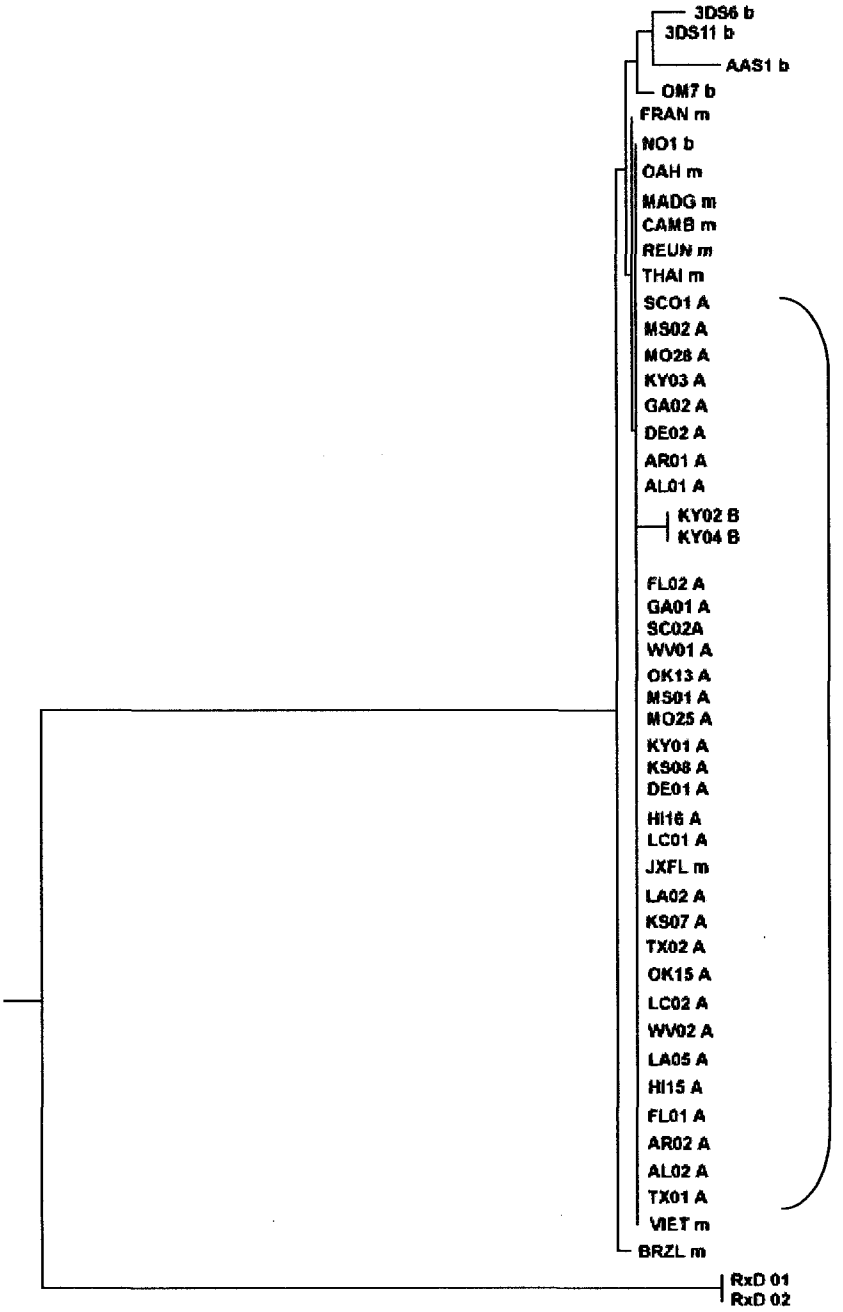
ID	(bp)	SEQUENCE
LC01A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
LC02A	405	TAAAAAAGGTATACCACATAAAGCTAAATTTGCAACATTAAAACATCTACAAGTTAAAGGTATTCTTATTCTTAAATTTC CTATAAAACGAATATCTTGAGCATTTTTTGTATTGTGAATAATTACCCCAGCACATATAAATAATAATGCTTTAAATAAA GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAAAATCCTATTGATAAAATTCTTATTATTAAACCTAATTGACTTAA TGTAGATAAAGCAATAATCTTTTTTAAATCAAATTCAAAATTAGCTCCTAATCCTGCTATAAATATAGTTATTCCGGATA TTAATAATAAAAATTGACCTAATGTAGAATTTTCTAATAAAATATTAAATCGAATTAATAAATAAACCCCTGCAGTTACT AAAGT
RXD1	405	CAAAAAAGGTATCCCACATAAAGCTAAATTAGCAATATTAAAACAACTACATGTTAAAGGTATTCTTATACTTAAACCCC CTATAAATCGAATATCTTGAGCATTTTTTGTATTATGAATAATTACTCCAGCACATATAAATAATAAAGCCTTAAAGAGT GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAATAACCAATAGATAAAATACTTATTATTAAACCTAATTGACTTAA AGTAGATAAAGCAATAATTTTTTTTTAAATCAAACTCAAAATTAGCCCCTAATCCAGCTATAAATATTGTTAATCCAGAAA CTAATAATAAAAATTGTCCTAACTTAGAATTATCTAATAAAATATTAAATCGAATTAATAAATAAACTCCAGCAGTTACT AATGT
RXD2	405	CAAAAAAGGTATCCCACATAAAGCTAAATTAGCAATATTAAAACAACTACATGTTAAAGGTATTCTTATACTTAAACCCC CTATAAATCGAATATCTTGAGCATTTTTTGTATTATGAATAATTACTCCAGCACATATAAATAATAAAGCCTTAAAGAGT GCATGTGTTAATAAATGAAAAAAAGCTAACTTATAATAACCAATAGATAAAATACTTATTATTAAACCTAATTGACTTAA AGTAGATAAAGCAATAATTTTTTTTTAAATCAAACTCAAAATTAGCCCCTAATCCAGCTATAAATATTGTTAATCCAGAAA CTAATAATAAAAATTGTCCTAACTTAGAATTATCTAATAAAATATTAAATCGAATTAATAAATAAACTCCAGCAGTTACT AATGT

Figure 14 – Phylogenetic Analysis of U.S. *Ae. albopictus* Field Collections - NJ



Neighbor Joining (NJ) tree of ND5 sequences among U.S. field collections of *Ae. albopictus*. All *Ae. albopictus* samples abbreviated by U.S. state and include 2 samples from each haplotype (e.g. Arkansas, individual 1, haplotype A = AR01A; Kentucky, individual 4, haplotype B = KY04B). *Ae. aegypti* outgroup control (RxD) includes 2 samples.

Figure 15 – Phylogenetic Analysis of Global *Ae. albopictus* Field Collections - NJ



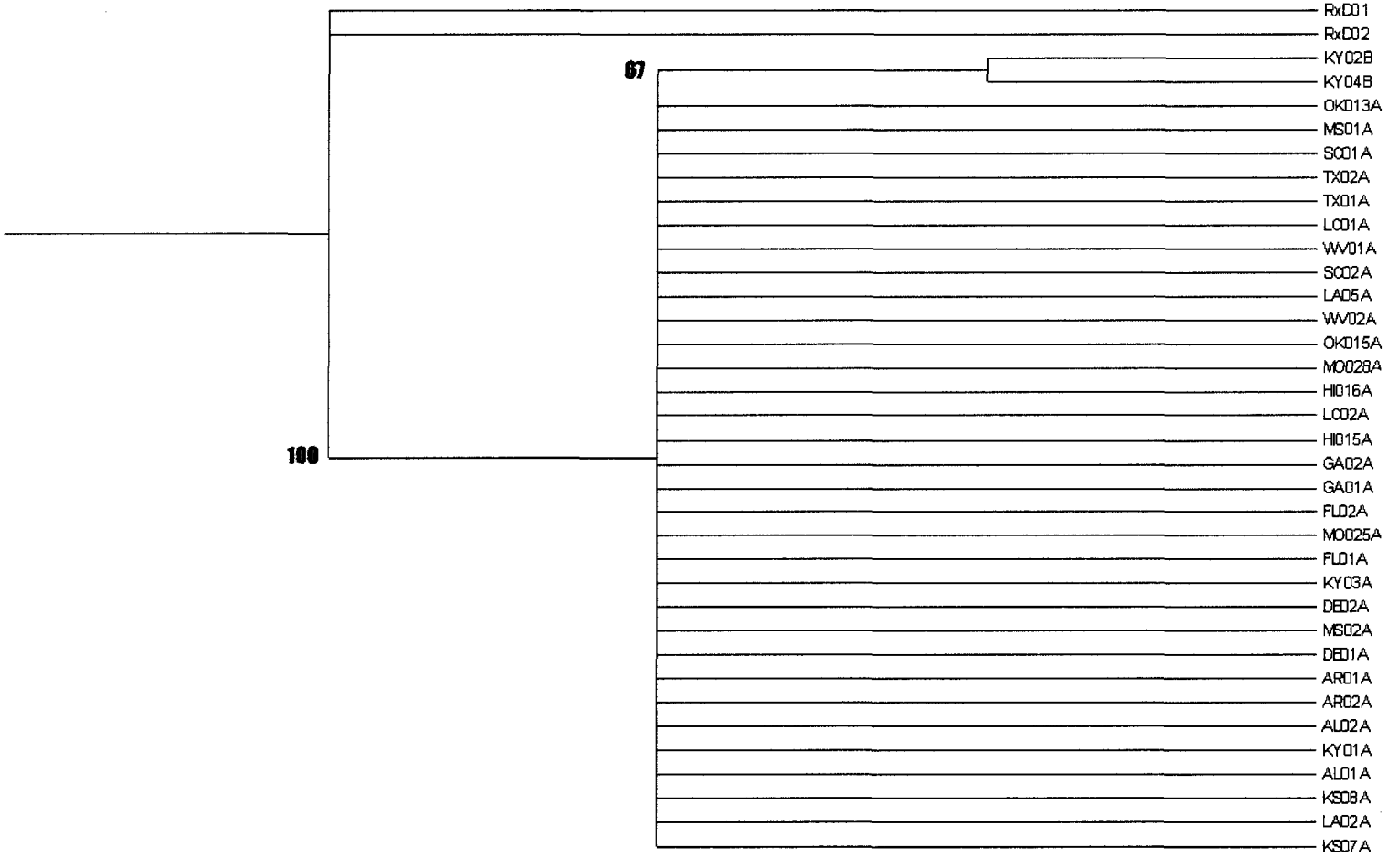
Neighbor Joining (NJ) tree of ND5 sequences among published and recent U.S. field collections of *Ae. albopictus*. *Aedes aegypti* (RxD) is this study's outgroup control. Bracketed samples are from this study's collections and are representative of either haplotype A or B (e.g. Arkansas, individual 1, haplotype A = AR01A; Kentucky, individual 4, haplotype B = KY04B). All others are representative of global samples from previous studies: Mousson et al. 2005 (m), and Birungi and Munstermann 2002 (b). An *Ae. albopictus* population from Brazil represented by BRZL m.

