LARVAL ECOLOGY AND ADULT VECTOR COMPETENCE OF INVASIVE MOSQUITOES *Aedes albopictus* AND *Aedes aegypti* FOR CHIKUNGUNYA VIRUS

By

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To my husband and daughter, Bill and Seneca Turechek

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Abiotic and biotic features of the mosquito larval environment shape life history traits important in arbovirus dynamics (e.g., fecundity, life span, biting rate) and can directly affect characteristics that influence adult arbovirus susceptibility. Chikungunya virus (CHIKV) has recently emerged as an important agent of human arboviral disease, and the invasive container mosquitoes *Aedes albopictus* and *Ae. aegypti* are the epidemic vectors.

When the effect of larval rearing temperature on *Ae. albopictus* growth and susceptibility to CHIKV was investigated, results showed that temperature had a significant effect on size, development time, and CHIKV infection and dissemination rates. Adult females produced from the coolest temperature, had the largest mean body size, took the longest to mature, and were 6 times more likely to be infected with CHIKV than females reared at the highest temperature.

In a separate experiment, *Ae. aegypti* larvae were reared at different temperatures and food levels to explore relationships among attributes of the larval habitat, body size, and CHIKV susceptibility. Larval temperature and food availability had significant effects on mean adult body size, and female size and quantity of CHIKV ingested were

positively correlated. Larval temperature, but not food quantity nor the temperature x food level interaction, had a significant effect on CHIKV infection, but temperature, food level, and their interaction had a significant effect on dissemination. Significant wing length - infection correlations disappeared after the extrinsic incubation period, suggesting that mosquito size alone may not be a good predictor of viral susceptibility.

Larval competition between *Ae. aegypti* and *Ae. albopictus* within the container habitat is modulated by temperature, and these factors may interactively influence adult susceptibility to CHIKV. The outcome of competition between the two species did not change with temperature, and *Ae. aegypti* was found to be the superior competitor under these experimental conditions. Temperature and larval competition did not affect the likelihood of infection or disseminated infection with CHIKV. However, mean body titer of CHIKV-infected *Ae. albopictus* females was significantly affected by larval rearing temperature, with females reared at lower temperatures having higher mean CHIKV body titers than counterparts from the highest temperature.

CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

Introductory Statement

Fluctuating temperatures, limited food, and high inter- and intraspecific competition are common features in container habitats occupied by the aquatic larvae of many holometabolous insects, including the Asian tiger mosquito, *Aedes albopictus* and the yellow fever mosquito, *Aedes aegypti* (Juliano 2009). The effect of the mosquito larval environment on adult life history traits and other characteristics such as growth rate, age at maturity, biting rates, gonotrophic cycle lengths, vector size, fecundity, and life span are well documented (Madder et al. 1983, Briegel 1990, Rueda et al. 1990, Scott et al. 1993a). However, the larval environment may also influence adult vector-pathogen interactions, by altering a vector's competence for a virus resulting in changes in the distribution and transmission intensity of an arbovirus. Vector competence is the capacity of an arthropod to acquire a pathogen and transmit it to a subsequent host. For successful transmission to occur arbovirus taken in from an infectious blood meal must overcome internal barriers in the mosquito midgut, disseminate into other organs, and then pass through an additional barrier into the salivary glands in order to infect a new host during the next blood feeding (Hardy et al. 1983). Vector competence can vary greatly among individuals and between mosquito populations (Lorenz et al. 1984, Tabachnick et al. 1985, Turell et al. 1992, Paupy et al. 2001) and has been shown to be influenced by both the genetic background of a vector and environmental conditions (Davis 1932, Hurlbut 1973, Hardy et al. 1990, Kilpatrick et al. 2008).

Most research has focused on the adult environment with fewer studies exploring the influence of larval ecology on mosquito-arbovirus interactions. There is evidence

that larval habitat variables, such as temperature, food resources, density and competition can affect vector competence in certain mosquito-arboviral systems (Baqar et al. 1980, Grimstad and Walker 1991, Turell 1993, Sumanochitrapon et al. 1998, Alto et al. 2005, Alto et al. 2008a, Bevins 2008). However, the strength of the effect and the direction seems to vary with different species of mosquitoes and different viruses. Furthermore, previous research has primarily focused on the effect of a single factor in the larval environment without consideration for the potential effects of interactions between variables.

The research presented in this dissertation explores the interactions of larval ecological factors and their impact on the transmission of chikungunya virus (CHIKV) by invasive container mosquitoes *Aedes albopictus* and *Ae. aegypti*. The hypothesis tested here is that variation in environmental factors during larval development affects the physical and physiological characteristics of adults to alter their ability to become infected and/or transmit an arbovirus. Experiments were conducted manipulating: (1) larval rearing temperature, (2) larval rearing temperature and larval food levels, and (3) larval rearing temperature and intra- and inter-specific larval competition in *Ae. albopictus* and/or *Ae. aegypti* from South Florida. To determine the effect of larval habitat quality on life-history characteristics of these mosquitoes, juvenile mortality, development time, and adult body size were measured. Adult females were given a blood meal containing CHIKV, and susceptibility to the virus was assessed by measuring infection and dissemination rates and whole mosquito body viral titer. This research is novel for exploring the interactions among multiple factors in the larval environment and their impact on transmission potential of an arbovirus by adult

mosquitoes. Furthermore, this work is particularly relevant in light of predicted alterations in temperature due to global climate change and the ongoing ecological and public health problems caused by the continued intercontinental dispersal of invasive vectors and pathogens.

The following literature review provides information on the mosquito vectors, CHIKV, and the influence of the environment on vector competence. Chapters two, three, and four contain original research on the influence of *Ae. albopictus* and *Ae. aegypti* larval ecological factors on adult CHIKV susceptibility, followed by chapter five which provides conclusions and recommendations for future research directions.

Mosquitoes: *Aedes aegypti* and *Aedes albopictus*

As the volume of global trade and travel has greatly increased in the past fifty years, so have the number of accidental introductions of exotic organisms to new geographic areas (Mooney and Hobbs 2000). Invading organisms can have grave detrimental effects on indigenous communities, but when the invader is also a potential vector of an exotic human pathogen then the introduction and spread of the organism can also have a major public health consequence (Juliano and Lounibos 2005). The container mosquito species, *Ae. aegypti* and *Ae. albopictus* have globally invasive ranges. *Aedes aegypti* is believed to have traveled to the New World from Africa in water storage jars aboard slave ships (Christophers 1960), while the spread of *Ae. albopictus* from its native Asian range has been a more recent phenomenon (Hawley et al. 1987). Arboviruses that are transmitted by *Ae. aegypti* and *Ae. albopictus* threaten the health of millions of people worldwide, and the continued range expansion of these species will add to the populations at risk.

Invasion Biology of Disease Vectors

Aedes (*Stegomyia*) *aegypti* (L.), the yellow fever mosquito, is the primary epidemic vector of yellow fever virus (YFV), dengue virus (DENV) CHIKV. The preferred habitat of *Ae. aegypti* is the urban environment and although it is limited to tropical and subtropical regions, within these limits it has a very cosmopolitan distribution. In East Africa *Ae. aegypti* exists in two forms: (1) *Aedes aegypti formosus*, a sylvan and likely ancestral form with darker scales which oviposits primarily in treeholes and is often found away from human habitats and (2) *Ae. aegypti aegypti*, a domestic form, which exhibits a preference for human habitats (Tabachnick and Powell 1979). In coastal Kenya, the sylvan and domestic *Ae. aegypti* represent two distinct gene pools, maintained through habitat selection, where sympatry exists gene flow between the two subspecies is limited (Tabachnick and Powell 1979).

Though *Ae. aegypti* formosus has been found in other regions of subSaharan Africa, it is the domestic form, *Ae. aegypti aegypti*, through its ability to exploit human water storage containers and human habitats, that has spread from Africa to tropical and subtropical regions across the globe (Christophers 1960, Tabachnick 1991). Key behavioral characters of *Ae. aegypti aegypti* are: oviposition in human water storage containers, feeding on human blood, and entering into homes in search of hosts, mates and oviposition and resting sites (Trpis and Hausermann 1975). Oviposition containers, such as water cisterns, aboard New World bound slave ships during the 15th to the 19th centuries are believed to be a major mode of introduction for this mosquito (Tabachnick 1991). The spread of *Ae. aegypti* across the globe likely involved the movement of multiple life stages of the mosquito: aquatic larvae, adults, and eggs (Christophers

1960). For the remainder of this dissertation *Ae. aegypti* will be used as a synonym for *Ae. aegypti aegypti*.

Aedes (*Stegomyia*) *albopictus* (Skuse), the Asian tiger mosquito, is also an efficient and important vector of DEN and CHIKV and, like *Ae. aegypti*, *Ae. albopictus* has successfully spread across the globe through the exploitation of man-made environments. Although common in suburban and rural settings, this mosquito originated in the forest edges of Southeast Asia, but human migration towards the Malay peninsula and the Indian Ocean islands, including Madagascar, may have led to an early movement of *Ae. albopictus* out of its native Asian range (Paupy et al. 2009).

In the late nineteenth century *Ae. albopictus* began spreading onto the Pacific islands, such as Hawaii and Guam (Lounibos 2002). In 1985 it was discovered in the United States, where it was identified as the most abundant artificial container-inhabiting mosquito in Houston, Texas (Sprenger and Wuithiranyagool 1986). Since its discovery, *Ae. albopictus* has become established in most states in the eastern part of the USA, extending as far north as Illinois, Indiana, Ohio, Pennsylvania and New Jersey (Moore 1999). Between 1985 and 1998, *Ae. albopictus* was recovered from many countries in the Americas and the Caribbean (Benedict et al. 2007). In Europe, it was first recorded in Albania in 1979, Italy in 1990, then France in 1999, and currently *Ae. albopictus* is present in at least 12 European countries (Knudsen et al. 1996, Vazeille et al. 2008). Established populations were recorded in Nigeria in 1991 (Savage et al. 1992) and, since, *Ae. albopictus* has spread to other nations in West and Central Africa. The establishment of this mosquito in numerous countries, in Africa, the Middle East, Europe

and the Americas is primarily due to the trade and movement of used tires that contain *Ae. albopictus* eggs (Hawley et al. 1987, Benedict et al. 2007).

Host Preference and Feeding

Humans are the most common bloodmeal source of *Ae. aegypti* (Scott et al. 1993a, Ponlawat and Harrington 2005), and much work has been done to determine how the consumption of human blood compares to blood from other vertebrate hosts in terms of fecundity and longevity in this mosquito (Briegel 1985, Harrington et al. 2001). Females of most mosquito species require both blood and sugar (Foster 1995), and both male and female *Ae. aegypti* have been observed feeding on plant nectars (Christophers 1960). However, certain populations of *Ae. aegypti* in Thailand have adapted to an environment low in sugar, but where human blood is readily available. These females seldom feed on plant sugars and take more frequent blood meals (Edman et al. 1992). Laboratory studies on *Ae. aegypti* suggest that a diet of human blood without sugar increases the reproductive success of this mosquito through a greater age-specific survival (l_x), estimated reproductive output per day (m_x) and the net replacement rate (R_o), although total egg production was greater in mosquitoes fed blood and sugar (Harrington et al. 2001).

Aedes albopictus is considered an opportunistic feeder, with a preference for mammals (Savage et al. 1993). In suburban North Carolina humans (24%), cats (21%) and dogs (14%) were the primary hosts (Richards et al. 2006). However, in villages in Thailand and Singapore humans were the primary sources of blood (Colless 1959, Ponlawat and Harrington 2005). This species is thought to have progressively adapted to anthropogenic changes in the environment, moving from a zoophilic host feeding regime to a greater dependence on blood meals from humans and domesticated

animals (Paupy et al. 2009). Laboratory studies on *Ae albopictus* also suggest that a diet of human blood without sugar increases the reproductive success of this species, and thus the benefit of feeding exclusively on blood is not limited to the highly anthropophilic *Ae. aegypti* (Braks et al. 2006).

Aedes aegypti and *Ae. albopictus* females often take multiple blood meals during a single gonotrophic cycle (MacDonald 1956, Scott et al. 1993b, Scott et al. 2000, Delatte et al. 2009). This behavior, known as multiple feeding, can lead to an exponential increase in the vectorial capacities of these two mosquitoes because the daily probability of being fed upon is a squared function in the vectorial capacity equation resulting from the product of host preference index and the frequency of feeding (Black and Moore 1996). Furthermore, multiple feeding can increase the probability of concurrent infection and viral genetic mixing (Kuno and Chang 2005).

Aedes aegypti preferentially feeds in the daytime, but individuals will on occasion feed at night if a suitable host is present (Christophers 1960). *Ae albopictus* is also a diurnal feeder with peak feeding times occurring at daybreak and two hours before sunset (Delatte et al 2009). Nocturnal feeding sub-populations of *Ae. aegypti* in the Ivory Coast (Diarrassouba and Dossou-Yovo 1997) and in Trinidad (Chadee and Martinez 2000) are documented. In Trinidad nocturnal feeding was recorded at the urban site and not at the rural site and accounted for approximately 10% of urban indoor feeding totals and 9.4% of outdoor urban feeding totals. It was suggested that the nocturnal extension of *Ae. aegypti* feeding may be an adaptation to increased electrical lighting in and around houses, and also explains the absence of nocturnal feeding in the poorly lit rural site (Chadee and Martinez 2000).

Short-Range Dispersal and Longevity

The movement of mosquitoes has been studied because of the important role of dispersal and flight range in vector-borne disease. Many studies support the theory that *Ae. aegypti* takes fairly short flights and does not disperse over far distances. Muir and Kay (1998), in a mark-release-recapture study in northern Australia found the mean distance *Ae. aegypti* traveled was 56 meters (m) for females and 35 m for males. Harrington et al. (2005), in an 11 year study, involving 21 mark-release-recapture experiments in Puerto Rico and Thailand, concluded that the mean dispersal distance for *Ae. aegypti* ranged from 28 to 199 m and Maciel-de-Freitas et al. (2007) found a similar pattern in Rio de Janeiro, Brazil where the average distance travelled ranged between 81 to 86 m. In contrast a few studies have reported, through assaying rubidium labeled eggs, longer distance dispersal of up to 800 m in Rio de Janeiro (Honorio et al. 2003) and means ranging between 221-279 m in Puerto Rico (Reiter et al. 1995). A major difference in the studies assaying labeled eggs is that they measured *Ae. aegypti* dispersal during an oviposition cycle and this may account for some of the differences between the two types of studies.

Dispersal has been investigated in *Ae albopictus* and compared to *Ae. aegypti, Aedes albopictus* generally takes longer flights and disperses over farther distances. In a mark-release-recapture study in Missouri the maximum distances *Ae albopictus* traveled were 525 m for females and 225 m for males (Niebylski and Craig 1994). Through ovitrapping of rubidium labeled eggs, dispersal by *Ae albopictus* of up to 800 m was found in Rio de Janeiro and did not differ in distance from *Ae. aegypti* in this study (Honorio et al. 2003). In Singapore *Ae albopictus* rubidium labeled eggs were found at distances as far as 640 m from the release site (Liew and Curtis 2004).