A maximum likelihood phylogenetic (MLP) tree of ND5 mtDNA for field collections of *Ae. albopictus* from this study is given in Figure 16, and a MLP tree combining sequencing data from this study and previously published *Ae. albopictus* ND5 sequences is given in Figure 17. Comparison to global and previously sequenced ND5 mtDNA, revealed a predominant identity conserved across populations. The U.S. and Brazilian collections were genetically divergent (Figures 15, 17), supporting previous observations (Birungi and Munstermann 2002, Hawley et al. 1987). Within the U.S., multiple haplotypes are only observed within collections from 2 states, Florida and Kentucky (Figures 14, 16).

From the 32 *Ae. albopictus* mtDNA sequences, 2 unique haplotypes were observed (Table 14), and nucleotide diversity, π, was 0.00031. The average haplotype diversity was 0.114 for the *Ae. albopictus* populations and 0.272 for the combined collections consisting of sequence data from this study and previously published sequences (Table 15). The overall estimates of molecular diversity for *Ae. albopictus* populations are low ($k=0.114\pm0.035$ and $\theta=0.245\pm0.060$).

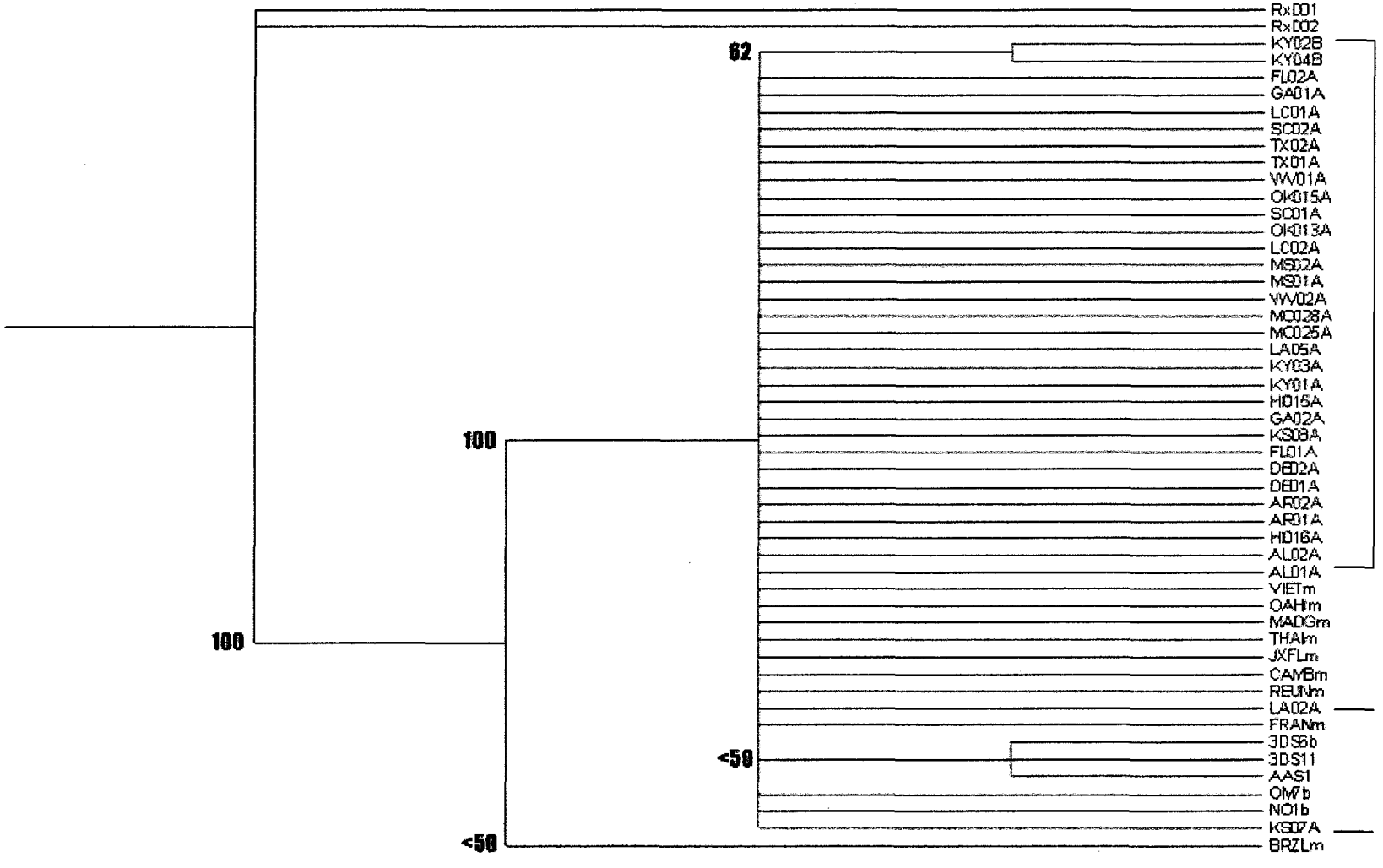
Hierarchical AMOVA analysis (Table 16) of *Ae. albopictus* collections among the 16 samples revealed a significant variance component 'among populations within groups' ($F_{ST}=0.323$, $p<0.05$). This indicates that 1 of the collections (Kentucky samples, KY02B and KY04B) significantly differs from the other sample groups.

Figure 16 – ML Phylogenetic Analysis of U.S. Field Collections of *Ae. albopictus*



Maximum Likelihood (ML) phylogenetic tree of ND5 mtDNA for field collected *Ae. albopictus*. Bold numbers are 100 replicate bootstrap value. All *Ae.* albopictus samples abbreviated by U.S. state and include 2 samples from each haplotype (e.g. Arkansas, individual 1, haplotype A = AR01A; Kentucky, individual 4, haplotype B = KY04B). *Ae. aegypti* outgroup control (RxD) includes 2 samples.

Figure 17 – ML Phylogenetic Analysis of Global Field Collections of *Ae. albopictus*



Maximum Likelihood (ML) phylogenetic tree of ND5 mtDNA for Published and Field Collected *Ae. albopictus*. Bold numbers are 100 replicate bootstrap values. *Aedes aegypti* (RxD) is this study's outgroup control. Bracketed and individual samples marked with lines are from the current collections. All others are representative of global samples from previous studies: Mousson et al. 2005 (m), and Birungi and Munstermann 2002 (b). An *Ae. albopictus* population from Brazil is represented by BRZL m.

Table 14 - Haplotype Analysis of Field Collected *Ae. albopictus*

ND5 Polymorphism and Haplotype Analysis for *Aedes albopictus* field collections	
Number of Sequences	34
Number of Sequences Used	34
Selected region	1-405
Number of Sites	405
Total Number of Sites	366
Number of variable sites(S)	1
Total Number of Mutations (Eta)	1
G-C Content	
at noncoding positions	0
at coding positions	0.219
total	0.219
Haplotype/Nucleotide Diversity	
Number of Haplotypes	2
Haplotype(gene) diversity (Hd)	0.114
Variance of Hd	0.00509
STDDEV of Hd	0.071
Nucleotide Diversity per site (π)	0.00031
Sample variance of π	0
STDDEV of π	0.00019
Average number of nucleotide differences (k)	0.11408
Theta (per sequence) from S, θ-W	0.24457
Theta (per site) from S, θ-W	0.00067
Neutrality Tests	
Tajima's D	-0.79933
not significant (p>0.10)	
Fu and Li's D test statistic	0.5804
not significant (p>0.10)	
Fu and Li's F test statistic	0.22484
not significant (p>0.10)	
Strobeck's S statistic	0.929
Haplotype Distribution	
Number of Haplotypes (h)	2
Haplotype diversity (Hd)	0.1141
h 1	2 (1-2)
h 2	32 (3-34)

Table 15 – Combined Haplotype Analysis of Field Collected and Published *Ae. albopictus*

ND5 Polymorphism and Haplotype Analysis for Field and Published *Aedes albopictus*	
Number of Sequences	48
Number of Sequences Used	48
Selected region	1-405
Number of Sites	405
Total Number of Sites	366
Number of variable sites(S)	6
Total Number of Mutations (Eta)	6
G-C Content	
at noncoding positions	0
at coding positions	0.219
total	0.219
Haplotype/Nucleotide Diversity	
Number of Haplotypes	7
Haplotype(gene) diversity (Hd)	0.272
Variance of Hd	0.00713
STDDEV of Hd	0.084
Nucleotide Diversity per site (π)	0.00111
Sample variance of π	0.0000002
STDDEV of π	0.00042
Average number of nucleotide differences (k)	0.4078
Theta (per sequence) from S, θ-W	1.35197
Theta (per site) from S, θ-W	0.00369
Neutrality Tests	
Tajima's D	-1.7928
not significant (0.10>p>0.05)	
Fu and Li's D test statistic	-1.38132
not significant (p>0.10)	
Fu and Li's F test statistic	-1.77036
not significant (p>0.10)	
Strobeck's S statistic	0.999
Haplotype Distribution	
Number of Haplotypes (h)	7
Haplotype diversity (Hd)	0.2722
h 1	2(1-2)
h 2	41 (3-33,35-42,46,48)
h 3	1 (34)
h 4	1 (43)
h 5	1 (44)
h 6	1 (45)
h 7	1 (47)

Table 16 – Analysis of Molecular Variance (AMOVA) Results for ND5 mtDNA of Field Collected *Aedes albopictus*

AMOVA

Source of variance	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among groups	3	1.060	-0.00026 Va	-1.16
Among populations within groups	11	4.335	0.00758 Vb	33.56
Within populations	735	11.220	0.01527 Vc	67.61
Total	749	16.615	0.02258	

Fixation Indices	
F_{SC}	0.33169
F_{ST}	0.32392
F_{CT}	-0.01164

Significance Tests (1023 permutations)	
Vc and F_{ST}	
P (rand. Value < obs. Value)	0.00000
P (rand. Value = obs. Value)	0.00000
P (rand. Value ≤ obs. Value)	0.00000 + -0.00000
Vb and F_{SC}	
P (rand. Value < obs. Value)	0.00000
P (rand. Value = obs. Value)	0.00000
P (rand. Value ≤ obs. Value)	0.00000 + -0.00000
Va and F_{CT}	
P (rand. Value < obs. Value)	0.20528
P (rand. Value = obs. Value)	0.24731
P (rand. Value ≤ obs. Value)	0.45259 + -0.01318

D. Discussion

Polymorphisms in the mtDNA marker, ND5, were used in this study to provide insight into the geographical distribution and genetic diversity for U.S. collections of *Ae. albopictus.* Little genetic variation was observed for 16 U.S. populations representing 14 mainland states, Hawaii, and one long-colonized laboratory strain from LA. Indeed, of all collections examined, just 2 haplotypes were observed, with only samples from KY exhibiting a unique haplotype. The lack of mtDNA polymorphism in the *Ae. albopictus* collections tested here remarkably contrasts with the extensive variation previously observed in other vector species (Szlanaski et al. 2006, Bosio et al. 2005, Thelwell et al. 2000). Additionally, early allozyme studies in Asian populations of this vector revealed relatively high levels of variation (Black et al. 1988b). However, global surveys sampling only a few U.S. collections have also demonstrated low levels of sequence variation within *Ae. albopictus* populations of the U.S. and New World (Mousson et al. 2005, Birungi and Munstermann 2002). This loss of genetic variation may be conditioned by multiple, possibly overlapping factors.