The life span of adult mosquitoes in the field is determined by factors such as quality of the environment, climate, predation, and genetic background. Laboratory studies in which *Ae. aegypti* and *Ae. albopictus* are given ample sugar and blood meals while being held at a constant and suitable temperature with a high level of humidity, record maximum longevity for females at over 100 days and the mean life spans between 4-6 weeks (Christophers 1960, Hawley 1988). Daily survival probabilities of adult females are an important element of vectorial capacity, and, to transmit an arbovirus, mosquitoes must survive longer than the time prior to taking an infectious blood meal combined with the extrinsic incubation period (EIP) of the pathogen, which is the time interval between ingestion of an infective blood meal and oral transmission of a virus (Davis 1932). The EIP for CHIKV in *Ae. aegypti* and *Ae. albopictus* can be as short as two days (Dubrelle et al. 2009).

The assumption can be made that wild mosquito individuals at the mercy of biotic and abiotic factors in the environment experience greatly reduced longevity; the difficulty is measuring it. Mark-release-recapture experiments have been the primary method of assessing probability of daily survival (PDS), and in Rio de Janeiro, Brazil female *Ae. aegypti* PDS varied from 0.71 to 0.87 depending on the site and season (Maciel-de-Freitas et al. 2007) and in Kenyan field studies PDS was 0.77 for male *Ae. aegypti* which corresponds to a 4.4 day life span, and 0.89 (9.2 days) for females (McDonald 1977). For *Ae. albopictus* in a scrap tire yard in Potosi, Missouri PDS was 0.89 (8.2 days) for females and 0.77 (3.9 days) for males (Niebylski and Craig 1994). Very low recapture rates in survival studies make the reliability of PDS estimates

questionable, and predictions of mosquito age in future studies may make use of newer technologies such as gene transcription profiling (Cook et al. 2006).

Oviposition

Gravid female *Ae. aegypti* and *Ae. albopictus* oviposit desiccation resistant eggs that can survive in a dried state for several months or longer until submersion in water triggers hatching. A common feature of oviposition containers selected by both female *Ae. aegypti* and *Ae. albopictus* is clean water with a high organic content (Clements 1992, Delatte et al. 2008). A gravid *Ae. aegypti* female will select both indoor, because of its close association with man, and outdoor containers for oviposition (Christophers 1960) and for *Ae. albopictus* peridomestic and rural oviposition sites are frequently used (Hawley 1988).

Egg-laying in both these mosquitoes is diurnal with the majority of eggs laid two hours after sunrise and two hours before sunset (Corbet and Chadee 1990, Delatte et al 2009). Based on the results of field studies on *Ae. aegypti* in Puerto Rico and Trinidad and on *Ae. albopictus* in Honolulu, Hawaii, it was determined that females deposit their eggs from the same batch at several oviposition sites, a behavior known as “skip oviposition” (Rozeboom et al. 1973, Chadee and Corbet 1987, Apostol et al. 1994, Reiter et al. 1995). Skip oviposition may benefit the species by decreasing sibling competition and spreading risk of mortality over multiple sites.

Aedes albopictus is remarkable for the wide range of natural and artificial containers in which it is found, ranging from tree holes, bamboo stumps, rock holes, leaf axils, flower plates, pots, catch basins, and discarded tires (Hawley 1988, Sota et al. 1992, Delatte et al. 2008). On the island of Reunion, where it is the most common *Aedes* species, *Ae. albopictus* showed a strong ecological plasticity. In the wet season

this mosquito occured most frequently in small artificial disposable containers and in the dry winter season, natural containers (bamboo stumps and rock holes) were clearly important (Delatte et al. 2008). In North America, the most common microhabitat for *Ae. albopictus* has been discarded tires (Sprenger and Wuithiranyagool 1986). However in Florida *Ae. albopictus* immatures are found in the water-holding tanks and axils of ornamental bromeliads, although in southern Florida their use of this phytotelmata is kept in check by two endemic *Wyeomyia* spp. of mosquitoes (Lounibos et al. 2003). In Florida *Ae. albopictus* is also found sharing tree holes with the native inhabitant *Ae. triseriatus* (Lounibos et al. 2001).

Thermal Tolerance

No life stage of *Ae. aegypti* undergoes diapause, and 16ºC seems to be close to the lower limit for adult activity of this species (Christophers 1960). Larvae continue to develop and pupate at temperatures as low as 15ºC, but the duration of the larval stage is approximately 31-32 days, and at temperatures between 8.2-10.6 ºC development completely ceases (Kamimura et al. 2002). In terms of upper thermal limits, *Ae. aegypti* larvae do not thrive in water temperature much above 34ºC and adults begin to die if air temperatures exceed 40 ºC. Desiccation resistant eggs are also susceptible to temperature extremes. Prolonged exposure to a low temperature of 10ºC and a high temperature of 40ºC resulted in 100% egg mortality (Christophers 1960).

In its native Asian range, *Ae. albopictus* is abundant in both tropical and temperate regions and, as a result, this mosquito species can survive over a broad spectrum of temperatures and relative humidity. At 11ºC Udaka (1959) found that larval development ceased for a Japanese strain of *Ae. albopictus.* A similar result was recorded with a strain of *Ae. albopictus* from Reunion island in the Indian Ocean, where

no development beyond first instar took place at 5ºC or 10ºC (Delatte et al. 2009). At temperatures as low as 12ºC *Ae. albopictus* larvae developed and pupated with an 28 day larval duration, but two-thirds of adult females did not mature eggs or died (Briegel and Timmermann 2001). Adults from a Reunion strain fared worse with no egg laying observed among females at 15ºC (Delatte et al. 2009). The limiting upper temperature of *Ae. albopictus* larval development is 35ºC (Monteiro et al. 2007, Delatte et al. 2009), and at 43.3ºC 100% adult mortality was observed after approximately 30 minutes (Smith et al. 1988). Analysis of life tables, combining developmental rates, reproduction and mortality, suggest maximum population growth (*r*) between 25 and 30ºC for *Ae. albopictus* tested at eight constant temperatures (5, 10, 15, 20, 25, 30, 35 and 40º C) (Delatte et al. 2009).

Strains of *Ae. albopictus* found in temperate regions are sensitive to short day-lengths during the pupal and adult stage leading to the production of diapause eggs (Hawley et al. 1987). Not long after *Ae. albopictus* was found in the United States, this species was also identified in Brazil (Forattini 1986), but the lack of a photoperiodic egg diapause in Brazilian populations suggested distinct origins for the two populations (Hawley et al. 1987). Sequence data from the mitochondrial ND5 gene confirmed that the North American populations of *Ae. albopictus* originated from Asian temperate regions, while Brazilian populations are tropical in origin (Birungi and Munstermann 2002). This was in contrast to an earlier allozyme study inferring a common Northern Asian (Japan) origin for both populations (Kambhampati et al. 1991). Future use of recently identified polymorphic microsatellite loci (Porretta et al. 2006) in population

genetic studies may help to further elucidate the structure and relationships among invasive *Ae. albopictus* populations.

Eradication and Control

In 1947 the countries comprising the Pan American Health Organization (PAHO) proposed a resolution to eradicate *Ae. aegypti*, primarily to control yellow fever. At the time of the resolution *Ae. aegypti* was known from all of the western hemisphere except Canada and Bolivia. Between 1958 and 1965 eradication was accomplished in 17 of 23 targeted countries, primarily through perifocal application of DDT insecticide to infested containers (Schliessmann and Calheiros 1974). However, countries and territories in the Caribbean had a more difficult time achieving eradication and, by the 1970s with depleting financial resources, and subsequent social upheaval in countries such as El Salvador, re-infestations became widespread.

Over the past 50 years, *Aedes* control methods, often for dengue control, have not greatly changed. Larval source reduction, remains the primary method of *Ae. aegypti* and *Ae. albopictus* control and is accomplished through the removal of disposable containers, or the treatment of water storage containers with one of three common larvicides: (1) temephos (an organophosphate), (2) methoprene (insect growth regulator), or (3) BTI (*Bacillus thuringiensis* var.*israelensis*). For adult control, which becomes a focus when an outbreak of *Aedes*-vectored disease is already underway, ultra-low volume aerosols of insecticide, such as malathion, are applied with either truck mounted units or airplanes (World Health Organization 1997)

Competition Between *Aedes Albopictus* and *Ae. Aegypti*

Aedes albopictus and *Ae. aegypti* currently have sympatric distributions in many parts of the world, and the co-occurrence of larvae and pupae of both species within the

same container is common (MacDonald 1956, Fontenille and Rodhain 1989, O'Meara et al. 1992, Braks et al. 2003). In larval competition experiments on North American and Brazilian populations of the two species, *Ae. albopictus* exhibited a competitive advantage over *Ae. aegypti* under field conditions (Juliano 1998, Braks et al. 2004). These results support a role for interspecific competition in the observed decline or local extinction of *Ae. aegypti* in a large portion of the United States now inhabited by *Ae. albopictus* (O'Meara et al 1995). Nevertheless, there are regions in Asia, North and South America, and Africa where these two *Aedes* species successfully coexist and much effort has gone into elucidating the mechanisms behind their sustained co-occurrence (Sota and Mogi 1992, Juliano et al. 2002, Costanzo et al. 2005, Lounibos et al. 2002, Leisnham and Juliano 2009, Leisnham et al. 2009).

A probable process at work is condition-specific competition, where seasonal and spatial variation in environmental conditions cause a change in which competitor is favored (Costanzo et al. 2005, Leisnham and Juliano 2009). Although *Ae. albopictus* is a better larval competitor, superior desiccation resistance in the egg stage by *Ae. aegypti* allows greater numbers of this mosquito to survive during the dry season (Sota and Mogi 1992, Juliano et al. 2002). Therefore, early in the wet season as eggs that persisted during the drying period hatch, *Ae. albopictus* populations are at a low due to greater egg mortality (Leisnham and Juliano 2009). Condition-specific competition can also explain the spatial partitioning of the two mosquitoes in the environment, the distribution of *Ae. aegypti* populations is associated with lower humidity and higher temperatures common in more urbanized settings. In contrast, *Ae. albopictus*, is negatively associated with hot, dry climate and is more common in suburban, rural, and

forest edge areas where vegetation is more abundant and there is an excess of humid and shaded resting and oviposition sites (Hawley 1988, Sota and Mogi 1992, Juliano et al. 2002, Braks et al. 2003, Rey et al. 2006, Reiskind and Lounibos 2009).

Chikungunya Virus

In parts of their geographic ranges both *Ae. aegypti* and *Ae. albopictus* are important epidemic vectors of CHIKV, a single stranded enveloped, positive sense RNA virus. In its native African range, CHIKV is a zoonosis, with a transmission cycle involving wild primates and sylvatic *Aedes* species. However, in the invasive range of CHIKV, the viral transmission cycle is urban or suburban, and the primary vectors are *Ae. aegypti* and *Ae. albopictus* with humans as the host. Since the first isolation and identification of the virus in Africa in the 1950s, CHIKV has spread into new geographic areas with human epidemics documented on multiple continents.

Discovery of Virus and Vectors

Chikungunya is an *alphavirus* in the Family Togaviridae and, based on serological cross-reactivity, CHIKV is grouped more specifically into the Semliki Forest virus (SFV) antigenic serocomplex (Powers and Logue 2007). The prototype virus was isolated by Ross during the 1953 dengue epidemic in the Newala distict of Tanzania (formerly Tanganyika) from the blood of a febrile patient (Ross 1956). The name chikungunya is derived from the Makonde word meaning “that which bends up” or “walking bent over” in reference to the stooped posture developed as a result of the incapacitating arthralgia that can last for months (Sourisseau et al. 2007). Although CHIKV is rarely fatal, symptoms of the disease include high fevers, rashes, headache, photophobia, vomiting, and excruciating joint and muscle pain. Clinical symptoms follow an incubation period of

2 to 7 days, and acute illness is short in duration, lasting 3 to 5 days with recovery in 5 to 7 days (Ligon 2006, Robinson 1955).

The vectors of the virus are *Aedes* mosquitoes in the subgenera *Diceromyia*, *Stegomyia* and *Aedimorphus*. Sylvan transmission cycles have been documented in tropical Africa in moist forest and semiarid savannah–woodland involving sylvatic *Aedes* species, such as *Ae. africanus*, *Ae. furcifer*, *Ae. luteocephalus*, *Ae. neoafricanus* and *Ae. taylori* and wild primates, such as vervet monkeys (*Cercopithecus aethiop*s) and baboons (*Papio ursinus*) (Jupp and McIntosh 1988, Jupp and McIntosh 1990, Diallo et al. 1999). In monkeys and baboons infection is characterized by a short incubation period and a subsequent viremia lasting approximately five days. There is no mortality and resulting immunity is likely life-long (de Moor and Steffens 1970). The average life expectancy of *C. aethiop*s is between three and four years and it is possible that CHIKV sylvan epidemics follow a 3-4 year cycle that correspond with the renewal of non-immune wild primate populations (de Moor and Steffens 1970; Powers and Logue 2007). Studies in the Zika forest of Uganda have also provided data suggesting that a 5 to 7 year cycle in CHIKV activity may correspond with the replacement of non-susceptible red-tailed monkey (*C. ascanius schmidti*) with susceptible individuals (Macrae et al. 1971).

How the virus is maintained long-term in the wild is unknown. No field or laboratory data can confirm that the virus is maintained transovarially in mosquito eggs (Vazeille et al. 2009). Computer simulated epidemiological models generated by de Moor and Steffens (1970) suggest that CHIKV could exist endemically by continuous transmission in the vertebrate (*C. aethiop*s) population. Urban epidemic transmission of CHIKV is

sustained by the mosquitoes *Ae. aegypti* and/or *Ae. albopictus* in a human-mosquito-human cycle (Jupp and McIntosh 1988).

Viral Characterization and Phylogenetics

Chikungunya virus, like all known *alphaviruses*, is arthropod-borne with mosquitoes being the predominant vector. *Alphaviruses* are enveloped particles, and their genome consists of a single stranded, positive sense RNA molecule of ~12,000 nucleotides (nt). The 5 prime end is capped with a 7-methylguanosine while the 3 prime end is polyadenylated. The CHIKV genome is approximately 11.8 kb and is divided into two major regions: the first two-thirds of the genome which forms the 5 prime end encodes the four non-structural proteins (nsP 1-4) and a structural domain encoding the three structural proteins of the virus (capsid (C), E2 and E1). The non-structural proteins are translated directly from the genomic RNA into a polyprotein that through proteolytic cleavage produces nsP1, nsP2, nsP3, and nsP4 in addition to important cleavage intermediates (Khan et al. 2002). The structural proteins are translated through a subgenomic mRNA intermediate called the 26S RNA into a single polyprotein that is also cleaved to produce the three structural proteins as well as two small polypeptides E3 and 6K (Strauss and Strauss 1994). Thus the genome of CHIKV is 5 prime cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6K-E1-poly(A) 3 prime.

CHIKV appears to have originated in Central/West Africa and spread to other parts of the world based on chronology of outbreaks, their infrequency and high morbidity in Asia (Carey 1971), the absence of a vertebrate reservoir and a sylvan transmission cycle outside of tropical Africa (Jupp and McIntosh 1988), and phylogenetic data (Powers et al. 2000). Phylogenetic analysis based on E1 sequences groups CHIKV into

three distict genotypes: (1) Asian, (2) East/Central/South African, and (3) West African (Parola et al. 2006; Schuffenecker et al. 2006).

Historical and Current Epidemics

Since its initial discovery in 1953, epidemics of CHIKV have occurred on multiple continents. In the 1960s and 1970s, outbreaks were recorded in Thailand, India, Vietnam, Cambodia, Myanmar and Sri Lanka (Jupp and McIntosh 1988; Rao 1971). In the 1962 to1964 outbreak in Bangkok, Thailand, seroprevalence rates were between 70-85% in 20 to 70 year olds (Halstead et al. 1969). Outbreaks from 1963 to 1973 in India sickened hundreds of thousands of people (Rao 1971). From the late 1950s through the 1990s CHIKV was isolated from multiple countries in central and southern Africa as well as from West Africa (Powers et al. 2000). More recent outbreaks include the 1999 to 2000 epidemic in the Democratic Republic of Congo (Pastorino et al. 2004) and the 2001-2003 outbreak in Indonesia and Malaysia (Kit 2002; Laras et al. 2005).

A large-scale epidemic of CHIKV began in 2004 on Lamu Island, Kenya and then spread to Mombasa (Chretien et al. 2007; Sergon et al. 2008). In 2005 and 2006 CHIKV moved onto the Indian Ocean island nations starting with the Comoros, then Reunion, and on to the Seychelles, Mauritius, and Madagascar. Extrapolation from seroprevalence data suggests that on Grande Comore island (population 341,000) nearly 215,000 people (63% of the population) may have been infected during the outbreak (Sergon et al. 2007) and on the island of Reunion (population 770,000) alone approximately 241,000 clinical cases (31% of the population) were reported (Paquet et al. 2006). As the epidemic continued, 1.39 million suspected cases were reported in India in 2006 and tens of thousands of additional suspected cases were identified in 2007 (Arankalle et al. 2007; NVBDCP 2007). Local transmission was reported in two

small towns in the Italian province of Ravenna in the summer of 2007 resulting in 200 human cases (Rezza et al. 2007; Watson 2007). The epidemic continues with additional cases reported throughout South East Asia, India, and Sri Lanka where cases were reported through 2009 and into 2010 (International Society for Infectious Diseases 2009-2010). Furthermore, multiple cases have been imported into other areas of Europe, the United States, Canada and many other countries through the movement of infected travelers (Lanciotti et al. 2007, International Society for Infectious Diseases 2009-2010)

Isolates from the current outbreaks of CHIKV are most closely related to strains in the East/Central/South African genotype (Parola et al. 2006, Schuffenecker et al. 2006, Arankalle et al. 2007, Njenga et al. 2008). Since the outbreak, full genome sequences have been published for multiple isolates from Kenya, Indian Ocean islands, India, and South East Asia and are available in GenBank.

CHIKV Interactions in Epidemic Vectors: *Aedes aegypti* and *Ae. albopictus*

In past CHIKV outbreaks, *Ae. aegypti* has been implicated as the dominant vector, with virtually all Asian mosquito isolates coming from this species (Powers and Logue 2007). Early on in the recent outbreaks (2004-2005), both in Kenya and on the Comoros islands, *Ae. aegypti* was the vector responsible for CHIKV transmission (Sang et al. 2008, Njenga et al. 2008). However, as the epidemic moved onto Reunion Island *Ae. albopictus* was the only vector present (Delatte et al. 2008). *Aedes albopictus* was also the sole vector on the Lakshadweep islands in the Indian Ocean (Samuel et al. 2009), and in Ravenna, Italy (Rezza et al. 2007), which all experienced CHIKV outbreaks between 2005 and 2008. Similar patterns are documented in DENV epidemics, in that in

the absence of *Ae. aegypti*, *Ae. albopictus* acts as the primary vector of DENV (Ali et al. 2003; Effler et al. 2005; Xu et al. 2007). There were also some outbreak localities where although *Ae. aegypti* is common, *Ae. albopictus* was still the primary vector of CHIK as was the case in the West African country of Gabon (Pages et al. 2009).

In other recent CHIKV outbreak regions where *Ae. aegypti* and *Ae. albopictus* distributions overlap there is evidence that both mosquitoes are transmitting the virus. In Singapore, in 2008, larval surveys identified *Ae. albopictus* as the predominant species in certain locations, with *Ae. aegypti* also present and adult mosquito surveillance yielded both *Ae. albopictus* and *Ae. aegypti* adult females positive for CHIKV (Ng et al. 2009). Similar patterns were found in Thailand, where wild-caught adults of both species were positive for CHIKV (Thavara et al. 2009). Entomological surveys done during the 2006 CHIKV outbreak in the southern Indian state of Kerala found high densities of *Ae. albopictus*, and in 2007 in some of the worst CHIKV-affected districts (Alappuzha, Kottayam and Pathanamthitta) of Kerala state, *Ae. albopictus* constituted 85-92% of the total mosquito juveniles collected and in a follow-up survey this mosquito made up 58-76% of the totals (Kumar et al. 2008). In the north eastern Indian state of Orissa, entomological surveys revealed the presence of both *Aedes* species with *Ae. albopictus* having a slightly higher density than *Ae. aegypti* (Dwibedi et al. 2009).

The increased global presence of *Ae. albopictus,* along with a largely non-immune population, likely fueled the magnitude and speed of the CHIKV outbreaks. However, there is evidence that the acquisition of a mutation in the viral gene coding the E1 envelope protein, during the outbreak may have also played a role in acceleration of the spread of CHIKV (Schuffenecker et al. 2006). In the laboratory this mutation, which

results in a substitution of valine for alanine at the 226 amino acid position (A226V) of E1, significantly increases the susceptibility of *Ae. albopictus* to the virus. The mechanism causing increased viral fitness in *Ae. albopictus* is unknown, but there may be some association with cholesterol dependence (Tsetsarkin et al. 2007). *Aedes albopictus* became infected with, disseminated, and transmitted a Reunion island isolate with the E1-A226V isolate at much higher rates at all blood meal titers, while there was no change in susceptibility in *Ae. aegypti* when compared with a back mutated isolate with alanine at the E1-226 position (Tsetsarkin et al. 2007). Tsetsarkin et al. (2007) then took a historic West African CHIKV isolate, mutated it to contain the same valine E1-226 substitution, and found when compared to the original West African isolate there was increased susceptibility in *Ae. albopictus*, but not *Ae. aegypti*.

Previous laboratory studies with East African and Thai isolates of CHIKV had already indicated that in the laboratory *Ae. albopictus* was a significantly more competent vector than *Ae. aegypti* (Mangiafico 1971; Turell et al. 1992). Jupp and McIntosh (1988) suggest that since historic human viremias do not circulate much above 7.0 $\log_{10}TCID_{50}$/ml, some populations of *Ae. aegypti* would be inefficient vectors in human-to –human transmission. The superior laboratory competence of *Ae. albopictus* over *Ae. aegypti* is also evident in the Tsetsarkin et al. (2007) study with the emergent E1-A226V CHIKV isolate. This pattern was again confirmed in another laboratory study in which first generation *Ae. albopictus* and *Ae. albopictus* from South Florida were given one of four blood meals each with a 10-fold reduced titer of the emergent E1-A226V CHIKV. *Aedes aegypti* showed an overall susceptibility

significantly lower than *Ae. albopictus,* and only *Ae. albopictus* individuals were infected at the two lowest viral doses (Pesko et al. 2009).

Early on in the epidemic in 2004 and 2005 the A226V mutation was absent from Kenyan and Comoros isolates, but the mutation then appeared in CHIKV isolates from Reunion island in 2005 and 2006 (Njenga et al. 2008, Gould and Higgs 2009). Isolates from the 2006 epidemic in India did not have the mutation, but isolates sequenced from 2007 Indian outbreaks did have the A226V (Yergolkar et al. 2006, Arankalle et al. 2007, Santhosh et al. 2008). Chikungunya viral samples from the 2007 Ravenna, Italy outbreak did have the A226V mutation where, as mentioned earlier, *Ae. albopictus* was the sole vector, and the virus was introduced to Italy by an Indian national visiting Ravenna (Rezza et al. 2007). In Singapore, CHIKV strains from the 2008 epidemic were mixed, isolates from *Ae. aegypti* abundant areas did not have the A226V mutation, but the mutation was present in virus identified from *Ae. albopictus* adults or in human blood samples from *Ae. albopictus* dominated neighborhoods. According to Gould and Higgs (2009), during the 2004-2009 epidemics the only mosquitoes that tested positive for CHIKV with the E1-A226V mutation were *Ae. albopictus,* and all positive *Ae. albopictus* tested were infected with this mutant. How the continuing geographic spread of *Ae. albopictus* combined with the E1-A226V mutation in CHIKV contributed to the magnitude of the epidemic of the past five years is currently unknown.

Vector Competence

After taking a viremic blood meal a mosquito will only be capable of orally transmitting the virus after a series of barriers to infection are overcome (Hardy et al. 1983). Assuming there is ingestion of a sufficient quantity of virions to exceed the infectious dose threshold (Chamberlain and Sudia 1961), the virions that enter the

midget lumen generally bind to the membrane of the midgut epithelial cells, enter the cell cytoplasm and replicate. Infectious virions must then disseminate from the midgut epithelial cells to the haemocoel and infect other tissues. Finally, for transmission to occur, virions must infect and replicate in salivary gland tissue and then be secreted in saliva during feeding on a subsequent host. The relationship between mosquito species and virus is often specific and the presence of one or more of the barriers in the described processes results in a mosquito that is an incompetent vector for a given virus.

However, even when a mosquito is determined to be a 'competent vector', both intrinsic and extrinsic factors can affect the susceptibility of a vector for a pathogen (Hardy et al. 1983). A vector's susceptibility to and ability to transmit an arbovirus can vary both inter- and intra-specifically, as is the case with *Ae. albopictus* and *Ae. aegypti* susceptibility for certain strains of CHIKV (Turell et al. 1992). Intrinsic (genetic) factors of the mosquito that influence vector competence include: behavior, physiology, and metabolism and thus, vector competence is thought to be a complex phenotypic trait under the control of multiple genetic loci (Bosio et al. 1998).

The Environment and Vector Competence

Extrinsic (environmental) factors can also exert a strong influence on mosquito vector competence, with temperature studies accounting for the majority of research on the topic. Within limits, increased temperature during the adult life stage has a positive influence on vector competence. In studies with West Nile Virus (WNV) (Dohm et al. 2002, Reisen et al. 2006, Richards et al. 2007), Western equine encephalomyelitis (Reisen et al. 1993) and multiple other viruses, temperature is shown to increase the number of individuals infected with or transmitting the virus. In addition, temperature has

an inverse relationship with an arthropod vector's EIP (Chamberlain and Sudia 1955), shortening the time between infection and the subsequent ability to transmit the virus.

The Larval Environment, Vector Competence, and Mosquito Size

Arboviral diseases are ecologically complex, and interactions between larval mosquitoes and their aquatic environment can influence adult transmission dynamics. Biotic and abiotic variables such as temperature, larval nutrition, and inter- and intra-specific competition can alter the life history and the intrinsic physiology of mosquitoes in ways that affect the ease with which individuals become infected and transmit a virus.

Previous studies have shown that larval rearing temperature can affect mosquito competence for several arboviruses, including *Ae. taeniorhynchus* for Rift Valley fever (RVFV) and Venezuelan equine encephalitis (VEEV) (Turell 1993), *Culex annulirostris* for Murray Valley encephalitis (MVEV) (Kay et al. 1989a), *Cx. tritaeniorhynchus* for Japanese encephalitis (JEV) (Takahashi 1976), and *Cx. tarsalis* for western equine encephalitis (WEEV) (Hardy et al. 1990) viruses. Unfortunately, none of these studies reared mosquitoes individually to separate temperature and density effects. In contrast, no rearing temperature effect was found in *Cx. tritaeniorhynchus* for WNV (Baqar et al. 1980) and *Aedes vigilax* for Ross River virus (RRV) (Kay and Jennings 2002). With the exception of *Cx. tritaeniorhynchus* for JEV (Takahashi 1976), adult females showed reduced vector competence with increased rearing temperature.

Larval nutrition can also influence the ability of adult mosquitoes to become infected with and transmit virus. *Cx. tritaeniorhynchus* and *Ae. triseriatus* nutrient-deprived larvae were more susceptible than their well-fed counterparts, for WNV (Baqar et al. 1980) and better transmitters of La Crosse virus (LACV) to suckling mice (Grimstad and Haramis 1984; Grimstad and Walker 1991). However, in other studies,

nutritional deprivation of larvae had no effect on vector competence of *Cx. annulirostris* for MVEV (Kay et al. 1989b) or *Ae. vigilax* for RRV (Jennings and Kay 1999). When an effect was detected, low nutritional resources produced adult mosquitoes more susceptible to virus.

Aedes triseriatus adults produced in low intra- and interspecific competition treatments with *Ae. albopictus* had higher infection and dissemination rates for LACV (Bevins 2008). The opposite was found with *Ae. albopictus* reared in highly competitive larval environments, wherein adults were smaller and had higher rates of infection and dissemination for Sindbis virus and DENV, while within the same studies a competitive larval environment showed the same trends but did not have a significant effect on vector competence in *Ae. aegypti* for the two viruses (Alto et al. 2005; Alto et al. 2008a).

In a number of the studies investigating the influence of larval environmental factors on vector susceptibility to arboviruses, mosquito body size is measured in addition to infection outcomes. Mosquito body size is an easily measurable physical manifestation of larval habitat quality, and larvae reared at low temperatures are generally larger as adults, while juveniles reared with low food availability and/or in a competitive environment will be smaller as adults. In a few studies larval factors were varied just as a means to produce mosquitoes of different size classes. Sumanochitrapon et al. (1998) produced three size classes of adult *Ae. aegypti,* by varying both the quantity of food and density of larvae, and found that large *Ae. aegypti* females showed higher rates of oral infection with dengue virus (DENV) compared to small and medium mosquitoes. Similar findings were reported when two size classes of *Ae. aegypti* were generated through variation in larval diet, and larger individuals were

more susceptible to RRV (Nasci and Mitchell 1994). In contrast, smaller *Ae. triseriatus* adults generated from field collected pupae were more likely to transmit LACV to suckling mice (Paulson and Hawley 1991), and when Alto et al. (2008b) carried out additional analyses in which size was examined independent of rearing conditions, small *Ae. aegypti* females were more susceptible to DENV; however it should be noted that the size range of individuals measured in this study was extremely narrow.