An influencing factor is the possibility that the colonization and rapid expansion of *Ae. albopictus* throughout the U.S. may have been conducted by founding populations of low mtDNA variability. The geographic range of *Ae. albopictus* has spread dramatically in the last 100 years, from its native southeast Asia to all continents except Antarctica (Ritchie et al. 2006, Gratz 2004). Although genetically similar, *Ae. albopictus* populations of the U.S. and Brazil lack shared haplotypes; suggesting an allopatric origin for populations in the two countries (Birungi and Munstermann 2002, Hawley et al.

1987). In both instances, separate introductions may have resulted in a distribution of haplotypes based on the original mtDNA variability of the founding population. Thus, the relatively recent introduction of this vector into North and South America might be characterized by the preponderance of one or two common haplotypes representative of founder populations. This can be facilitated through trade and modern transportation, particularly given *Ae. albopictus*' desiccation resistant eggs and oviposition affinity for manmade containers (Charrel et al. 2007, Tatem et al. 2006, Hawley 1988).

Population bottlenecking can occur repeatedly and over long periods of time, resulting in loss of mtDNA variation (Nei et al. 1975). Control programs, particularly those successfully utilizing integrated pest management (IPM) techniques such as source reduction and larviciding, can be crucial to control and reduction of *Aedes* populations (Yan et al. 1998, Nasci 1995). Each control method drives the removal of susceptible individuals while conversely fostering the retention and promulgation of resistant or refractory individuals. This is a form of directional selection (Black and Tabachnick 2005). Thus, selection pressure from control strategies may be an element facilitating a more homogeneous population.

A unique haplotype was found only within the KY collection. This haplotype has not been previously described and may represent a novel mutation in the established U.S. populations. However, since prior work also observed low variability with multiple unique haplotypes being found only in 1 state (FL), additional sampling replicates would be useful in identifying rare or uncommon polymorphisms (Birungi and Munstermann 2002).

Cytoplasmic incompatibility (CI) may also play a role in the observation of low mtDNA variability. *Wolbachia* are obligate intracellular, rickettsia-like bacteria known to infect a broad number of arthropods (Jiggins et al. 2001). Part of their survival strategy involves the manipulation of host reproduction. CI results in a form of sterility with unviable embryos being produced by matings between infected and uninfected individuals. However, mating between two *Wolbachia* infected mosquitoes produces normal, viable progeny. Thus, in *Ae. albopictus* populations that include both infected and uninfected individuals, CI provides a replicative advantage to the infected (Dobson et al. 2004). Specifically, infected females are longer lived, have higher rates of egg hatching, and are more fecund with respect to uninfected females. This can quickly lead to population replacement, with the infected cytotype driving into the host population and removing the uninfected cytotype (Hoffman and Turelli 1997). Indeed, cage experiments with *Ae. albopictus* found complete population replacement within two generations (Kambhampati et al. 1992). A recent field survey of over 1,500 adult *Ae. albopictus* specimens collected in Thailand found the field prevalence rate of *Wolbachia* to be 100% (Kittayapong et al. 2002). This was one of the first reports of *Wolbachia* prevalence in a field population of an insect disease vector. Furthermore, a global survey of 18 *Ae. albopictus* populations (including 3 mainland U.S. states and 1 from Hawaii) found all to be infected with *Wolbachia*. Of those, 7 disparate populations were chosen for sequencing of two *Wolbachia* gene markers (a 356 and 474bp region). All sequences among these populations were identical (Armbruster et al. 2003). Thus, *Wolbachia* infection status may play a role in homogenizing populations and reducing *Ae. albopictus* mtDNA variability.

The results here support previous studies demonstrating remarkably low genetic variation between populations of *Ae. albopictus* (Mousson et al. 2005, Birungi and Munstermann 2002). 93.75% of regional strains of U.S. *Ae. albopictus* collected in this study express the identical sequence for the ND5 mtDNA marker. Thus, U.S. *Ae. albopictus* populations examined here express little to no local differentiation for ND5 mtDNA. Given the rare expression of alternate or multiple haplotypes, future studies should include additional markers, adult nested spatial sampling, as well as more collection localities. Such techniques would increase the chance of detecting unusual or rare mtDNA variants and provide insight into local genetic differentiation within the U.S.. Additional work characterizing the *Wolbachia* field prevalence and infection status of collections may also aid in our understanding of this complex and intriguing relationship.

CHAPTER 4: DEVELOPMENT OF TOT SUSCEPTIBLE AND REFRACTORY STRAINS OF *AE. ALBOPICTUS*

A. Introduction

The first conclusive evidence of vertical transmission of an arbovirus in a mosquito was the demonstration of transovarial transmission of La Crosse virus (LAC) by *Aedes triseriatus* (Watts et al. 1973). This mechanism serves as the means by which the virus evades vertebrate immunity in the natural vertebrate hosts and survives during the winter season (Pantuwatana et al. 1974, Watts et al. 1974). *Ae. triseriatus* is the primary vector of LAC and is very efficient at vertically transmitting this virus. Rates for both filial infection (FIR, proportion of infected progeny) and transovarial transmission (TOT, proportion of infected females transmitting to progeny) can exceed 70% (Miller et al. 1977). Indeed, after a single infectious bloodmeal, little variation was observed in FIR over eight subsequent generations, maintaining an average of 71% for each generation.

The varied LAC FIRs of diverse laboratory and geographic populations of *Ae. triseriatus* suggests a possible heritable basis to this trait (Woodring et al. 1998, Miller et al. 1982). Experiments on the selection of 2 strains of Wisconsin *Ae. triseriatus* for high and low FIR and TOT rates also supported this suggestion (Graham 1999). The FIRs were reduced from 18% to 3% and TOT rates were lowered from 72% to 14% within only 3 generations of selection. This study revealed that TOT has a significant heritable component, with a pronounced response to selection in the downward direction. Subsequently, it was demonstrated that three quantitative trail loci (QTL) on *Ae. triseriatus'* chromosomes II and III cumulatively account for approximately 53% of the phenotypic variance in TOT (Graham et al. 2003). TOT of LAC by *Ae. triseriatus*

remains one of the best characterized systems of vertical transmission among disease vectors (Beaty and Bishop 1988).

Comparatively little work has been performed exploring the genetic basis of TOT of LAC by *Ae. albopictus*. These mosquitoes can transovarially transmit the California Group Orthobunyaviruses LAC and San Angelo virus (SAV) (Tesh 1980, Tesh and Gubler 1975). *Ae. albopictus* and SAV provide a valuable experimental model for the investigation of factors modulating transovarial transmission efficiency of California Group viruses. Differences were observed in the TOT rate of SAV among seven distinct Pacific-Asian strains of *Ae. albopictus*, with FIRs ranging from approximately 23% in a Taiwanese strain to 3% in a Japanese strain (Tesh 1980). Furthermore, subsequent selection experiments yielded an *Ae. albopictus* line with San Angelo virus FIRs of 100% within six generations of selection (Tesh and Shroyer 1980). However, once selection pressures were removed, a rapid reduction in SAV FIRs occurred (Shroyer 1986). The FIR decline in each following generation was attributed to the spontaneous appearance of uninfected progeny and the advent of transovarially infected females that are inefficient transovarial transmitters. Strain differences in SAV FIRs provided direct evidence that mosquito host genetic factors influenced the decline in SAV FIRs in randomly reproducing *Ae. albopictus*.

In Chapter 2, variation was demonstrated in LAC FIRs among different geographic populations of U.S. *Ae. albopictus*. Similar variation has also been observed with *Ae. triseriatus* and LAC and *Ae. albopictus* and SAV (Miller et al. 1982, Tesh 1980). This intraspecific variation suggested that the efficiency of LAC TOT in *Ae. albopictus* might also be genetically determined. In order to test this hypothesis, studies

were conducted to select TOT susceptible and refractory strains of *Ae. albopictus*. A strong and consistent response to selection is an indication that the trait has a significant genetic basis (Falconer and Mackay 1996). Selection for susceptibility and refractoriness to TOT of LAC was attempted in two strains of *Ae. albopictus*; FIR was the phenotype under selection. Production of such strains would also provide valuable research stocks for future studies elucidating the molecular and genetic mechanisms conditioning TOT. Understanding these mechanisms could have profound and far-reaching effects, with the ultimate hope of leading to novel methods of disease control or management. This could include the production of mosquitoes with genetically augmented resistance to TOT or LAC itself.

B. Materials and Methods

Mosquitoes. Starting colonies for these experiments were based on recently colonized field populations ($\leq$F2). Based on vector competence results (Chapter 2), two candidate strains were chosen for selection. These strains were derived from populations collected in Mobile, Alabama (MOBAL) and Galveston, Texas (GALTX) (see Chapter 2, Table 2 and Figure 8). Initial data on F1 FIRs from these strains were available from previous experiments. The FIRs for these 2 populations differed significantly from the average FIR of the other tested *Ae. albopictus* populations. The MOBAL strain FIR was significantly higher (FIR of 31%, $P<0.005$), while the GALTX strain FIR was significantly lower (FIR of 3%, $P<0.05$) (see Chapter 2, Table 6, and Figure 10). Therefore, selection continued for a high average FIR in the MOBAL strain (i.e. TOT susceptible strain development), and a low average FIR in the GALTX strain (i.e. TOT

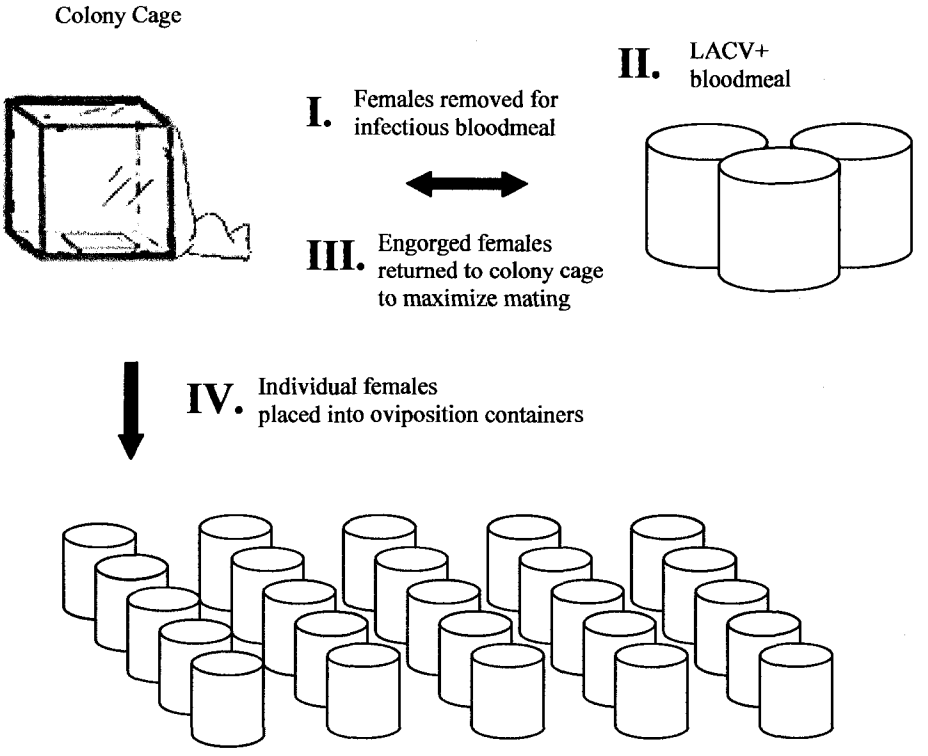
refractory strain development). Each colony was reared under standard insectary conditions of ≥80% RH, 14:10 light-dark cycle and 28°C (Gerberg et al. 1994). Adult mosquitoes were provided sugar cubes and water *ad libitum*. Forty-eight hours before artificial membrane feeding, sugar cubes were removed, and mosquitoes were provided only water.