However, body size may not be a good predictor of how an individual mosquito may respond to viral challenge in the form of an infectious blood meal. There may be more critical, but not as easily measurable physiological and physical features of adult mosquitoes that vary with larval conditions and are more correlated than size with viral susceptibility. Furthermore, the type of larval condition that is being manipulated may influence the strength and even possibly the direction of the response. For example, variations in larval temperature or food level may produce mosquitoes of a similar size range, but their responses to infection may be very different.

CHAPTER 2
LARVAL ENVIRONMENTAL TEMPERATURE AND THE SUSCEPTIBILITY OF *Aedes albopictus* SKUSE (DIPTERA: CULICIDAE) TO CHIKUNGUNYA VIRUS

Introduction

Climate is one of the principal determinants of the distribution of vector-borne diseases (van Lieshout et al. 2004). In particular, both vector development and survival and arbovirus replication are greatly influenced by temperature. In mosquito vectors, temperature can influence larval development time, larval and adult survival, biting rates, gonotrophic cycle lengths, and vector size (Madder et al. 1983, Rueda et al. 1990, Scott et al. 1993). Ambient temperature can affect arboviral dynamics within the mosquito vector by altering the length of the extrinsic incubation period (EIP), which is the time between ingestion of an infectious blood meal to when transmission to a subsequent host is possible (Chamberlain and Sudia 1955, Reeves et al. 1994, Patz et al. 1996). Furthermore, temperature often defines the latitudinal and altitudinal ranges of a vector. Species range may limit the distribution of disease when pathogens are species specific. Temperature may also limit viral transmission in areas where the vector is present but the temperature precludes efficient transmission (Purse et al. 2005).

Vector competence, which is the capacity of an arthropod to acquire an infection and transmit it to a subsequent host, can greatly vary among individuals and between populations (Lorenz et al. 1984, Turell et al. 1992) and is influenced by genetics (Mercado-Curiel et al. 2008) as well as by climate variables, such as temperature (Davis 1932, Turell 1993, Dohm et al. 2002). An increase in environmental temperature for adult mosquitoes reduces the EIP (Davis 1932, Chamberlain and Sudia 1955), most

likely due to an increase in the metabolism of the adult mosquito and the replication speed of the virus.

Although the majority of research on mosquito-virus interactions has focused on adult mosquitoes, temperature changes experienced in the immature stages of holometabolous vectors prior to infection, may affect vector-virus interactions by changing physical and physiological characteristics of midgut and salivary gland barriers, which could have direct consequences on viral infection, replication, and transmission. Previous studies have shown that larval rearing temperature can affect mosquito competence for several arboviruses, including Murray Valley encephalitis (MVEV) (Kay et al. 1989a), Japanese encephalitis (Takahashi 1976), and western equine encephalitis (Hardy et al. 1990) viruses. In a specific study with *Aedes taeniorhynchus*, mosquitoes reared at 19°C had higher infection rates for Rift Valley fever and Venezuelan equine encephalitis viruses than counterparts reared at 26°C (Turell 1993). In view of global climate change models, which predict changes in temperature that will directly impact larval mosquito habitats, this study, which investigates a previously unexplored relationship between *Aedes albopictus* larval environmental temperature and chikungunya virus (CHIKV) susceptibility, could help increase the predictability of disease transmission patterns and future outbreaks.

The intercontinental dispersal of invasive arbovirus vectors, such as the Asian tiger mosquito *Ae. albopictus*, is accompanied by an increase in human vulnerability to the exotic diseases vectored by these invaders (Juliano and Lounibos 2005). In 2005-2006 CHIKV, a single stranded positive sense RNA enveloped *Alphavirus* in the family Togaviridae, emerged as an important pathogen in the Indian Ocean Basin. On the

island of Reunion alone, 241,000 clinical cases of chikungunya fever, representing 31% of the population, were reported (Paquet et al. 2006). A sylvan transmission cycle of CHIKV involving mosquitoes, such as *Ae. furcifer* and *Ae. luteocephalus*, and wild primates is limited to tropical Africa, and epidemic transmission of the virus is sustained though infection of the mosquitoes *Ae. aegypti* and *Ae. albopictus* in urban and peridomestic environments (Jupp and McIntosh 1990, Diallo et al. 1999).

An unusual feature of the South West Indian Ocean CHIKV outbreak was the increased importance of *Ae. albopictus* as a vector. The enhanced role of *Ae. albopictus* as a CHIKV vector on Reunion island was in part due to the rarity of the primary vector *Ae. aegypti* (Delatte et al. 2008), however, an amino acid substitution in the Reunion CHIKV isolates, from alanine to valine at the 226 position of the E1 envelope structural protein, has been shown to increase *Ae. albopictus*, but not *Ae. aegypti*, susceptibility to the virus in the laboratory (Tsetsarkin et al. 2007). Following the Indian Ocean Basin epidemic, a strain of CHIKV nearly identical (99.9% nucleotide identity) to isolates from La Reunión emerged in India where 1.3 million human cases were reported in 13 states in 2005-2006 (Arankalle et al. 2007). This widespread epidemic was not restricted to the tropics, with autochthonous transmission reported in northern Italy in 2007 (Rezza et al. 2007). The epidemic continued to spread to Indonesia, Sri Lanka, and Singapore where cases were reported through 2008 and into 2009 (Seneviratne et al. 2007, International Society for Infectious Diseases 2008-2009).

In this study we explore how variation in temperature during larval development affects the susceptibility of *Ae. albopictus* for CHIKV by measuring infection and dissemination rates, and viral titer. We also assess how larval temperature affects

growth and survivorship by measuring larval mortality, development time to adulthood, and adult body size.

Materials and Methods

Mosquitoes and Viruses

Aedes albopictus used in this experiment were generated from field collections of approximately 4000 larvae and/or eggs made from June to August 2007 in Palm Beach County, Florida. This population of *Ae. albopictus* was previously shown to be highly susceptible to the Reunion (LR2006-OPY1) CHIKV strain (Reiskind et al. 2008). Females reared from field-collected immatures were given 20% sucrose *ad libitum*, blood fed weekly on live chickens, and kept in cages at 14 L:10 D, 26°C (+/- 1˚C SD) and >80% rh. Chicken care followed federally mandated animal use and care policies (University of Florida, IACUC Protocol VB-17). Eggs (F_1) were hatched in tap water and, within 24 hours after hatching, individual larvae were placed in 50 ml Falcon® conical tubes with 35 ml of tap water and 0.0105g 1:1 yeast:albumin food. Based on preliminary studies, 0.0105g of food at the beginning of the experiment was adequate for the completion of individual development but did not increase mortality at the higher temperature. Larvae were reared at 18°C, 24°C and 32°C with a 14L:10D cycle in a Percival (Percival Corporation, Perry, IA) incubator. The experimental treatment units in this study were different incubators which were identical in all respects except for rearing temperature and, thus, it was assumed that differences in treatments were caused by rearing temperature. These temperatures are within the range encountered in the treehole environment occupied by *Ae. albopictus* in Florida (Lounibos 1983). Larvae in each temperature treatment were from the same cohort of eggs whose hatch was staggered to synchronize adult emergence among all three temperature

treatments. After the final larval instar, pupae were removed from rearing tubes, sexed and stored in groups of 10 in water-filled vials to record adult emergences. After emergence, all adults were held at 24°C, 95-99% rh with a 14L:10D cycle in a biosafety level-3 facility and given 20% sucrose *ad libitum*.

The LR2006-OPY1 CHIKV strain, (GenBank accession number DQ443544) was isolated in France from a febrile patient who had been infected on the island of Reunion (Parola et al. 2006). This recently emergent strain of CHIKV contains the alanine to valine substitution at the 226 position of the E1 envelope structural protein that has been identified as a feature in many current CHIKV epidemics (Rezza et al. 2007) and has been shown to increase *Ae. albopictus* susceptibility (Tsetsarkin et al. 2007). To produce virus for infectious blood meals, a T-75 cm^2 flask with a confluent monolayer of Vero cells in 10 mL of cell culture media (M199 media supplemented with fetal bovine serum, antibiotics and antimycotics (Invitrogen®, Carlsbad, CA) was inoculated with 150 µL of previously frozen stock virus, and allowed to incubate in a 5% CO_2 and 35°C atmosphere for 24 hours.

Vector Competence

Groups of 50 five to seven day-old mosquitoes were placed in 1-L cylindrical, waxed cardboard containers (Dade Paper Co., Miami, FL) with mesh screening. Mosquitoes were starved for 24 hours prior to being offered an infectious blood meal of 1:1000 dilution of freshly propagated CHIKV in citrated bovine blood (Hemostat Laboratories, Dixon, CA) supplemented with ATP [5mM] as a phagostimulant. Water-jacketed glass membrane feeders (Rutledge et al. 1964) covered with Edicoll® collagen film (Devro, Sandy Run, SC) and connected to a Haake Series F water circulator (Thermo Haake, Paramus, NJ) were used to maintain the blood meal at 37°C.

Mosquitoes were given 30 minutes to feed. Low feeding success of *Ae. albopictus* made it necessary to conduct three consecutive days of feeding for individuals from all three temperature treatments. The blood meals from the three consecutive feeding days were assayed using qRT-PCR, with copy number standardized to plaque forming unit (pfu) by plaque–assay performed on 10-fold serial dilutions of virus stock (Bustin 2000). Virus titers in blood meals were $\log_{10}$ 4.7, 4.5, and 3.4 pfu/mL for the three feeding days, respectively. After feeding, mosquitoes were cold anesthetized, and fully engorged mosquitoes were removed, placed in new cages, and given 20% sucrose *ad libitum*.

After a 10 day EIP at 24°C, surviving *Ae. albopictus* females were killed by freezing. Females were stored at -80°C and, after thawing, wings were removed for measurements, bodies were assayed to determine infection status and titer, and legs were tested to check for a disseminated infection (Turell et al. 1984). Wing length was measured as an indicator of body size (Blackmore and Lord 2000), in millimeters, from alula to wing tip, using digital images and a computer imaging and measurement program (i-Solution lite®, AIC Inc., Princeton, NJ).

Bodies and legs were triturated separately in 2 mL microcentrifuge tubes containing 900µL of BA-1 media (Lanciotti et al. 2000) and two zinc-plated beads. Samples were homogenized at 25 Hz for 3 min using a Tissuelyzer® tissue homogenizer (Qiagen Inc., Valencia, CA) and then clarified by centrifugation (3,000 x g for 4 min). Viral RNA was extracted from 250 µL of the sample with Trizol-LS (Invitrogen®, Carlsbad, CA) following the manufacturer's instructions and using a final elution volume of 50 µl in DEPC treated water.

One-step qRT-PCR was used to determine infection and dissemination status and body titer by previously established protocols (Reiskind et al. 2008). Primers from the CHIKV E1 gene were designed with the following sequence: forward: 5'-ACC CGG TAA GAG CGA TGA ACT-3'; reverse: 5'-AGG CCG CAT CCG GTA TGT-3'. The probe sequence was: 5'-/5Cy5/CCG TAG GGA ACA TGC CCA TCT CCA /3BHQ_2/-3' (IDT DNA, Coralville, IA).

Statistical Analysis

Kruskal-Wallis tests followed by Dunn's pairwise multiple comparisons (family $\alpha = 0.05$) were used to determine differences among treatments in distributions of wing lengths and development times to adulthood (SAS 9.1, SAS Institute, Inc., Cary, NC). Males and females were analyzed separately because of gender-specific sizes and developmental times in this species. Chi-squared tests of independence were used to determine the effect of temperature on survivorship to adulthood. If significant effects were detected, post-hoc pairwise comparisons were made with an alpha adjusted (Bonferroni) correction to account for multiple comparisons (Minitab 15, Minitab Inc., State College, PA).

A generalized linear mixed model (PROC GLMMIX) was used to describe relationships among temperature (fixed effect), feeding day (random effect), infection rate (# with virus/# fed), dissemination rate (# with virus in their legs/# with virus), and population dissemination rate (# with virus in their legs/# fed) specifying a logistic link function and a binomial error distribution. Odds ratios (OR) and 95 % confidence intervals for infection at each temperature treatment were also calculated (SAS 9.1). A linear mixed model (PROC MIXED) was used to test for effects of temperature treatments (fixed) and feeding day (random), which were both class variables on body

titer, a continuous variable. Titer data did not fit the model assumptions of normality, but approximate normality was achieved through a log transformation of titer values (SAS 9.1). Wing length comparisons, pooled across all temperature treatments, between binomial variables: infected versus uninfected and disseminated versus non-disseminated were analyzed with t-tests (Minitab 15).

Results

Growth and Mortality

Wing lengths and development times to adulthood were significantly affected by temperature for both females (wing length: H= 417.5; df = 2; P<0.0001, development time: H= 1279.6; df = 2; P <.0001) and males (wing length: H= 2127.4; df = 2; P <0.0001 and development time: H= 1697.3; df = 2; P <0.0001) (Table 2-1). There was an inverse relationship between wing length and temperature with the largest adult mosquitoes produced at 18°C and the smallest mosquitoes produced at 32°C. Mosquitoes reared at the lowest temperature (18°C) took over two times longer to develop than those at 24°C and 32°C. Juvenile mortality rates at 18°C, 24°C, and 32°C were 9.16% (n = 1454), 7.34% (n = 1520), and 16.90% (n = 1473), respectively. There was a significant relationship between survivorship to adulthood and temperature (χ^2 = 54.123, df = 2, P<0.0001). *Aedes albopictus* reared at 32°C were significantly more likely to die as larvae when compared with individuals reared at 18°C and 24°C, with no differences between the two lower temperatures.

Chikungunya Infection and Dissemination

There was a significant temperature treatment effect on the percentage of females that developed CHIKV infections (F=16.92, df = 2, P<0.0001) (Figure 2-1). Infection was 6 times more likely in adult females reared at 18°C than at 32°C (Odds Ratio (OR) =

6.052; 95% CI 3.22-11.373), and females reared at 24°C were 2.7 times more likely to be infected than those reared at 32°C (OR = 2.722; 95% CI 1.385-5.351). Females reared at 18°C were 2.2 times more likely to be infected than those reared at 24°C (OR = 2.223; 95% CI 1.328-3.722). Among the infected individuals the proportion of females that developed disseminated infections did not vary significantly among larval rearing temperatures ($F = 0.85$, $df = 2$, $P > 0.4293$), however there was a significant temperature effect on population dissemination rate ($F=6.20$, $df = 2$, $P<0.0022$) (Figure 2-1). Population dissemination rate was approximately 5 times higher in adult females reared at 18°C than at 32°C (OR = 4.905; 95% CI 1.814-13.271) and 2.3 times higher in females reared at 18°C than at 24°C (OR = 2.291; 95% CI 1.088-4.827). There was no significant difference in population dissemination rates between the 24°C and 32°C treatments.

After the 10-day EIP, no significant variation in body titer of virus positive mosquitoes was observed among the temperature treatment groups ($F = 1.14$, $df = 2$, $P>0.4300$). *Aedes albopictus* females that were positive for CHIKV infection, in all three temperature treatments were significantly larger than uninfected females ($t = -3.59$, $df = 327$, $P = 0.0004$) as measured by mean wing length. There was no significant difference in mean wing lengths between females with disseminated and non-disseminated infections ($t = -0.41$, $df = 94$, $P = 0.6830$).

Discussion

Due to the impact of climate on vector ecology, mosquito-borne diseases will be sensitive to projected changes in global temperatures. In this laboratory study we demonstrated that larval rearing temperature can influence survival, development time, and wing length, and may directly impact disease transmission by influencing the

likelihood of infection with CHIKV. Although the proportion of infected females that developed disseminated infections did not differ significantly among the three larval rearing temperatures, the population dissemination rates were significantly higher at 18°C, when compared to the two higher temperatures of 24°C and 32°C. Disseminated infection is generally accepted as a measure of a mosquito's ability to transmit a virus through biting (Turell et al. 1984). The rate of dissemination, when expressed as a percentage of the number of mosquitoes infected, may provide information about the effect of a "midgut escape barrier" moderating whether gut infections are able to disseminate into the hemolymph. In this study, individuals reared at 32°C had significantly lower infection rates, but no significant difference was found in dissemination rate. Thus, it can be speculated that there may be a reduced "midgut escape barrier" in mosquitoes derived from the higher rearing temperatures. On the other hand, the population dissemination rate, expressed as a percentage of the total number of mosquitoes tested, was greater for mosquitoes from 18°C, and is epidemiologically more important in that it gives an estimate of the vector competence or the transmission potential of a population. Body titers did not differ among temperature treatments for mosquitoes with disseminated infections. Although this result was somewhat surprising, it is possible that after the 10-day EIP virus titers stabilized to the extent that treatment effects on titer were diminished. Additionally, only a limited number of mosquitoes developed disseminated infections and, although the mean titer of disseminated mosquitoes from 18°C was higher, it was not significantly different from the other treatments.

The higher temperature of 32°C decreased survivorship, when compared with 18°C and 24°C. This was unexpected because results from preliminary experiments showed no difference in survivorship between the three temperature treatments. The increased mortality could have been due to the stress of high temperature or the interaction of high temperature and excess nutritional resources resulting in the proliferation of detrimental micro-organisms in the larval environment. Larvae took longer to develop at cooler temperatures which produced larger adults. Mean wing length differences between successive temperature treatments were approximately 0.2 mm, confirming for *Ae. albopictus* that adult body size, within limits, exhibits an inverse relationship with larval rearing temperature (Briegel and Timmermann 2001). Upper and lower thermal limits for the growth of *Ae. albopictus* larvae are approximately 11°C and 35°C, at which temperatures larval development is inhibited, eventually resulting in mortality (Hawley 1988, Monteiro et al. 2007).

Our data indicate that at lower larval rearing temperatures there is an increased likelihood of an adult female becoming infected with CHIKV virus. This may have had a positive effect on CHIKV infection rates in locations such as highlands of Reunion island, where entomological surveys recovered *Ae. albopictus* at elevations of 1200 meters and temperatures as low as 12.6°C (Delatte et al. 2008). However, 12.6°C is a lower larval environmental temperature than what was investigated in this study and therefore it is uncertain whether the relationship between reduced temperature and higher infection rates would hold true at this temperature. The ability of *Ae. albopictus* to tolerate low temperatures and adapt to diverse ecological environments combined with their vector competence for currently circulating CHIKV isolates may help to explain the

2007 northern Italy CHIKV outbreak and increases the potential for future epidemics in other temperate areas where *Ae. albopictus* is abundant.

This study focused only on the influence of larval temperature, and adults were maintained at a common temperature of 24°C. How combinations of different adult and larval temperatures may affect vector competence was not addressed. It is likely that adults maintained at the lower temperature will have decreased virogenesis and a longer EIP resulting in a decreased probability of transmission. Therefore, the maintenance of low adult temperatures may result in a reduction or elimination of any benefit low rearing temperature may have on increasing vector competence.

Results from this study are consistent with other systems where arboviral vector competence was reduced in female mosquitoes that were reared at higher compared to lower temperatures (Kay et al. 1989a, Hardy et al. 1990, Turell 1993). Unfortunately, none of the previous studies that explored larval temperature effects on adult arboviral susceptibility, reared mosquitoes individually to separate temperature and density effects, nor did they measure variables such as wing or body size and survivorship, as we have done in this study.

In our work, lower rearing temperature not only produced mosquitoes that were more susceptible to viral infection but were also significantly larger. Blood consumption by females is a function of size and large females are known to imbibe more than twice as much blood as smaller females (Briegel 1990). Thus, in smaller mosquitoes reared at the higher temperature, the imbibing of a lower blood volume would decrease the initial viral dose and, in combination with a low CHIKV titer in the blood meal, limit the establishment of infection. Low initial exposure to virus may not affect dissemination,

which requires post-infection virus replication. If this explains why large mosquitoes derived from cooler temperatures have higher infection rates than smaller mosquitoes then we would expect that titers of freshly engorged mosquitoes will increase with body size, which will be tested in future studies. We also predict that different sized mosquitoes derived from different temperatures and fed an infectious blood meal with a high virus titer would overcome a threshold infectious dose and may result in similar infection rates.

Because the variation in size was achieved through different temperature treatments it is difficult to separate the effect of temperature from the response variable body size. There may be other temperature dependent phenotypic traits that vary in a way so as to cause an increase in infection when adults are subjected to a lower temperature larval environment that we did not measure. Previous studies show a lack of consistency in relationships between vector size and pathogen transmission. Large adult *Ae. aegypti* females from low density larval conditions showed higher rates of oral infection with dengue virus (DENV) compared to two other size classes from higher density larval conditions (Sumanochitrapon et al. 1998), and similar findings were reported for *Ae. aegypti* and Ross River virus (Nasci and Mitchell 1994). In contrast, *Ae. albopictus* adults reared in competitive larval environments were smaller and had higher rates of infection and dissemination for Sindbis virus and DENV, while within the same studies a competitive larval environment did not have a significant effect on vector competence in *Ae. aegypti* for the two viruses (Alto et al. 2005; Alto et al. 2008a). In *Ae. aegypti*, when size was examined independent of rearing conditions small adults were more susceptible to DENV, however the size range of individuals measured was

extremely narrow (Alto et al. 2008b). In contrast, larger *Ae. triseriatus* adults produced through variation in competitive treatments had higher infection and dissemination rates for La Crosse virus (LACV) (Bevins 2008). In nutritional deprivation studies with *Cx. tritaeniorhynchus* and *Ae. triseriatus*, smaller mosquitoes derived from nutrient-deprived larvae were more susceptible than their well-fed, larger counterparts, for West Nile Virus (Baqar et al. 1980) and better transmitters of LACV to suckling mice (Grimstad and Haramis 1984; Grimstad and Walker 1991). Smaller *Ae. triseriatus* adults generated from field collected pupae were more likely to transmit LACV to suckling mice (Paulson and Hawley 1991). However, nutritional deprivation which led to small mosquitoes had no effect on vector competence in *Cx. annulirostris* for MVEV (Kay et al. 1989b) and *Ae. vigilax* for Ross River virus (Jennings and Kay 1999). These inconsistencies in the effect of size on vector competence could be based on the intrinsic differences between vector-viral systems, however it is also possible that larval conditions, such as high temperature and low nutrients or competition produce small mosquitoes by different mechanisms that differently effect their competence as vectors.

In summary, cooler rearing temperatures produced mosquitoes that were larger, had higher survival, and were more likely to become infected with CHIKV, emphasizing the importance of the mosquito larval environment in determining adult vector-virus interactions. Future studies should explore the connection between larval rearing temperature-infection patterns observed in the laboratory to patterns in the field and how climate and climate change may continue to impact the mosquito larval environment and the epidemiology of CHIKV.

Table 2-1. *Aedes albopictus* treatment medians and interquartile ranges (IQR= 25th percentile -75th percentile) for development time to adulthood and wing length. Medians with different letters in the same row are significantly different from one another.

	18ºC	24ºC	32ºC
Female time to adulthood (days)	24.5a	11.0b	9.0c
Interquartile range	23.0-26.0	10.0-11.5	8.5-9.5
(n)	(515)	(556)	(445)
Male time to adulthood (days)	22.5a	10.0b	8.0c
Interquartile range	21.5-24.0	9.5-10.5	7.5-8.5
(n)	(817)	(860)	(791)
Female wing length (mm)	3.40a	3.14b	2.91c
Interquartile range	3.29-3.51	3.00-3.26	2.80-3.01
(n)	(347)	(150)	(207)
Male wing length (mm)	2.83a	2.61b	2.44c
Interquartile range	2.77-2.90	2.55-2.66	2.37-2.51
(n)	(745)	(810)	(740)

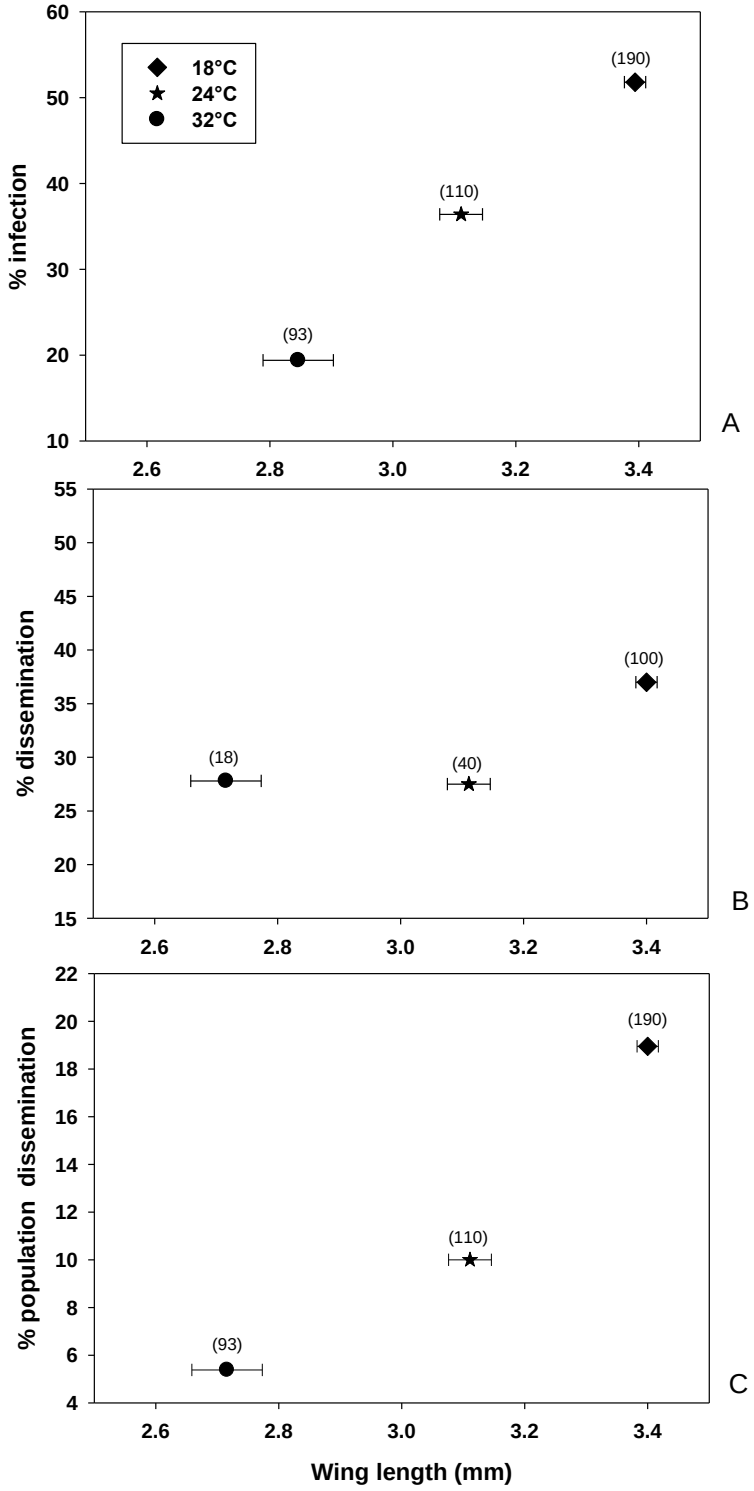

Figure 2-1. Bivariate plots of mean wing lengths (±SE) and CHIKV susceptibility A) percent infection, B) percent dissemination, and C) percent population dissemination grouped by treatment. Numbers in parentheses above symbols represent number of mosquitoes tested.

CHAPTER 3
LARVAL TEMPERATURE AND NUTRITION ALTER THE SUSCEPTIBILITY OF *Aedes aegypti* L. (DIPTERA: CULICIDAE) MOSQUITOES TO CHIKUNGUNYA VIRUS

Introduction

In recent years chikungunya virus (CHIKV) has emerged as an important agent of human arboviral epidemics sickening millions of people worldwide (National Vector Borne Disease Control Programme (NVBDCP) 2007, International Society for Infectious Disease 2005-2010). Chikungunya, a single stranded enveloped, positive sense RNA *alphavirus* (Family Togaviridae), was first isolated by Ross in 1953 from the blood of a febrile patient in Tanzania (Ross 1956) and, although endemic CHIKV was known to be rarely fatal, symptoms of the disease include high fevers, rashes and severe and debilitating arthralgia (Robinson 1955, Ligon 2006). In its native African range, CHIKV is a zoonosis, with wild primates serving as hosts and sylvatic *Aedes* spp. as vectors. However, in the invasive range of CHIKV, humans are the main host and *Ae. aegypti* and *Ae. albopictus* are the vectors (Jupp and McIntosh 1988). *Aedes aegypti,* which through the exploitation of man-made habitats spread from Africa to tropical and subtropical regions across the globe, is the primary epidemic vector of dengue, yellow fever, and, historically, CHIKV (Tabachnick 1991, Powers and Logue 2007). During previously documented Asian CHIKV epidemics all mosquito isolates were solely from *Ae. aegypti* (Powers and Logue 2007) and, although *Ae. albopictus* has recently risen in importance as a CHIKV vector, *Ae. aegypti* has continued to play an important role in recent CHIKV outbreaks.

An epidemic of CHIKV began in Kenya in 2004 (Chretien et al. 2007) and spread in 2005 and 2006 to the African island nations of the Comoros, Reunion, Seychelles, Mauritius, and Madagascar in the Indian Ocean (Sergon et al. 2007). Chikungunya then

moved into India, where 1.39 million suspected cases were reported in 2006 and tens of thousands of additional cases were identified in 2007 (Arankalle et al. 2007; NVBDCP 2007). Local transmission of CHIKV was reported in 2007 in the northern Italian province of Ravenna (Rezza et al. 2007), and the epidemic continues with additional infections confirmed in 2010 in the Maldives, Madagascar, Sri Lanka, and many South East Asia countries, such as Indonesia, Malaysia, Thailand, and Myanmar (International Society for Infectious Diseases 2009-2010). Furthermore, multiple cases have been imported into other areas of Europe, the United States, Canada and many other countries through the movement of infected travelers (Lanciotti et al. 2007, International Society for Infectious Diseases 2009-2010).