Infectious Bloodmeal. Mosquitoes were infected with an artificial bloodmeal as previously described (Chapter 2, page 35). Fully engorged females were returned to their respective colony cages for 24 hours to maximize the likelihood of mating (Figure 18).

Virus Titration Protocol. Virus was tittered as previously described (Chapter 2, page 36).

Oviposition and Egg Collection. Females were removed from the colony cage and placed into individual, 500mL oviposition containers. Eggs were oviposited in 25μL ovicups lined with moist strips of brown paper towel (OP liners). One batch of eggs was collected from the first (1^{st}) ovarian cycle and another was collected from the second (2^{nd}) ovarian cycle. The OP liners were partially dried and individually labeled according to mother. The 1^{st} ovarian cycle was induced from the infectious bloodmeal. The 2^{nd} ovarian cycle was induced by a non-infectious bloodmeal consisting of a 1:1 solution of defibrinated sheep blood and DMEM. This second bloodmeal was administered separately to each female by use of artificial membrane feeders (Figure 18).

Figure 18 – Schema for LACV Infection and Collection of Eggs for Selection



V. Individual mothers:
- 1st cycle eggs collected, labeled, stored
- Offered non-infectious bloodmeal
- 2nd cycle eggs collected, labeled
- Mothers headsquashed
- Progeny from infected mothers hatched, reared for FIR determination

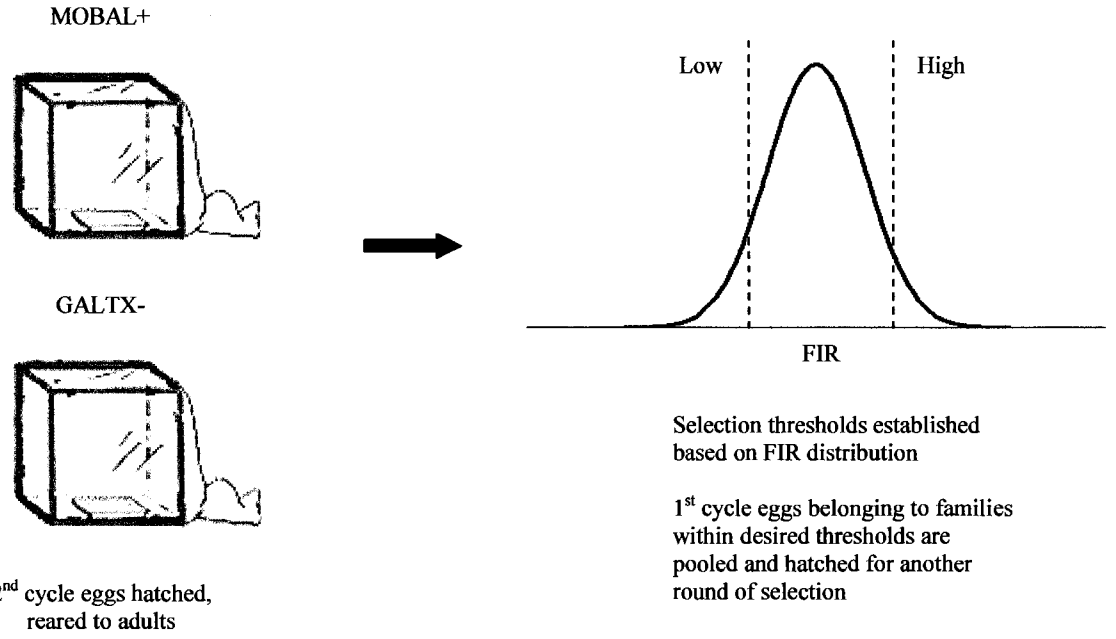
Mosquito Viral Infection Assay. To induce egg hatching, the 2nd cycle OP liners were placed in ~1L of water containing 0.1% brain heart infusion broth. After larval hatching, progeny mosquitoes were fed (Purina Mouse Chow and Tetramin fish food) and raised under conditions identical to that of their parents. Larvae from the 2nd ovarian cycle were reared to pupae then transferred to individual 500mL oviposition containers labeled according to mother. Within 24 hours of ecdysis, adults were collected and sacrificed, heads were removed and squashed onto microscope slides and assayed for the presence of LAC antigen (Ag) using an anti-La Crosse antibody conjugated with fluorescein isothiocyanate (FITC), and a fluorescent microscope (Chapter 2, page 38) (Beaty and Thompson 1975). Adult mosquito mothers were assayed for disseminated infection by immunofluorescent assay of head tissues for detection of viral Ag (Beaty and Thompson 1975). Distinct fluorescence foci in tissues indicated LAC infection.

Selection Scheme. Only the progeny of mothers with disseminated infections were retained for selection. This method ensured that the selection focused on potential barriers to ovarian infection instead of barriers to disseminated infection.

For each infected mother, progeny from the 2nd ovarian cycle were reared by family to adults and assayed for infection. The FIR was determined for females with more than 10 offspring. Based on the distribution of FIRs for each mosquito strain, a selection cut-off was chosen. This cut-off point was determined through the balancing of the ability to apply sufficient selection intensity while also retaining enough individuals to propagate the strains. The 1st ovarian cycle eggs from mothers whose FIRs fell within

the desired selection cut-off were pooled, hatched, and used for another round of selection (Figure 19).

Figure 19 – Family Based Selection Scheme for *Ae. albopictus* FIR



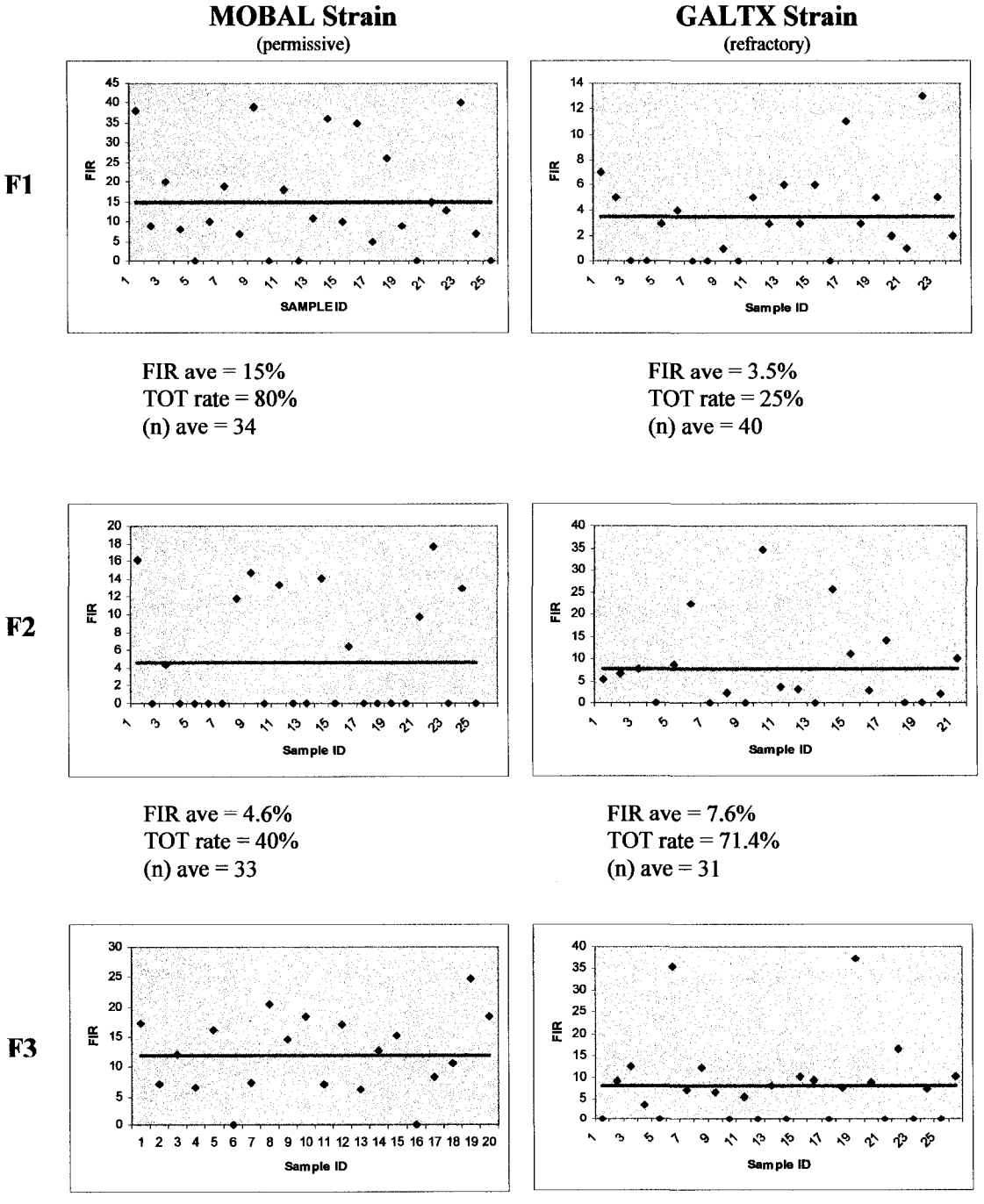
Analysis. Tests of significance for response to selection on FIR as well as tests for the uniform distribution of FIRs among individuals was subject to Chi-square analysis. Overall trends and FIRs between generations of selection were subject to a Pearson test for determination of multivariate correlation coefficient values (R^2) (Moore and McCabe 1993).