Arboviral diseases, such as CHIKV, are ecologically complex, and the interaction between immature mosquitoes and factors in their aquatic environment can influence the ability of adult mosquitoes to transmit an arbovirus. *Aedes aegypti* larvae feed on microorganisms and organic detritus available in their container habitats. Containers hold all the nutrients needed by developing larvae, whose resources are often limited leading to nutritionally stressed adult populations of *Ae. aegypti* (Barrera et al. 2006). Abiotic factors such as temperature also influence such factors as larval development and adult body size, and many variables interact with food availability to alter mosquito life history traits (Padmanabha unpublished data).

Previous studies have shown that temperature and food availability during the immature stages can exert a strong influence on adult mosquito vector competence, which is the capacity of an arthropod to acquire a pathogen and transmit it to a subsequent host (Hardy et al. 1990). Larval rearing temperature has been shown to

affect mosquito competence for viruses of Rift Valley fever (RVFV), Venezuelan equine encephalitis (VEEV) (Turell 1993), Murray Valley encephalitis (MVEV) (Kay et al. 1989a), Japanese encephalitis (JEV) (Takahashi 1976), and western equine encephalitis (WEEV) (Hardy et al. 1990), while nutritional deprivation has been shown to affect vector competence for West Nile Virus (WNV) (Baqar et al. 1980) and La Crosse virus (LACV) (Grimstad and Haramis 1984, Grimstad and Walker 1991).

It is well established that a larval environment with high temperatures and/or low food availability will produce smaller adult mosquitoes (Keirans and Fay 1968, Briegel 1990, Rueda et al. 1990). Thus, mosquito body size is an easily measurable physical manifestation of larval habitat quality, which has been documented in many studies investigating larval environmental factors and arboviral susceptibility. In a few studies larval factors were varied specifically to produce mosquitoes of different size classes to test the effect of adult body size on arboviral susceptibility to viruses of dengue (DENV) (Sumanochitrapon et al. 1998) and Ross River (RRV) (Nasci and Mitchell 1994). Overall, the relationships among larval habitat quality, body size, and vector competence are not well worked out, and results from different experiments are conflicting. More controlled and well-designed investigations into unexplored vector-viral systems and diverse combinations of larval ecological factors will add to a growing understanding of this subject.

This study explores how features of larval habitat shape *Ae. aegypti* competence for CHIKV, an important emerging arbovirus causing human disease. Specifically investigated are the relationships among larval rearing temperature, food availability, adult body size, and *Ae. aegypti* susceptibility to CHIKV. In Chapter Two research was

done investigating the influence of temperature at a few discrete levels in *Ae. albopictus*, while in this experiment two factors are crossed to express more realistically the variation experienced in the field by developing *Ae. aegypti*, another key vector of CHIKV. A further objective of this work was to establish the effect of rearing temperature and food availability on larval mortality and development time to adulthood. Since the first isolation and identification of CHIKV in Africa in the 1950s, this virus has spread to new geographic areas with human epidemics documented on multiple continents. Understanding how larval ecological factors can affect the interaction of adult *Ae. aegypti* with CHIKV may help in making predictions as to the direction and magnitude of future outbreaks.

Materials and Methods

Mosquitoes and Viruses

Aedes aegypti used in this study were first generation progeny of approximately 3000 field collected eggs and larvae, which were collected from April to July 2008 in Palm Beach County, Florida. Field collected females were given 20% sucrose ad libitum, blood fed weekly on live chickens, and kept in cages under constant environmental conditions (26 ±1˚C, 14:10 L:D photoperiod, >80% rh). Chicken care followed federally mandated animal use and care policies (University of Florida, IACUC Protocol VB-17). First generation eggs were hatched in tap water and, within 24 hours (h) after hatching, individual larvae were placed in 50 ml Falcon™ (BD Biosciences, Franklin Lakes, NJ) conical tubes with 35 ml of tap water and 10.5 mg or 3.0 mg 1:1 yeast:albumin food. Based on preliminary studies, 3.0 mg given to a larva at the beginning of the experiment was the lowest level of food that allowed for the completion of development to adulthood without significant reduction in mortality, and 10.5 mg was

the highest level of food that could be given to an individual without a marked increase in mortality due to fouling of the aquatic environment.

Larvae were individually reared at 20, 27, and 34˚C with a 14L:10D cycle. Thus, the experiment was a 3x2 factorial design with temperature as one factor at three levels and food as the second factor at two levels. Larvae in each temperature and food treatment were from the same cohort of eggs whose hatch was staggered to synchronize adult emergence among all treatments. After the final larval instar, pupae were removed from rearing tubes, sexed and stored in groups of 20 in water-filled vials to record adult emergences. After emergence, all adults were held at 27˚C, 95-99% relative humidity (rh) with a 14 L:10 D cycle in a Percival (Percival Corporation, Perry, IA) incubator in a biosafety level-3 facility and given 20% sucrose *ad libitum*.

The LR2006-OPY1 CHIKV strain, (GenBank accession number DQ443544) was isolated in France from a febrile patient who had been infected on the island of Reunion in 2006 (Parola et al. 2006). Previously tested *Ae. aegypti* individuals from Palm Beach County were shown to be highly susceptible to the Reunion (LR2006-OPY1) CHIKV strain (Reiskind et al. 2008), which contains the alanine to valine substitution at the 226 position of the E1 envelope structural protein (E1 A226V) that has been identified as a dominant genotype in many current CHIKV epidemics (Rezza et al. 2007). Virus for infectious blood meals was produced by inoculating a confluent monolayer of Vero cells in a T-75 cm^2 flask with 250 µL of previously frozen stock virus, and incubating them in a 5% CO_2 atmosphere (atm) at 35°C for 48 h. After 48 h, infectious blood meals were made by combining freshly recovered media-viral suspension with citrated bovine blood (Hemostat Laboratories, Dixon, CA) in a 1 to 20 ratio. Concentration of virus in fresh

blood meals was 6.3 Log_{10} plaque-forming units (PFU)/mL, which was measured by plaque assay performed in duplicate 6-well plates of confluent Vero cells. Ten-fold serial dilutions (to the 10^{-9} dilution) of infectious blood meal samples were prepared by combining 0.1 ml of the CHIKV infected blood meal with 0.9 ml BA-1 media (Lanciotti et al. 2000) and repeating the process. Each cell well was inoculated with 0.1 ml of a dilution, plates were incubated for 1 h at 5% CO_2 atm at 35°C, before a first overlay of agarose was applied to the cell monolayer. The second overlay was applied two days later, the plate was read the following day, plaques were counted, and final viral concentrations were expressed in PFUs per ml of blood meal.

Mosquito Infection

Groups of 100 five to seven day-old *Ae. aegypti* mosquitoes were placed in 1-L cylindrical, waxed cardboard containers (Dade Paper Co., Miami, FL) with mesh screening. Mosquitoes were starved for 24 hours and then offered an infectious blood meal using a water-jacketed glass membrane feeder (Rutledge et al. 1964) covered with Edicoll® collagen film (Devro, Sandy Run, SC) and connected to a Haake Series F water circulator (Thermo Haake, Paramus, NJ) used to maintain the blood meal at 37°C. Mosquitoes were given 30 minutes to feed. Immediately after feeding, mosquitoes were cold anesthetized, and 10 fully engorged mosquitoes were removed, from each temperature-food treatment, frozen in individual microcentrifuge tubes at -80°C for subsequent wing removal and measurement, trituration, viral RNA extraction, and quantitative RT-PCR. Wings were removed from each mosquito using forceps that were sterilized with 100% ethanol followed by intense flaming using a portable one-touch burner (Daigger®). Wing length was measured in millimeters as an indicator of body size (Blackmore and Lord 2000) from the alula to wing tip, excluding wing fringe. Digital

images of the wing were captured and measured using a computer imaging and measurement program (i-Solution lite®, AIC Inc., Princeton, NJ). These individuals were used to determine the relationship between mosquito size and quantity of virus initially ingested by freshly feed females.

The remainder of the engorged mosquitoes were held for a 10 day EIP at 27°C and provided with 20% sucrose *ad libitum*, after which surviving *Ae. aegypti* females were killed by freezing. Females were stored in individual microcentrifuge tubes at -80°C and, after thawing, wings were removed for measurements, bodies were assayed to determine infection status and titer, and legs were tested to check for a disseminated infection. An assayed mosquito could be, (1) uninfected, have an (2) isolated infection, which specified a CHIKV positive body, but legs negative for the presence of the virus or have a (3) disseminated infection, which meant virus was found in the legs signifying the infection had spread beyond the midgut and on to secondary organs (Turell et al. 1984). Both wings and legs of individual mosquitoes were removed using the sterilized forcep technique described previously. Samples were homogenized at 25 Hz for 3 min using a Tissuelyzer® tissue homogenizer (Qiagen Inc., Valencia, CA). RNA was extracted separately from bodies and legs. Mosquito bodies were homogenized in TRI Reagent® (Molecular Research Center, Inc., Cincinatti, OH) and then RNA was extracted according to the manufacturer's protocol using 50 µl of DEPC treated water as the final elution volume. Mosquito legs were homogenized in 250 µl BA-1 media (Lanciotti et al. 2000) with two zinc-plated BBs (Daisy®), which was then added to 750 µl of TRI Reagent-LS® (Molecular Research Center, Inc., Cincinatti, OH) for RNA

extraction, following the manufacturer's protocol and also with a final elution volume of 50 µl in DEPC treated water.

One-step quantitative RT-PCR was used to determine infection status and body titer of samples. Primers were designed from the E1 gene and had the following sequences: forward: 5'-ACC CGG TAA GAG CGA TGA ACT-3'; reverse: 5'AGG CCG CAT CCG GTA TGT-3'; and probe: 5'-/5cy5/CCG TAG GGA ACA TGC CCA TCT CCA /3BHQ_2/-3' (IDT DNA, Coralville, IA). Reactions were performed in a 96-well reaction plate, with each reaction containing: 0.4µl SuperScript III RT/Platinum Taq mix (Invitrogen, Carlsbad, CA), 10µl 2x reaction mix (a buffer system, MgSO4, dNTPs and stabilizers), 1µl forward primer (10 µmol/L), 1µl reverse primer (10 µmol/L), 0.4µl fluorogenic probe (10 µmol/L), 2.2µl DEPC-treated H2O, and 5µl of the test sample RNA. Viral RNA was quantified using a Roche LC480 light-cycler (Roche Applied Sciences, Indianapolis, IN) with the following thermal conditions: 20 minutes at 48° C and 2 minutes at 95° C, followed by 40 cycles of PCR, 10 seconds at 95° C and 15 seconds at 60° C followed by a cool down for 30 seconds at 50° C. A negative control (DEPC-treated water in place of sample) and a positive control (CHIKV stock virus, 10^{-2} dilution) were included in each reaction run.

A standard curve was generated by assaying a full range of ten-fold serial dilutions of CHIKV virus stock (7.8 Log_{10} PFU/ml) by plaque assay which determined PFUs per dilution. Viral RNA was then isolated from three replicates of each dilution using TRI Reagent-LS® (as previously described for leg aliquots), and all dilutions were assayed using qRT-PCR. Viral concentrations and crossing point (Cp) values determined from qRT-PCR from dilutions 10^{-2} through 10^{-6} constituted the six values used to establish a

linear regression (Cp = -3.455*Log10(PFU) + 32.2, n=6, $p<0.0001$, $r^2 = 0.9985$). Mosquito body titers in each test sample were then calculated by comparing the test sample with standard curve values that had been transformed into plaque-forming unit (Cp) equivalents.

Statistical Analysis

All statistical analysis was performed using SAS software, version 9.2 (SAS Institute, Carey NC). CHIKV mosquito titer data for the engorged female mosquitoes killed directly after blood feeding did not fit assumptions, of normality and approximate normality was achieved through a log transformation of titer values. Product moment correlation analysis was then carried out between the log transformed CHIKV mosquito body titer and the wing lengths of the engorged females. Two-way analysis of variance (ANOVA) (PROC GLM) was used to compare the effects of temperature and food level and their interaction on CHIKV titer in engorged females. Main effects means (temperature and food) were compared by Tukey's studentized range tests. To determine differences among the mean wing lengths of the engorged females from the six larval treatment groups (temperature-food combinations) a two-way ANOVA (PROC GLM) was followed by Tukey's studentized range tests. Temperature and food were categorical variables in the two-way ANOVA analysis.

Two-way ANOVA (PROC GLM) followed by Tukey's studentized range tests was used to determine differences among the temperature and food treatments in distributions of the wing lengths of blood-fed females that were killed by freezing after the 10 day EIP. Two-way ANOVA (PROC GLM) followed by Tukey's studentized range tests was also used to detect development time differences among the temperature-food treatments. Mean development times were determined from all mosquitoes reared,

not just blood fed females and males and females were analyzed separately because of gender-specific developmental times in this species. The proportion of mosquitoes that died as larvae during rearing was analyzed for significant effects of the six treatments by maximum likelihood categorical analyses of contingency tables (PROC CATMOD). Comparisons of mortality rates between treatments were performed with maximum likelihood contrasts using a Bonferroni adjustment to maintain an experiment-wise $\alpha = 0.05$.

Logistic regression (PROC LOGISTIC) were used to model mosquito CHIKV body infection (# with virus/# fed) and disseminated infection (# with virus in their legs/# with virus) by temperature, food level, and temperature x food interaction, specifying a logistic link function and a binomial error distribution. Odds ratios (OR) and 95 % confidence intervals for infection and disseminated infection by treatment were also calculated.

Two-way ANOVA was used to test for effects of temperature, food level, and a temperature x food interaction on mosquito body titer following the 10 day EIP. Differences between main effect means (temperature and food) were further analyzed by Tukey's studentized range tests. Titer data did not fit the model assumptions of normality, but approximate normality was achieved through a log transformation. To determine if there was a difference in size among uninfected, infected (non-disseminated), and disseminated females a one-way ANOVA (PROC GLM) was used. The one-way ANOVA was used to compare differences in mean wing length among the females of the three infection status categories. These were females that blood fed,

completed the 10 day EIP and were then pooled across all larval treatments for the size analysis.

Results

Chikungunya Titer of Freshly Engorged Mosquitoes

Correlation analysis showed a significant positive correlation between mosquito wing length and log transformed-CHIKV body titer (r = 0.5787, P<0.0001, df = 56) (Figure 3-1). When tested by two-way ANOVA, the log-transformed mean titers of CHIKV in freshly engorged females were significantly affected by larval rearing temperature (F= 20.97, df = 2, P<0.0001), but not larval food level (F= 1.25, df = 1, P<0.2683), nor the temperature x food level interaction (F= 1.59, df = 2, P<0.2140) (Figure 3-2). Females reared at two lower temperatures of 20 and 27˚C had significantly higher CHIKV titer in mosquito bodies than those reared at 34˚C (Tukey's studentized range tests, P < 0.05). Wing lengths of freshly engorged females were significantly affected by larval rearing temperature (F= 103.88, df = 2, P<0.0001), larval food level (F= 33.69, df = 1, P<0.0001), but not the temperature x food level interaction (F= 1.50, df = 2, P<0.2316). When followed up with pairwise comparisons using Tukey's studentized range tests (P < 0.05) all temperature levels and food levels were significantly different from eachother (Figure 3-2).