C. Results

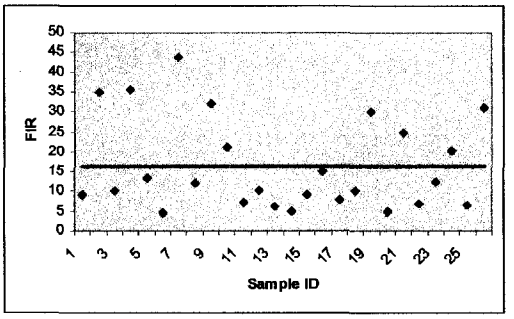
The results of selection through the F6 generation of the MOBAL (susceptible) strain and GALTX (refractory) strain are shown in Figure 20. Although there was a minor response for selection of a low FIR in the GALTX strain (R^2=0.3097), there was no corresponding response for selection of a high FIR in the MOBAL strain (R^2=0.1525). The average FIR in the GALTX strain was initially 3%, which increased to approximately 8% after selection in the F3 generation. This subsequently decreased to almost 2% by the F4 generation of selection and 1.2% by the F6 generation. For the MOBAL strain, the FIR was 31% in the parental generation, which decreased to 15% and subsequently to 4.6% in the F2 generation. FIR did not respond to selection in a consistent or predictable manner. Both strains under selection exhibited diverse FIRs after multiple generations of selection pressure. There was no significant response to selection for FIR in either strain (Table 17). Response to selection for FIR broadly fluctuated (Figure 21).

Tests of significance for response to selection on FIR in permissive individuals are shown in Table 18. Neither strain exhibited a uniform distribution of filial infection rates at any generation. Indeed, both strains displayed an excess of individuals in the lowest two deciles. While this would be expected when selecting for transovarial transmission refractoriness, the appearance of larger than average deciles in the lower range of filial infection rate distribution after multiple generations of high FIR selection was unanticipated.

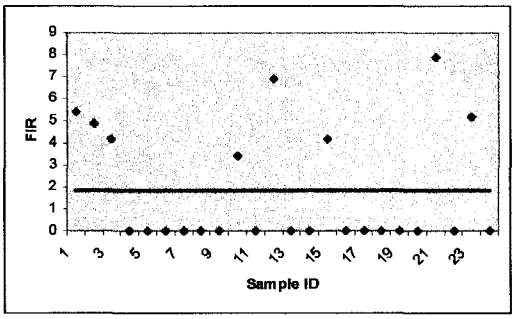
Figure 20 – Response to Selection on Filial Infection Rate in LACV Permissive and Refractory strains of *Aedes albopictus.*



F4

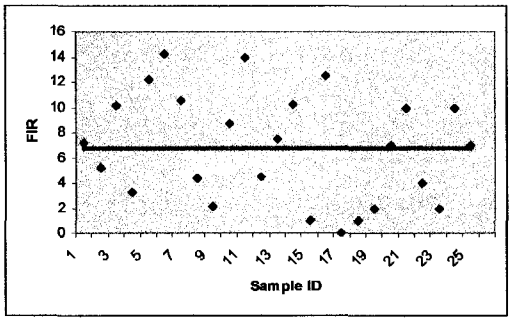


FIR ave = 16.3%
TOT rate = 100%
(n) ave = 51

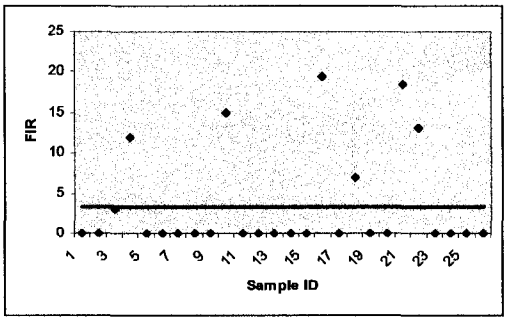


FIR ave = 1.8%
TOT rate = 36%
(n) ave = 35

F5

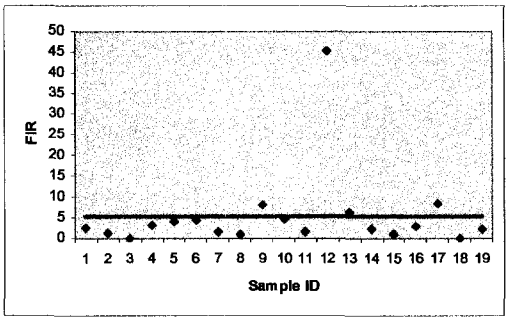


FIR ave = 6.8%
TOT rate = 92%
(n) ave = 48

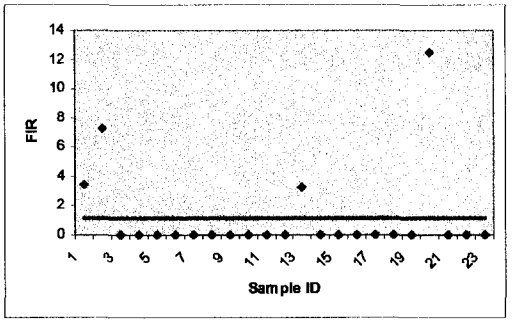


FIR ave = 3.4%
TOT rate = 32%
(n) ave = 34

F6



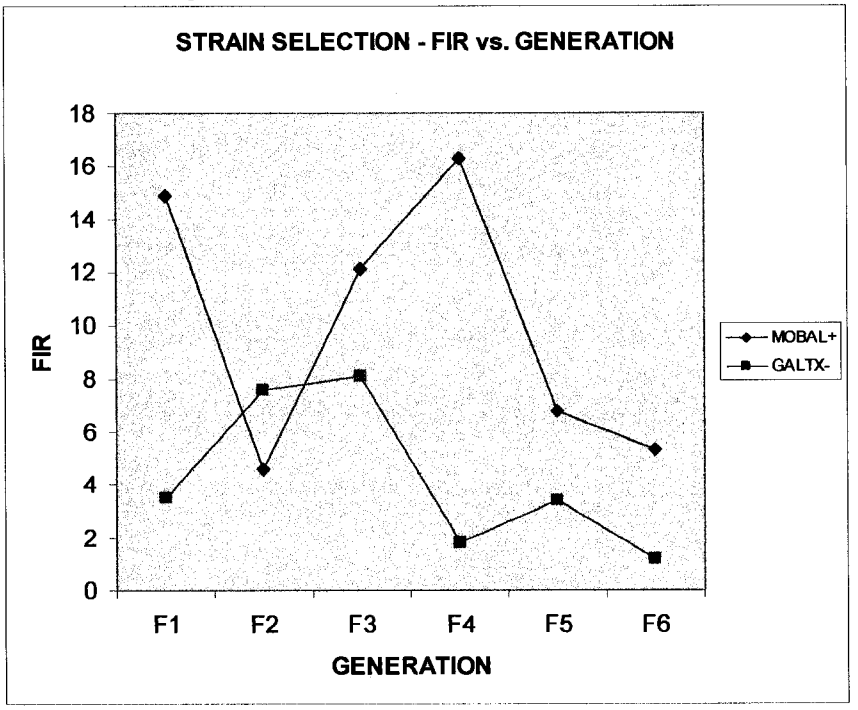
FIR ave = 5.3%
TOT rate = 89.5%
(n) ave = 52.3

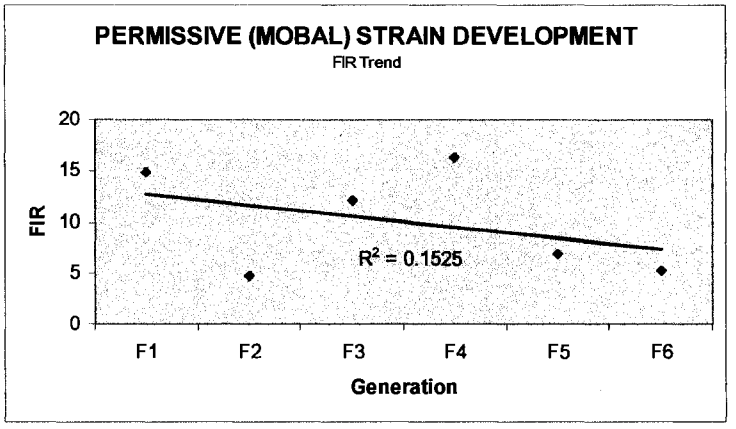


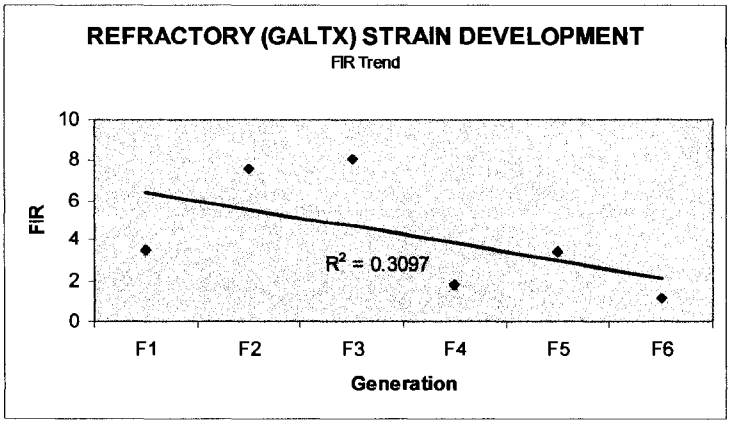
FIR ave = 1.2%
TOT rate = 17.4%
(n) ave = 25.3

FIR rates for each generation under selection for either a permissive (MOBAL) or refractory (GALTX) strain of *Ae. albopictus*. Each sample ID (1, 2, 3, etc.) represents an individual female mosquito mother per generation of selection and the FIR of her progeny.

Figure 21 – Trends in FIR Selection Response







FIR trends after selection for either permissive or refractory strains. FIRs were determined after each generation of selection. Trends were subject to a Pearson test for determination of multivariate correlation coefficient values (R^2) (Moore and McCabe 1993).

Table 17– Response to Selection on FIR Between Generations

RESPONSE TO SELECTION ON FIR		
	χ2 Value	
Generation	Permissive (MOBAL)	Refractory (GALTX)
F1 to F2	**5.55** (sig, p<0.025)	1.43 (p<1)
F2 to F3	**3.15** (sig, p<0.10)	0 (p<1)
F3 to F4	0.66 (p<1)	3.79 (p<1)
F4 to F5	**3.98** (sig, p<0.05)	0.21 (p<1)
F5 to F6	0.35 (p<1)	1.02 (p<1)
OVERALL	**9.6** (sig, p<0.05)	6.7 (p<0.2)

Table displays results of Chi-square analysis testing for significant change between generation of selection for FIR (i.e. is the change in FIR significantly increasing or decreasing after a given generation of selection) Bolded values indicate significant χ^2 values.