Growth and Mortality

Wing lengths of blood-fed females that were held through the 10 day EIP varied significantly due to temperature (F = 577.76; df = 2; P<0.0001), food (F = 235.66; df = 1; P<0.0001), and the temperature x food interaction (F= 7.58, df = 2, P= 0.0006). Development time to adulthood in females was also significantly affected by larval rearing temperature (F = 16187.6; df = 2; P<0.0001), food (F = 100.48; df = 1;

P<0.0001), and the temperature x food interaction (F= 46.34, df = 2, P<0.0001). There were also significant effects in male development due larval rearing temperature (F = 16614.9; df = 2; P<0.0001), food (F = 130.55; df = 1; P<0.0001), and the temperature x food interaction (F= 51.97, df = 2, P<0.0001) (Table 3-1). Larger mosquitoes were generated from the lower temperatures and higher food treatments.

Juvenile mortality rates at the low and high food levels within the three temperature treatments, 20°C, 27°C, and 34°C, respectively were 2.37% (n = 969), 2.05% (n = 975), 2.90% (n = 966), 2.53% (n = 1029), 2.89% (n = 1073), and 6.42% (n = 1028) (Figure 3-3). There was a significant difference in mortality among treatments (X^2 = 39.67, df = 5, P<0.0001). The maximum likelihood contrasts showed that the 34°C-high food level treatment at 6.42%, had a significantly higher juvenile mortality rate than all other treatments (Table 3-2).

Chikungunya Infection and Dissemination

There was a significant temperature (X^2=26.0248, df = 2, P<0.0001) effect on the likelihood of females developing CHIKV infections, but food level (X^2=1.1108, df = 1, P = 0.2919) and the interaction between temperature and food level (X^2=4.5452, df = 2, P = 0.1030) were not significant (Figure 3-4). Infection was 5.4 times more likely in adult females reared at 27°C than at 20°C (Odds Ratio (OR) = 5.428; 95% Confidence Interval (CI): 2.798-10.532) and females reared at 27°C were 4.7 times more likely to be infected than those reared at 34°C (OR = 4.768; 95% CI: 1.980-11.485). There was no significant difference in CHIKV infection between females reared at 20°C and at 34°C (OR = 1.138; CI: 0.525-2.468).

Among the infected individuals the proportion of females that developed disseminated infections was significantly affected by larval rearing temperature

(X^2=8.7265, df = 2, P = 0.0127), food level (X^2=5.0123, df = 1, P = 0.0252), and their interaction ($\chi 2$= 6.9914, df = 1, P = 0.0303) (Figure 3-5). Dissemination was 5.4 times more likely at 27°C compared to 34°C (OR = 5.466; 95% CI: 1.4515-20.585) and 2.1 times more likely at 27°C compared to 20°C (OR = 2.109; CI: 1.0380-4.2832). There was no significant difference in disseminated infections between 20 and 34°C. Dissemination was also 2.7 times more likely at the higher food level (OR = 2.7000; CI: 1.1317-6.4417).

After the 10-day EIP, when tested by a two-way ANOVA, log titer of CHIKV in infected females was not significantly affected by larval rearing temperature (F= 2.01; df = 2; P = 0.1353), food level (F= 0.45; df = 1; P = 0.5037), nor the temperature x food level interaction (F= 0.56; df = 2; P= 0.5706). A one-way ANOVA showed that there was no significant variation in wing lengths among CHIKV infection status categories (F = 1.66, df = 2, P = 0.192) (Figure 3-6).

Discussion

Ecological factors in the larval environment influence mosquito life history traits that are important in infectious disease dynamics (i.e. growth rate, life span, biting rate) and can directly affect traits that affect arbovirus susceptibility. Specific results from this experiment demonstrate that temperature and food availability influence body size, development time and CHIKV infection status, although the nature of the relationship between body size and viral susceptibility is not clear.

Among engorged *Ae. aegypti* females, assayed immediately after taking a blood meal, there was significant correlation between body size, as measured by wing length, and body titer (Figure 3-1). Prior to the experiment it was hypothesized that larger females would take in a greater volume of blood and, thus have a higher initial titer of

virus when assayed immediately after feeding and this hypothesis was partially supported by the correlation analysis results. However, when the effect of larval temperature and food quantity on freshly engorged female mosquito body titer was analyzed via two-way ANOVA it was found that only temperature and not food level had a significant effect on body titer, yet food level definitely had a significant effect on size (Figure3-2). Mosquitoes reared at 34°C, but given two different quantities of food were significantly different in size, but not in body titer, while the mean titer of mosquitoes reared at 34°C was significantly lower than the mean titers of mosquitoes from 27 and 20°C treatments. Thus how the body size was achieved, either by temperature differences or food differences, was an important factor in determining the amount of blood and virus ingested and that size alone was not the best predictor of ingested blood volume and initial viral dose.

Larval habitat features are important in regulating the growth of individuals and populations of mosquitoes (Rueda et al. 1990; Scott et al. 1993; Juliano 2009). In this experiment, temperature and food availability had measurable effects on mosquito development rate, size, and mortality. As expected, males had shorter development times than their female counterparts due to developmental dimorphism between the sexes. Growth rate was phenotypically plastic with respect to temperature and food level and at the lowest temperature of 20°C mosquitoes took the longest to develop with median development time to adulthood for the low level food females of 12 days and for the high level food treatment of 13 days. As treatments increased in temperature the differences between development time decreased so at 27°C mosquitoes only took approximately one day longer than individuals reared at 34°C to reach adulthood.

Surprisingly, mosquitoes (both male and female) reared at 20°C and at 34°C took longer to develop if they were given more food as larvae. Most studies show a decline in development time when food quantity per larvae is increased (Wada 1965, Black et al. 1989, Teng and Apperson 2000). However, *Ae. aegypti* reared at 60°F (15.6°C) fed a finely ground laboratory chow took 33 days to pupate at the full food treatment and 28 days to pupate at the half-food treatment (Keirans and Fay 1968), and among *Ae. aegypti* larvae given different daily amounts of Brewer's yeast larvae from the lowest food treatment did not differ from the other treatments in mean rate of pupation (Peters et al. 1969). It is possible that at 34°C the interaction between temperature and the higher food quantity produced a polluted environment leading to a longer development time, while the low temperature of 20°C is close enough to the lower thermal development limit of *Ae. aegypti* that resource utilization was unpredictable. At 27°C there was no significant difference in median development-time to adulthood between the food levels, but the interquartile range for the low food level is wider and skewed to include a longer development period. This temperature is probably close to the optimal physiological temperature for *Ae. aegypti* (Christophers 1960) and is commonly used in experiments, which may explain the more expected relationship of increased food availability and decreased development time.

The three temperature and two food level combinations produced six significantly different wing length classes of mosquitoes with differences between adjacent groups ranging from 0.06 mm to 0.33 mm (Table 3-1). At 27°C the two food levels, produced females with a wing length difference of approximately 0.25 mm, but no difference in development time to adulthood. The same difference in wing length, achieved through

different rearing temperatures also produced difference in development time to adulthood. The low temperature of 20°C had a more dramatic effect on size and development time, that the two higher temperatures.

The experiment was designed to maximize adult production at temperatures and food levels that would produce markedly different outcomes in size, development times and responses to virus. To that effect there was an attempt to keep larval mortality at a minimum and equal among all six temperature-food combinations. However, there was a significant difference in mortality between the highest temperature-food combination and all the other treatments (Figure 3-3). Although results from preliminary experiments showed no difference in survivorship between the larval treatments the very large sample size probably increased the ability to see even a small effect. The increased mortality could have been due to the interaction of high temperature and excess nutritional resources resulting in the proliferation of detrimental micro-organisms in the larval environment.

Only temperature had an effect on CHIKV infection status, with a significantly greater proportion of individuals infected when reared at the middle temperature of 27°C and no difference between the lower and higher temperature of 20 and 34°C. In similar studies with *Aedes vigilax*, larvae reared at 32°C and held at 25°C had lower RRV infection rates than counterparts reared at 18 and 25°C and held at 25°C (Kay and Jennings 2002), and in *Ae. albopictus* there was a reduction in CHIKV infection with increasing temperature (Westbrook et al. 2009). Non-linear responses to temperature are common in biological systems (Zhou et al. 2008) and, based on the pattern of the results in Figure 3-4, the *Ae. aegypti* response to the effect of larval rearing temperature

on CHIKV infection may not be linear. Sub-optimal temperature conditions, represented by the 20 and 34°C may lessen the susceptibility of *Ae. aegypti* to CHIKV. This study was designed to explore the general question of whether rearing temperature and food availability effect CHIKV infection status in *Ae. aegypti* and the use of a generalized linear model with temperature as a classed predictor variable was appropriate considering the limited number of temperatures used. However, now that an effect has been established future experiments will be designed with the intent of predictability. Increasing the number of temperature levels, reducing the difference between levels and treating temperature as a continuous variable would provide data to model the response pattern more thoroughly.

Temperature, food availability, and their interaction had an effect on the probability of having a disseminated infection. Significance in the interaction terms specified that temperature had a different effect on the probability of having a CHIKV disseminated infection dependent on food level. At 20 and 34°C mosquitoes generated from high food treatments were more likely to have disseminated infections, while the opposite pattern was found at 27°C. In the overall model disseminated infections were 2.7 times more likely in the high food treatments. This result is in contrast with other studies on larval nutrition and vector competence of *Cx. tritaeniorhynchus* for WNV and *Ae. triseriatus* for LACV which showed reduced susceptibility of adults generated from nutrient-deprived larvae (Baqar et al. 1980, Grimstad and Haramis 1984; Grimstad and Walker 1991).

Although wing length was positively correlated with the initial quantity of virus ingested, significant wing length-infection correlations disappeared after the extrinsic incubation period, suggesting that mosquito size alone in this vector-viral system is not

a good predictor of viral infection, dissemination or body titer (Figure 3-6). It was originally hypothesized that a larger initial viral dose, even after the ten day EIP, may lead to a higher proportion of larger mosquitoes with isolated infections and disseminated infections. There was support for this hypothesis in results from other studies. For example, large *Ae. aegypti* females, produced by varying food and density, showed higher rates of oral infection with DENV compared to small and medium sized individuals (Sumanochitrapon et al. 1998), larger *Ae. aegypti* generated through variation in larval diet which were more susceptible to RRV (Nasci and Mitchell 1994), and large *Ae. triseriatus* adults from competition treatments had lower infection and dissemination rates for LACV (Bevins 2008). In contrast, smaller *Ae. albopictus* generated from high competition larval environments had higher rates of infection and dissemination for Sindbis (SINV) and dengue (DENV) viruses (Alto et al. 2005; Alto et al. 2008a).

In this study rearing temperature and food level affected the ease with which *Ae. aegypti* became infected with and disseminated CHIKV which may impact the epidemiology of this disease. Failure to consider the importance of the larval environment may lead to incorrect estimates of vector susceptibility. Variations in different ecological factors in the larval habitat larval may produce mosquitoes of a similar size range, but with very different responses to infection. Thus, in this experiment body size was not a very good predictor of how a mosquito will respond to arboviral infection and there may be more critical, but not as easily measurable physiological and anatomical features of adult mosquitoes that vary with larval conditions and are more substantially correlated with viral susceptibility. This

experiment demonstrates the significant role of larval ecology in adult vector-viral interactions, but additional well designed experiments with predictability in mind are required to determine more quantitatively the effects of factors such as food, temperature, and interactions with other individuals or organisms during juvenile development on adult vector-viral interactions.

Table 3-1. *Aedes aegypti* LS means and standard error for development time to adulthood and female wing length.

Temperature	20ºC		27ºC		34ºC	
Food Level	Low	High	Low	High	Low	High
Male time to adulthood (days)	11.47±0.04a	12.18±0.04b	6.53±0.04c	6.48±0.04c	5.17±0.04d	5.55±0.04e
(n)	(459)	(488)	(547)	(526)	(550)	(513)
Female time to adulthood (days)	11.97±0.04a	12.68±0.04b	6.65±0.04c	6.55±0.04c	5.36±0.04d	5.75±0.04e
(n)	(487)	(467)	(391)	(477)	(492)	(449)
Female wing length (♀) (mm)	3.34±0.02a	3.67±0.02b	3.03±0.02c	3.28±0.01d	2.70±0.02e	2.87±0.02f
(n)	(116)	(60)	(90)	(91)	(33)	(58)

* Means from PROC GLM analysis with different letters in the same row are significantly different from one another

Table 3-2. Maximum likelihood (ML) contrasts for comparisons of mortality rates for 34ºC-high food treatment with all other temperature and food level treatment groups

ML Contrast	df	Chi square	*P*
34ºC-high food vs.			
20ºC-high food	1	17.72	<.0001
27ºC-low food	1	20.94	<.0001
27ºC-high food	1	13.07	0.0003
34ºC-Low food	1	17.02	<.0001
34ºC-high food	1	14.13	0.0002

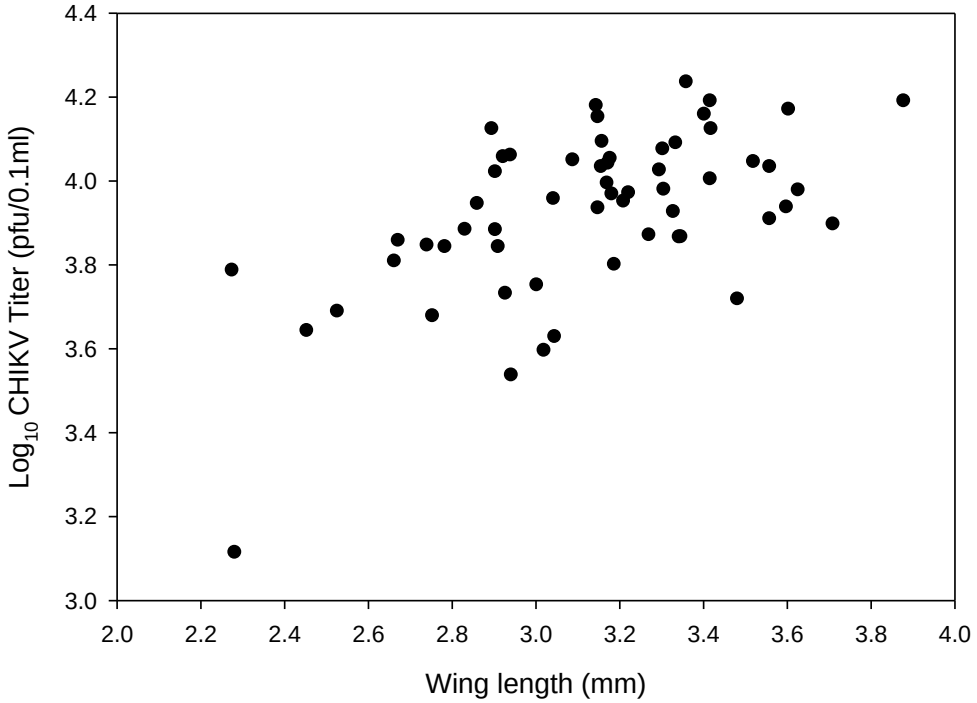

Figure 3-1. Correlation between log transformed whole mosquito body titers of CHIKV and wing lengths for engorged *Aedes aegypti* females killed immediately after feeding (r = 0.5787, P<0.0001).