Table 18 – Tests of Significance for Response to Selection on Filial Infection Rate

GENERATION	1-10%	11-20%	21-30%	31-40%	41-50%	51-60%	61-70%	71-80%	81-90%	91-100%	Total	χ^2 value	p-value
						Permissive (MOBAL) Strain							
F1	**8**	**6**	1	**5**	0	0	0	0	0	0	20	43	p<0.001
% Total	(40)	(30)	(5)	(25)	(0)	(0)	(0)	(0)	(0)	(0)			
F2	**3**	**7**	0	0	0	0	0	0	0	0	10	48	p<0.001
% Total	(30)	(70)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F3	**6**	**10**	2	0	0	0	0	0	0	0	20	54	p<0.001
% Total	(30)	(50)	(10)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F4	**13**	5	3	4	1	0	0	0	0	0	26	58.62	p<0.001
% Total	(50)	(19.2)	(11.5)	(15.4)	(3.8)	(0)	(0)	(0)	(0)	(0)			
F5	**18**	5	0	0	0	0	0	0	0	0	23	128.7	p<0.001
% Total	(78.3)	(21.7)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F6	**16**	1	0	0	0	0	0	0	0	0	17	225	p<0.001
% Total	(94.1)	(5.9)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
						Refractory (GALTX) Strain							
F1	2	**4**	0	0	0	0	0	0	0	0	6	27.33	p<0.01
% Total	(33.3)	(66.6)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F2	**10**	2	2	3	0	0	0	0	0	0	17	51.82	p<0.001
% Total	(58.8)	(11.8)	(11.8)	(17.6)	(0)	(0)	(0)	(0)	(0)	(0)			
F3	**12**	**6**	0	1	0	0	0	0	0	0	19	76.26	p<0.001
% Total	(63.2)	(31.6)	(0)	(5.3)	(0)	(0)	(0)	(0)	(0)	(0)			
F4	**9**	0	0	0	0	0	0	0	0	0	9	81	p<0.001
% Total	(100)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F5	**7**	**3**	0	0	0	0	0	0	0	0	10	48	p<0.001
% Total	(70)	(30)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			
F6	3	1	0	0	0	0	0	0	0	0	4	12	p<0.10
% Total	(75)	(25)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)			

Number of females in each non-zero filial infection rate decile as a percentage of the total number of transmitting females in a given generation. Bolded, underlined values indicate deciles which caused significant χ^2 values. The χ^2 analysis is a goodness-of-fit test to a uniform distribution. Expected values in a uniform distribution are the total number of TOT permissive females/10.

D. Discussion

The lack of a strong and uniform response to selection on FIR suggests that this trait is not under genetic selection. Even with six generations of selection, FIR fluctuated erratically across strains (MOBAL FIR $\bar{x}$ = 10, stddev = 5.10, GALTX FIR $\bar{x}$ = 4.25, stddev = 2.89, Figure 21, Table 17). A slightly negative trend was observed overall, and a significant number of FIRs were distributed in the two lowest deciles (1-20%, $p<0.001$) (Table 18). It would seem that for a given female, the frequency and extent of ovarian infection during TOT is conditioned by random persistence dynamics possessing modulatory effects on the replication and infectivity of LAC. These could involve multiple factors in both the host vector and the virus itself. Host determinants could include the relative location of ovarioles within an ovary and point of viral entry into an ovary, and replicating virus may be subject to random factors limiting infection and survival within ovarian tissue. Cells of insect organs may respond differently to virus infection and may well be a determinant of viral tropism. Indeed, *Ae. albopictus* tissues display a remarkable range of responses to Sindbis virus infection (including refractory, transient, cleared, and persistent) (Bowers et al. 1995).

The selection results for FIR confirms previous studies suggesting that this parameter may not have a significant heritable component. The observation that the prominent parameter responding to selection in the LAC and *Aedes triseriatus* system is the TOTr and not the FIR was previously described (Graham et al. 1999). Those investigators determined that the TOT refractory phenotype is caused by a homozygous recessive genotype, andTOT susceptibility is mediated by the presence of at least one dominant allele. Furthermore, the investigators hypothesized that the strong selection

response among refractory strains is due to the unambiguous resolution of genotype. That is, a FIR of 0% may be clearly identified as homozygous recessive. However, since the susceptible phenotypes could possess either homozygous dominant or heterozygous genotypes, it becomes difficult to screen out heterozygous individuals during selection for TOT susceptibility. Mating heterozygous adults would have a 25% chance of producing refractory progeny (homozygous recessive). Thus, TOT refractory individuals could continue to appear in susceptible strains in the face of multiple generations of selection. It would appear that the *Ae. albopictus* and LAC system may fit this model as well.

The continued appearance of transovarially transmitting females in the GALTX strain, each generation of which was composed solely of the offspring of refractory females, also fits the TOT model for La Crosse and *Ae. triseriatus* (Graham et al. 1999) TOT dominant alleles could encode some form of viral receptor within the ovaries (Graham et al. 1999). Homozygous recessive offspring would lack this proposed receptor, providing an anatomic barrier to infection. Those with heterozygous alleles would possess an intermediate affinity, and homozygous dominant individuals would most effectively bind with LAC, promoting more efficient and pervasive follicular infection. Nevertheless, specific mosquito viral receptors for LAC have not been identified (Wang et al. 1991, Schlegel et al. 1982). Infection of offspring from refractory mothers could occur through a vertical transmission mechanism. While inefficient (~1%), infection of the egg could occur through the micropyle as the egg passes through heavily virus-infected calyx and oviducts during oviposition (Chandler et al. 1998, Rosen et al. 1989).

Genetic studies could identify and confirm specifics of this system. This could involve genetic modeling, such as the Fisher-Single Locus Selection model to compare observed and expected allelic frequencies for the inference of the TOT relevant genotypes (Rouzine et al. 2001, Fisher 1958). Performance of a reciprocal cross between the MOBAL and GALTX strains and testing of the offspring for TOT susceptibility would also provide insight into the genetics underlying TOT by *Ae. albopictus* (Bennett et al. 2005, Graham et al. 2003).

The mosquitoes under selection were derived directly from field-acquired strains (≤F2). Notwithstanding attempts at optimizing light cycles, temperature, humidity, and other environmental variables, simply propogating mosquitoes in the laboratory may introduce unnatural selection pressures (Lima et al. 2004, Gargan et al. 1983, Lorenz 1981). The mosquitoes in this study were subject to a battery of laboratory manipulations and conditions (e.g. pooled and individual membrane feeding, single adult oviposition cages, retention of only fully-engorged females, static mating exposures, etc.). Thus, selection pressures may have been introduced that have no relationship to TOT. Future experiments may necessitate additional rounds of selection or the development of bi-directional TOT lines from laboratory strains. Finally, because FIR is poorly responsive to selection pressure, it would be prudent for future studies to assay pools of mosquitoes with TOTr as the phenotype under selection.

CHAPTER 5: SUMMARY

La Crosse encephalitis continues to be an important cause of pediatric arboviral encephalitis in the United States (U.S.) (Rust et al. 1999, Calisher 1994, Tsai 1991). The majority of La Crosse virus (LAC) infections result in an asymptomatic to mild illness, but those cases which do develop a clinical presentation are serious and often require hospitalization and intensive care (Beaty et al. 2000, Calisher 1994). This is in stark contrast to the effects of infection on the natural, invertebrate host. Remarkably, adults of *Aedes (Ochlerotatus) triseriatus* can develop persistent, lifelong infections with virus dissemination through virtually all tissues without any major deleterious effects (Borucki et al. 2002). Furthermore, this virus can then be efficiently transovarially transmitted (TOT) to the vector's progeny, promoting viral maintenance in nature (Watts et al. 1973).

In 1985, the first established breeding colonies of *Aedes albopictus* were discovered in the continental U.S. (Sprenger and Wuithiranyagool 1986). Since that time, this invasive vector has spread throughout the country and has become identified as a possible vector of LAC. The mosquito can transmit the virus orally and vertically as demonstrated by TOT, the virus has been isolated from field collected larvae, and encephalitis outbreaks have been epidemiologically associated with *Ae. albopictus* (Erwin et al. 2002, Gerhardt et al. 2001, Tesh and Gubler 1975). As *Ae. albopictus* has become established, there has been a concurrent emergence of La Crosse encephalitis in the Southeastern U.S. (Nasci et al. 2000, Jones et al. 1999, Szumlas et al. 1996). *Ae. albopictus* has also largely displaced *Aedes aegypti* (a U.S. resident for centuries and relatively poor vector of LAC), becoming the most abundant mosquito in artificial containers throughout the Southeast (Hughes et al. 2006, Moore 1999, O'Meara et al.

1995). With the ongoing dispersal of a non-native, aggressively catholic feeding mosquito throughout LAC endemic regions, it is important to further understand the fundamental vector-virus interactions of this new, medically important system.

A competent vector of at least 22 arboviruses (including Chikungunya, dengue, eastern equine encephalitis, Japanese encephalitis, and yellow fever), *Ae. albopictus* transmission has the potential to dramatically change the epidemiology of U.S. arbovirus diseases, including La Crosse encephalitis (Gratz 2004). The purpose of this dissertation was to study and broaden the understanding of TOT and the vector-virus dynamics between *Ae. albopictus* and LAC. To that end, this study investigated 4 components of this LAC and *Ae. albopictus* system: 1) determined the time course of dissemination and filial infection rates among recently colonized field strains, 2) investigated the anatomic pattern of virus dissemination in ovaries during the 1st gonotrophic cycle, 3) characterized mtDNA variation among geographically dispersed populations, and 4) developed transovarial transmission competent and refractory mosquito strains. During the course of these studies, approximately 19,490 mosquitoes were examined from across 20 states and 2 laboratory populations in order to add to the understanding of this evolving system.

All of the geographic strains of *Ae. albopictus* tested were susceptible to LAC oral infection and capable of TOT (Chapter 2). Time course studies of disseminated infections revealed an average dissemination rate of 85.4% on Day 7; 95.8% on Day 10; and 98.1% on Day 14. All of the geographic strains were potentially competent vectors. Although there were some differences in the replication and dissemination kinetics of La Crosse virus in some populations, no regional or geographic patterns emerged.

All strains studied were permissive for TOT of LAC. First gonotrophic cycle transmission was observed in 6 of the study populations; however, the filial infection rate (FIR) was low, averaging approximately 1%. This is not the first report of this phenomenon occurring with LAC and *Ae. albopictus* (Cully et al. 1992, Tesh and Gubler 1975). There was no detected geographic trend to this occurrence. Given such an inefficient rate of infection, it is likely the result of a form of vertical transmission; potentially involving infection through the micropyle during oviposition.

The average FIR of LAC resulting from 2nd gonotrophic cycle transmission was approximately 10%. Despite a wide range of 2nd gonotrophic cycle FIRs (3-31%), no geographic patterns were discerned. Recently colonized field strains (F1) did not differ significantly from the laboratory colonized control population of *Ae. albopictus*. The FIRs in the natural vector, *Ae. triseriatus*, were significantly higher than those in all populations of *Ae. albopictus* (FIR of 50% vs. an average of 10%, respectively) (Chapter 2). Nonetheless, this demonstrates that field populations are readily susceptible to disseminated infection and that geographic differences exist with respect to TOT. It remains to be established whether these differences have epidemiological significance, particularly within ranges of La Crosse virus endemicity.

LAC antigen (Ag) was detected in *Ae. albopictus* ovarian tissues as early as Day 2 post oral exposure (Chapter 2). LAC Ag was detected in ovarian tissue prior to dissemination from the midgut, as evidenced by the lack of Ag detection in head tissue before being found in ovarian tissue. Calyx tissue infections were observed more frequently than head tissue infections at Day 2 (23% vs. 0%), Day 3 (47% vs. 17%), and Day 4 (47% vs. 30%). Viral Ag was not detected in ovarian follicles through Day 7.

These results are consistent with prior findings of rapid dissemination to the ovaries following oral exposure in *Ae. triseriatus*. Ovaries were found to be infected as early as 1 day after an infectious bloodmeal and became infected by mechanisms other than dissemination from midgut infection (Kempf et al. 2006, Chandler et al. 1998). Several possible causes may underlie these observations.

A "leaky midgut" phenomenon may occur in conjunction with permeability-primed, receptive ovarian tissues to allow rapid egress of virus from the midgut into the ovaries (Hardy 1988). The ovarian infection observed in *Ae. albopictus* may be an artifact of artificial bloodmeals containing an unnaturally high viral titer. Viral transport by trachea, which permeate both midgut and ovarian tissues could also be involved. The conduit model of midgut dissemination proposes that tracheal cells may act as routes for early viral escape out of the midgut (Romoser et al. 2004). The lack of viral Ag in the follicles suggests that 1st gonotrophic cycle eggs become infected during oviposition. At this early developmental stage, virus can be taken into the egg as it progresses through the micropyle and infect the developing embryo. This mechanism occurs during vertical transmission of Flaviviruses and has been observed in this study (Rosen et al. 1989). More information on the kinetics and tropisms of the virus would be useful in determining the role of *Ae. albopictus* in the epidemiology of LAC.

Examination of variation in the ND5 mitochondrial DNA marker revealed remarkable uniformity across the tested populations of U.S. *Ae. albopictus* (Chapter 3). Only two haplotypes were observed from geographically dispersed states, including Hawaii, and only samples from Kentucky presented with unique haplotypes. This is the first study devoted exclusively to ND5 mtDNA variation among U.S. populations and

confirms previous observations of low genetic variability in U.S. populations of *Ae. albopictus* (Mousson et al. 2005, Birungi and Munstermann 2002). Possible explanations for such homogeneity range from the founder effect of low-variability in founding populations to population bottlenecking and random genetic drift. However, cytoplasmic incompatibility has been proposed to explain the perplexing observation of low mtDNA variability but average genomic DNA variability among *Ae. albopictus* populations (Armbruster et al. 2003). Cytoplasmic 'sweeps' mediated through maternal inheritance of infection with the rickettsial parasite, *Wolbachia*, may be acting to homogenize host *Ae. albopictus* mtDNA, while not affecting nuclear gene diversity (Ono et al. 2001, Shoemaker et al. 1999). Studies confirming the *Wolbachia* infection status of field populations would help determine if such a phenomenon is occurring. The use of additional marker sets, including nuclear gene markers as well a greater number of sampling locations for evidence of rare mtDNA haplotype variants could assist in the characterization of *Ae. albopictus* diversity in the U.S..

Ae. albopictus responded poorly to selection based on FIR for the development of LAC suceptible and refractory strains (Chapter 4). Despite 6 generations of selection, FIR varied widely and unpredictably across strains (Chatper 4, Table 17). Lack of a predictable, directional response to selection suggests this trait is not under genetic selection. Furthermore, random host and viral determinants could be modulating the replication and tropism of LAC (ovariole location, viral entry, infection barriers, etc.). This data supports previous work with the *Ae. triseriatus* and LAC system. In *Ae. triseriatus*, TOTr, not FIR, was responsive to selection. Refractoriness was determined to be caused by a homozygous recessive genotype (Graham et al. 1999). It is likely that the

same dynamic is occurring with TOT in *Ae. albopictus*. Additional studies could identify and confirm specifics of this system.

It is important to note that the *Ae. albopictus* selection results may have been confounded through the use of recently colonized strains. In addition to the selection scheme based on FIR, recently established field strains may have been subject to selection pressures derived from laboratory colonization. This potential complication could be ameliorated in future studies through either additional rounds of selection or strain development with laboratory-adapted colonies. Irregardless, TOTr should be assayed as the trait under selection.

The results of this study demonstrate the complexity of the relationship between *Ae. albopictus* and LAC. The study supports literature suggesting that *Ae. albopictus* may be an important secondary vector of LAC (Hughes et al. 2006, Erwin et al. 2002, Gerhardt et al. 2001). However, TOT efficiencies appear to be too low to play a major role in virus survival in nature. A mosquito species that may be of importance in the horizontal transmission of the virus to humans may not necessarily be essential to the trans-seasonal survival of the virus. Further studies will be necessary to clarify the role of TOT of La Crosse virus by *Ae. albopictus*. This should include determination of TOT under diapausing conditions, fitness costs associated with transovarial infection, and the mechanism of ovarian and follicular infection.

With increases in intercontinental trade and travel, geographic boundaries to invasive organisms continue to erode between nations. The U.S. will be increasingly exposed to novel vector and virus interactions with unforeseen epidemiologic outcomes. It is the responsibility of the public health and medical entomology community to

conduct investigations into field-relevant, vector-virus relationships in order to provide risk assessment information and effective control strategies to these emerging threats.

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APPENDICES

APPENDIX A. REAGENTS FOR IMMUNOFLUORESCENT ASSAY OF OVARIAN TISSUES

PBT-1 (1x PBS, 1% BSA, 0.1% Triton X-100) [0.1% Sol^n]
1mL 10X PBS
1mL 10% BSA
7.9mL ddH_2O
100µL 10% Triton X-100 (Sigma, 93443-100ML)

PBT-2 (1X PBS, 1% BSA, 0.2% Triton X-100) [0.2% Sol^n]
1mL 10X PBS
1mL 10% BSA
7.8mL ddH_2O
200µL 10% Triton X-100

4% Paraformaldehyde
Add 2mL of 16% paraformaldehyde (Electron Microscopy Sciences, #15700)
Add 800µL of 10X PBS
Bring total volume to 8mL with ddH_2O
Invert several times and aliquot in 1mL increments
Freeze 1mL aliquots at -20°C

10X PBS
Dissolve: 80g of NaCl
2.0g of KCl
14.4g of Na_2HPO_4
2.4g of KH_2PO_4
in 800mL distilled H_2O.
Adjust pH to 7.4 with HCl.
Adjust volume to 1L with additional distilled H_2O.
Sterilize by autoclaving.

10% BSA
Add 10g BSA (Sigma, A9418-10G) to 80mL of ddH_2O
Stir, do not vortex
Bring final volume to 100mL
Aliquot in 10mL increments, freeze at -20°C

APPENDIX B. LYSIS BUFFER FOR DNA EXTRACTIONS *

For 100mL Solution: 0.1M NaCl (584mg)
0.2M Sucrose (6.85g)
0.1M Tris HCL pH 9.1 or 9.2 (1.211g)
0.05M EDTA [1.861g (Na_2)EDTA]
0.5% SDS (5mL of a 10% SDS solution)

Bring to 100mL, adjust pH to 9.2

*a.k.a. Patt Roman's Buffer, Fly Grinding Buffer (Black and Krafsur 1984)

Appendix C. Detailed Protocol and Reagents List for Single Strand Conformation Polymorphism (SSCP) Gel Production.*

PREPARE THE GLASS PLATE

A. Examine the glass plate. Determine which side is best suited for adhering the gel to. This is the side you will clean and coat.

B. Spray the glass plate with deionized 18.3 M Ω (Ultra-pure) water and wipe dry with a clean large Kimwipe tissue. Allow to dry (about two minutes).

C. Spray the glass plate with 95% ETOH and wipe dry with a clean large Kimwipe tissue. Allow to dry (about two minutes).

D. Coat the glass plate. Bind silane (γ-methacryloxy-propyltrimethoxysilane, Sigma Chemical Co.) is used to insure that the gel will adhere thoroughly to the glass plate. Wear gloves when working with bind silane. Add 5 μL of bind silane to 1mL of 95% ETOH with 0.5% glacial acetic acid (solution prepared in advance – see appendix). Pour the bind silane solution onto the glass plate, and spread thoroughly and evenly over the entire surface using a small Kimwipe tissue. This must be done quickly because of the high evaporation rate. Allow to cure for five minutes.

E. Spray the glass plate with 95% ETOH and wipe dry with a clean large Kimwipe tissue. Allow to dry (about two minutes).

F. Repeat step E above two additional times. These washes will remove any excess bind silane that could later dissolve the gel or migrate to the IPC.

PREPARE THE INTEGRAL PLATE CHAMBER (IPC)

Warning: It is critical not to contaminate the IPC with bind silane. Discard the gloves you have been wearing and put on a fresh pair.

A. The IPC has a flat glass side. This is the surface you will clean and coat.

B. Spray the IPC with Ultra-pure water and wipe dry with a clean large Kimwipe tissue. Allow to dry (about two minutes).

C. Spray the IPC with 95% ETOH and wipe dry with a clean large Kimwipe tissue. Allow to dry (about two minutes).

D Coat the IPC. RainX is used to insure that the gel does not stick to the IPC. Wear gloves when working with RainX. Squirt 1 – 1.5 mL of RainX onto the IPC, and spread thoroughly and evenly over the entire surface using a small Kimwipe tissue. Continue wiping and spreading as long as there is any visible liquid. The objective is to achieve a uniform sheen over the entire IPC. Allow to cure for five minutes. Apply a second coat just like the first one. Allow to cure for five minutes.

PUT THE IPC ASSEMBLY TOGETHER

Note: It is critical that RainX not get on the glass plate. Either discard the gloves you have been wearing and put on a fresh pair, or be certain not to touch the treated side of the glass plate in the following steps.

A. With the IPC treated side up, place a long red vinyl spacer along each outside edge.
B. Position the glass plate treated side down on top of the IPC.
C. Align the IPC, the glass plate, and the red vinyl spacers so that they are all flush with each other at the bottom (the closed end) of the IPC.
D. Place side clamps into position and lock (if new style) or squeeze into place (if old style).

POUR THE GEL

Caution: Wear protective materials for the eyes, hands, and face.

A. Insert the bottom end of the assembly into a new style casting tray, and lock the casting tray to the assembly by rotating the white levers. Place the assembly on a flat working surface with the glass plate down / IPC up.
B. Into a graduated cylinder, pour 95 mL of 5% acrylamide gel mix (solution prepared in advance – see SSCP appendix). Add 95 µL of Temed and 95 µL of ammonium persulfate (APS) solution (see SSCP Note 2). Mix well.
C. Using a plastic syringe with tube attached, draw all the acrylamide gel mix from the graduated cylinder into the syringe. Insert the Luer taper on the end of the tube into the fitting at the bottom of the casting tray. Push the syringe plunger until the acrylamide fills the assembly and approaches the upper edge of the IPC. Ideally, the assembly will fill uniformly with acrylamide (no bubbles), and there will be little or no spillage off the top edge of the glass plate.
D. Insert a well forming comb (clear plastic) into the top of the assembly, between the IPC and the glass plate. The well forming comb is inserted with the non-slotted edge into the gel (in this application the slots of the well forming comb are not used). Push the comb into the gel just far enough so that the slotted edge of the comb extends about 2 mm beyond the top of the glass plate.
E. Allow the acrylamide to polymerize for at least 1 hour.
F. Remove syringe from bottom of casting tray. Acrylamide that has polymerized in the syringe and tube goes in the disposal bucket for acrylamide. Rinse outside of syringe with warm water. Force warm water through the syringe and tube to make sure no acrylamide remains. Rinse casting tray with warm water, making sure no acrylamide adheres to any of its surfaces.

SET UP AND LOAD THE GEL

A. Dilute 300 mL of 5x TBE (solution prepared in advance – see SSCP appendix) to get 1500 mL of 1x TBE (buffer). Pour 350-500mL of buffer into base.
B. Place the IPC assembly in the base and secure with stabilizer bar and lower safety cover.
C. Fill the buffer chamber of the IPC with buffer.
D. Clean any excess acrylamide off the upper end of the IPC. Remove the well forming comb, and use a small plastic pipette to clean the well of any acrylamide pieces and/or bubbles.
E. Into the wells of a v-bottom plate, aliquot the appropriate amount of denaturing loading mix (DLM) and PCR product (see SSCP Note 1). The quantities and proportion of DLM and PCR product will differ with different PCR product concentrations. Denature the samples in either the thermocycler or the heating block at 95° for 5 minutes, then place on ice for 5 minutes.
F. Insert sharkstooth comb(s) into the top of the gel. Load a 1Kb ladder into the first and last wells and samples into the remaining wells.

RUN THE GEL

A. Place the upper safety cover securely on the IPC apparatus.
B. Run the gel at a constant 15-16 milliamps. Record the starting time and starting electrical values (watts, volts, milliamps). A typical gel runs anywhere from 16 to 22 hours (until the bromophenol blue band has migrated ¾ down the plate).
C. After the electrophoresis is complete, record the ending time and ending electrical values. Shut down the power supply, and remove the upper and lower safety covers.
D. Pour off the buffer from the IPC buffer chamber, remove the clamps and separate the gel plate from the IPC.

STAIN THE GEL

Fixer Solution
1800 mL Ultra-pure water
200 mL Glacial Acetic Acid

A. Place the gel plate in a tray containing 2L of 10% solution of acetic acid (fixer solution). Place the tray on a shaker operating at 70 rpm for at least 20 minutes. Gel plate can be left in acetic acid for up to 2 or 3 days, if necessary.
B. Pour the fixer solution into a container, for later use.
C. Pour 2L deionized water into the tray and allow to shake at 70 rpm for at least 2 minutes. Pour off the water.
D. Repeat step C. above two additional times.

Silver Stain
2500 mL Ultra-pure water
1.5 g silver nitrate

The water and silver nitrate may be mixed in advance, but the formaldehyde is to be added no more than 5 or 6 minutes before use.

3.75 mL 37% Formaldehyde

Caution: Formaldehyde is a known carcinogen in humans. Skin contact can cause burns and sensitization in some individuals. Breathing the vapors can lead to respiratory problems.

E. Pour the silver stain into the tray and allow to shake at 70 rpm for 25-30 minutes.

DEVELOP THE GEL

A. Pour off the silver stain and rinse the gel briefly with deionized water for 10 seconds. Pour off the rinse water.

Developing Solution
2500 mL Ultra-pure water
75.0 g Sodium Carbonate

The water and sodium carbonate are mixed in advance and put in the cool room to be chilled. Add formaldehyde and sodium thiosulfate (STS) (see SSCP Note 3) no more than 5 or 6 minutes before use.

3.75 mL 37% Formaldehyde
50 µL STS

Caution: Formaldehyde is a known carcinogen in humans. Skin contact can cause burns and sensitization in some individuals. Breathing the vapors can lead to respiratory problems.

B. Pour the developing solution into the tray and allow to shake at 70 rpm.
C. When the bands have developed and before the background darkens, pour in the fixer solution saved earlier. This will stop development.
D. Once the developer/fixer solution stops bubbling, pour it off and add deionized water for a final rinse. Shake at 70 rpm for at least 20 minutes.
E. Set the plate up to dry.

CLEAN THE EQUIPMENT

Caution: Wear protective materials for the eyes, hands, and face.

IPC

Rinse the spacers, sharkstooth comb(s), and base with warm water. Scrub the flat glass side of the IPC with UB Solution (solution prepared in advance – see SSCP Appendix) and a green scrubbing pad. Rinse away the UB Solution with warm water. Rinse the buffer chamber of the IPC with warm water. Wash the molded polycarbonate panel of the IPC with soap and warm water. Rinse away the soap with warm water. Rinse the flat glass side of the IPC and the buffer chamber with deionized water.

Glass Plate

It is very important that the glass plate be free of all contamination so that the gel will bind fully. Soak the plate in a 5% sodium hydroxide solution for 24 - 48 hours. Scrape any gel adhering to the plate into the soak (to be disposed of later). Scrub the plate using UB Solution and a green scrubbing pad. Rinse the glass plate with deionized water on both sides and set aside to dry.

Developing tray

Clean the developing tray with soap and water. This will make it ready for the next use. However, over time stain and glycerin builds up in the developing tray. This must be removed by using Alconox, bleach (optional), and a green scrubbing pad.

DISPOSE OF WASTE

Hazardous Waste Disposal:
Excess acrylamide should be allowed to polymerize and then be discarded in the lab's hazardous waste collection site. The gel waste bucket must be labeled with the following: the start date of the bucket, 8-81/2% acrylamide solution, silver nitrate, 10M sodium hydroxide, filter paper, water. Silver nitrate is a concentration of 0.004 – 0.006% and can be put down the drain. The acetic acid and sodium carbonate solution is neutralized and can be disposed of down the drain.

SSCP NOTE

1. At any given time, there should usually one 1.5 mL tube of DLM in ready for use at 4°C. Also, there should be a stock box of pre-made tubes at -20°C. Use the DLM at 4°C first. Take tubes out of the freezer as needed. When the box in the freezer gets half empty, it is time to make a new batch - see SSCP Appendix.
2. If there is a fresh (no more than 30 hours old) tube of APS solution at 4°C, use that. Otherwise mix a new tube of solution by adding enough Ultra-pure water to a tube of APS powder to bring the total volume to 1 mL. Mix well. If there is any unused APS solution in a tube you mix, mark the tube with the current date

and place it in the refrigerator. 1.5 mL tubes with 250 mg APS powder should be made up in advance.

3. If there is a tube of STS solution ready at 4°C, use that. Otherwise mix a new tube of solution by adding enough Ultra-pure water to a tube of STS powder to bring the total volume to 1 mL. Mix well. If there is any unused STS solution, place it in the refrigerator. 1.5 mL tubes with 450 mg STS powder should be made up in advance.

SSCP APPENDIX - SOLUTIONS THAT ARE PREPARED IN ADVANCE:

95% ETOH with 0.5% Glacial Acetic Acid (page 145)
30% Acrylamide (page 145)
5% Acrylamide Gel Mix (page 145)
0.5 M EDTA (page 146)
5x TBE (page 146)
Denaturing Loading Mix (DLM) (page 147)
UB Solution (page 147)

95% ETOH with 0.5% Glacial Acetic Acid

Pour 100 mL of 95% ETOH into a 100 mL bottle. Add 0.5 mL of acetic acid.

30% Acrylamide (W/V, 49:1 acrylamide to bis) (Black and Duteau 1997)

Caution: Wear protective materials for the eyes, hands, and face.

For 1 L of solution: 500 mL Deionized 18.3 M Ω (Ultra-pure) water.
294.0 g acrylamide.
6.0 g N, N' – methylene bisacrylamide.

A. Stir until thoroughly dissolved.
B. Add additional Ultra-pure water to bring total volume up to 1 L.
C. Pour into 1 L Nalgene bottle and label.
D. Store at 4°C (solution is good for one year).

5% Acrylamide Gel Mix for SSCP (Black and Duteau 1997)

Caution: Wear protective materials for the eyes, hands, and face.

For 2 L of solution: 334 mL 30% Acrylamide.
400 mL 5x TBE.
1166 mL Deionized 18.3 M Ω (Ultra-pure) water.
100 mL glycerin.

A. Because glycerin is viscous, it is easiest to mix the first three components, and then add the glycerin slowly to the stirring acrylamide solution. Warming the glycerin in a microwave oven also helps.
B. Stir until thoroughly mixed.
C. Filter through a Buchner funnel lined with three layers of filter paper (Fisherbrand P5). Use a sidearm flask connected to a vacuum pump to speed the flow.
D. Pour into 2 L Nalgene bottle.
E. Write your name and the date in the appropriate spaces of chart:
30% Acrylamide – 5% Acrylamide gel mix.
Write (from the chart) the appropriate number on the 2 L bottle.
Bottle must also be clearly labeled: 5% Acrylamide gel mix.
F. Store at 4°C (solution is good for one year).

0.5 M EDTA, pH 8.0

This is a stock solution described in many manuals. The following is a description similar to that in "Preparation of Reagents and Buffers Used in Molecular Cloning" (Appendix B, page 11) in Molecular Cloning, A Laboratory Manual, Vol 3, Sambrook, Fritsch, Maniatis, 2nd Edition, 1989, Cold Spring Harbor Laboratory Press

Caution: Wear protective materials for the eyes, hands, and face.

For 2 L of solution:
1600 mL Deionized 18.3 M Ω (Ultra-pure) water.
42 g NaOH.
372 g EDTA (the EDTA will not initially go into solution).

A. Add additional Ultra-pure water to bring total volume up to 2 L
B. Stir for at least 30 minutes.
C. Slowly add additional NaOH until EDTA dissolves and pH equals 8.0.
D. Pour into 2 L Nalgene bottle and label.

5x TBE (Black and Duteau 1997)

Caution: Wear protective materials for the eyes, hands, and face.

For 10 L of solution:
1 L Deionized 18.3 M Ω (Ultra-pure) water.
540.0 g Tris (hydroxylmethyl) aminomethane
275.0 g Boric acid
200.0 mL 0.5 M EDTA, pH 8.0

A. Stir for several hours.
B. Pour into a carboy clearly marked for 10 L of volume.
C. Add additional Ultra-pure water to bring the total volume up to 10 L.
D. Testing pH: Draw off 10 mL of above solution.

Add 40 mL Ultra-pure water to bring concentration to what it will be in final product.
The pH should be 8.5-8.7.

Denaturing Loading Mix (DLM)

Wear gloves.

Into a 200 mL flask, mix:
95 mL Formamide
1 mL 1 M NaOH *
0.05 g Xylene Cyanole
0.05 g Bromphenol Blue
4 mL Ultra-pure water

A. From the 200 mL flask, aliquot 1 mL of DLM into each of 100 (1.5 mL) tubes. Place tubes in a box. Store at -20°C.

*For 100 mL batch by add 4 g of NaOH to 100 mL Ultra-pure water.

UB Solution

Caution: Wear protective materials for the eyes, hands, and face.

Into a 1000 mL beaker, mix: 1000 mL tap water
400g NaOH

The solution will become very hot due to the exothermic reaction of NaOH.

A. After the NaOH is dissolved, add: 58g sodium di-lauryl sulfate (SDS)
B. Pour the mixture into two clearly marked 500 mL bottles.

*From the laboratory of Dr. William C. Black IV (modified from Black and Duteau 1997).