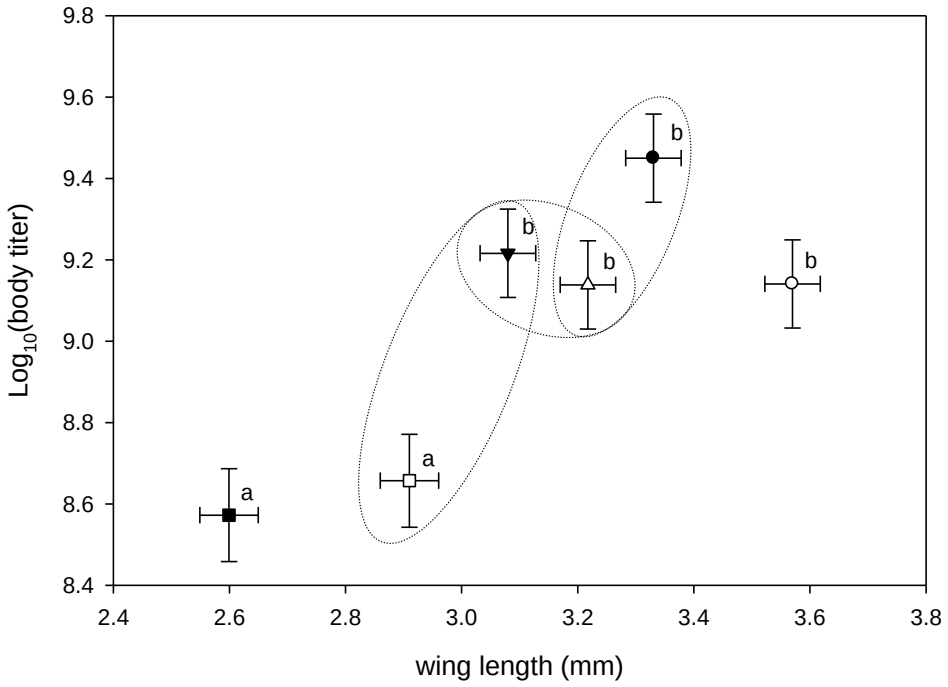

Figure 3-2. Bivariate plot of LS means (±SE) for wing lengths and log transformed CHIKV body titers for engorged *Aedes aegypti* females killed immediately after feeding. Filled symbols represent the low food treatment and open symbols the high food treatment, squares are 34°C, triangles 27°C, and circles 20°C. Different letters indicate significant differences among means of log transformed CHIKV body titers. Symbols within dashed ellipses do not have significantly different wing lengths.

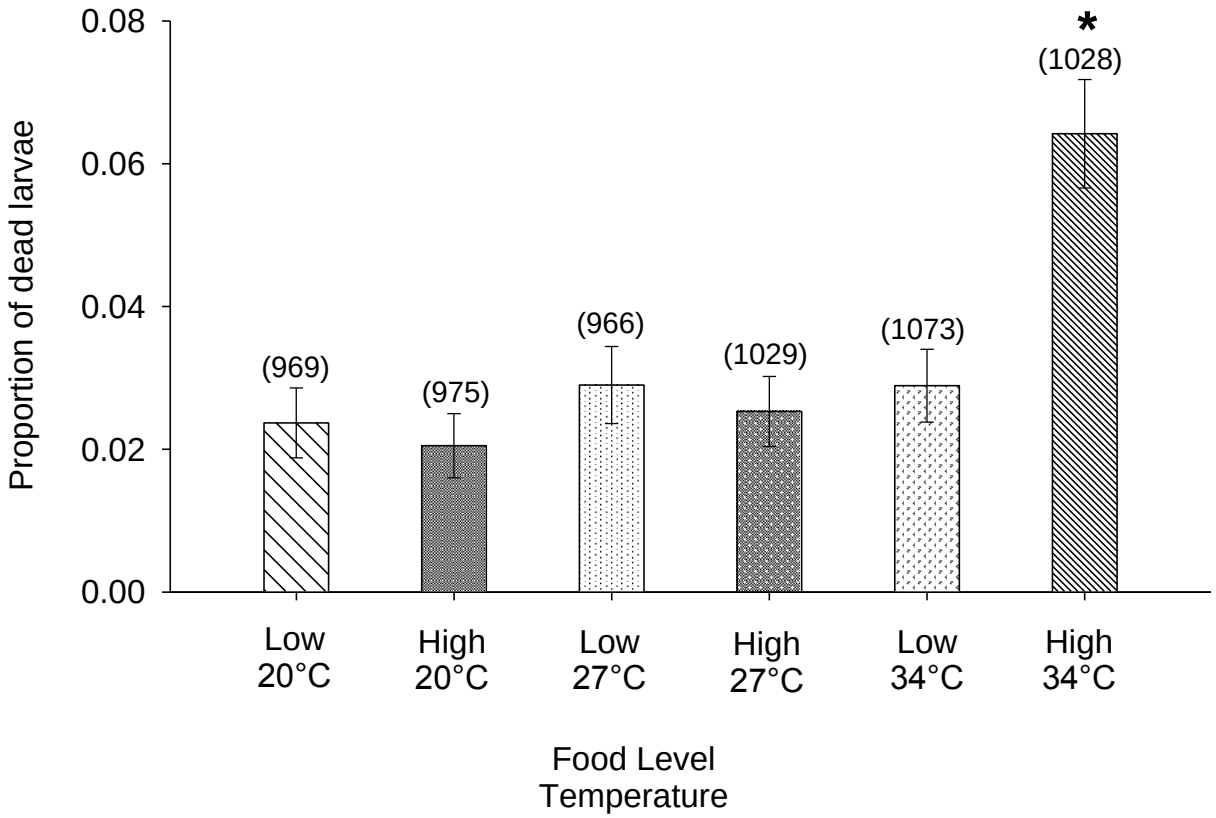

Figure 3-3. Juvenile mortality rates at the low and high food levels within the three temperature treatments for *Aedes aegypti*. *Significantly different from other five treatments based on results from maximum likelihood contrasts.

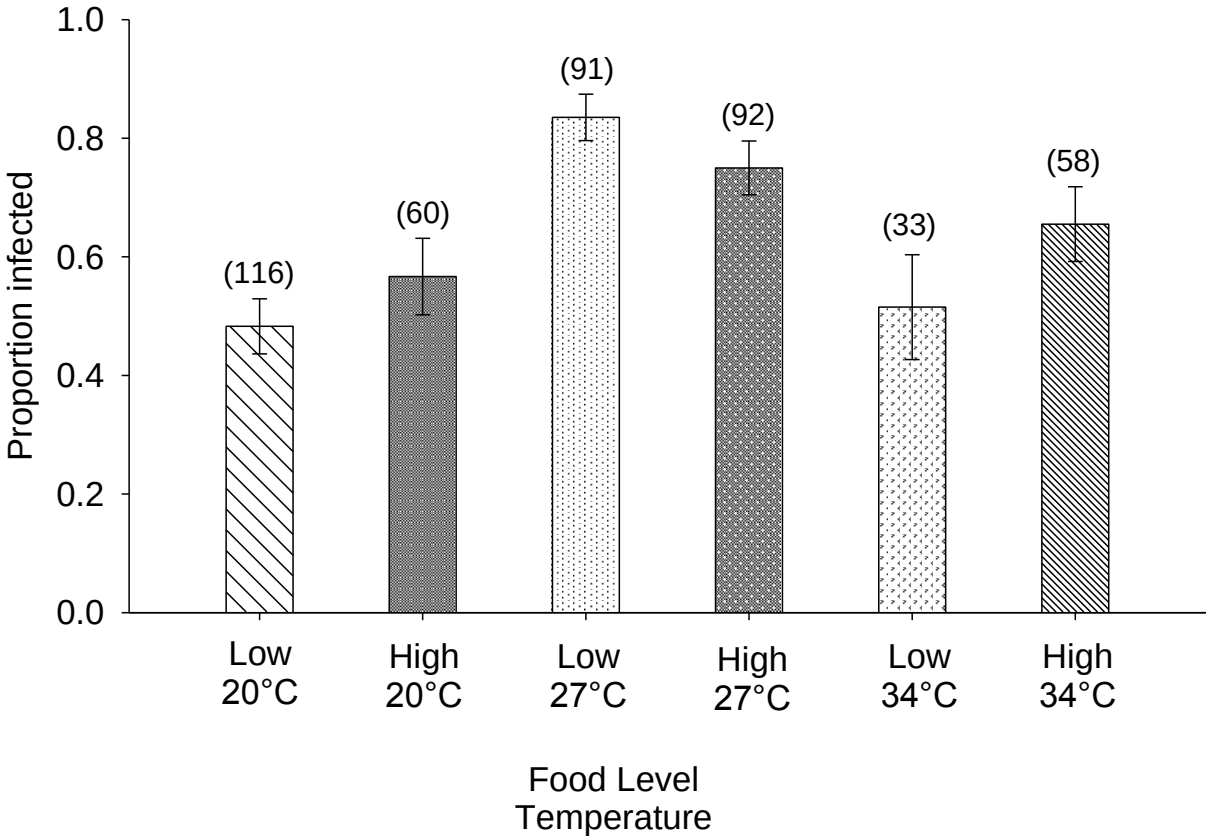

Figure 3-4. Proportion of *Aedes aegypti* females (±SE) in each temperature treatment infected with CHIKV. Numbers in parentheses are the number of blood fed females in that treatment group.

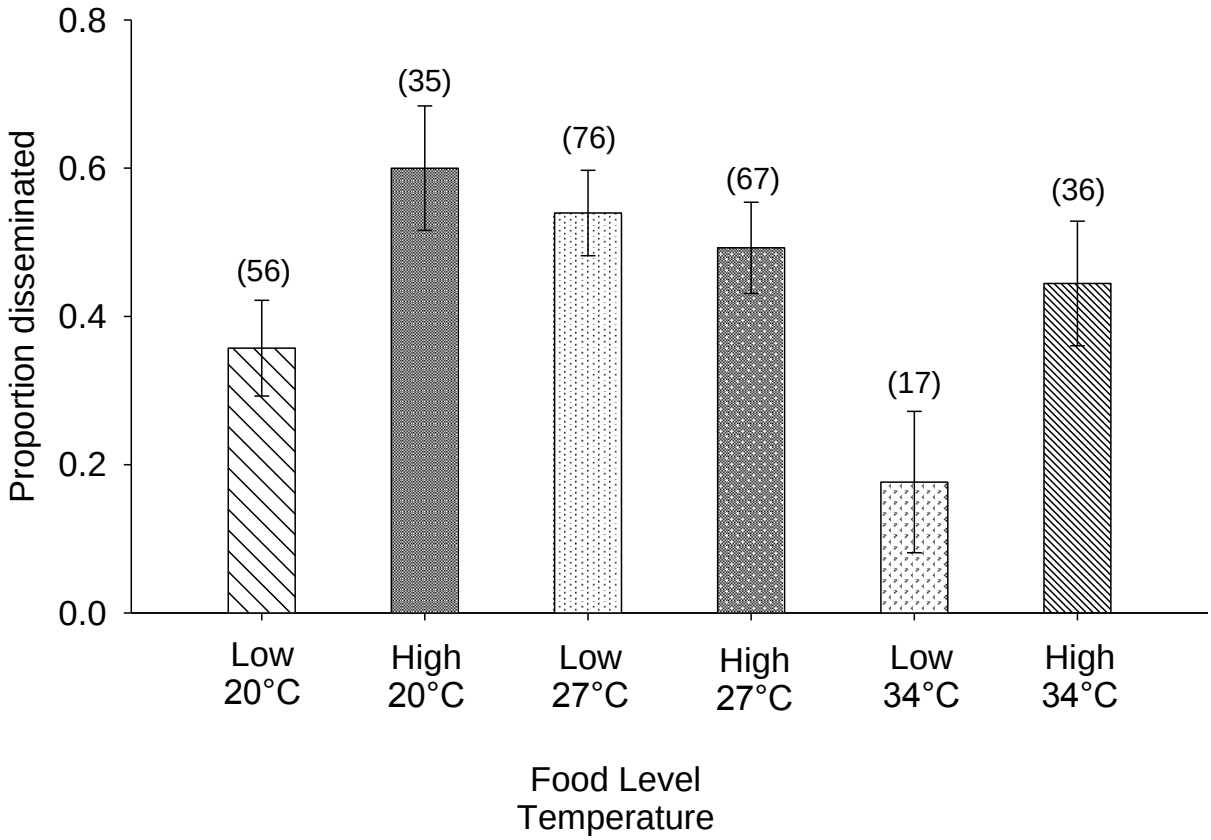

Figure 3-5. Proportions of infected *Aedes aegypti* females (±SE) from temperature and food level treatments with disseminated CHIKV infections. Numbers in parentheses are the number of blood fed females in that treatment group.

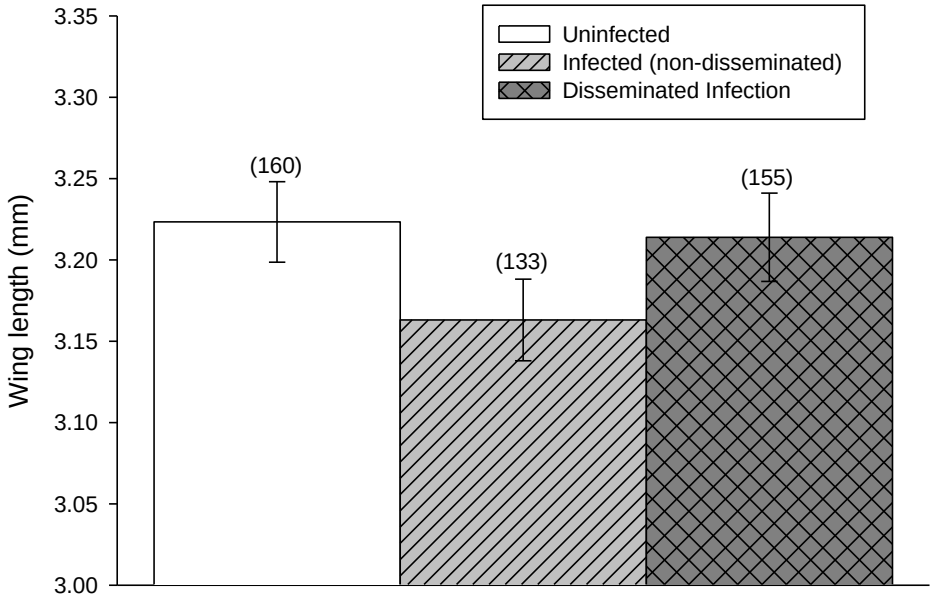

Figure 3-6. Least squared means (±SE) for sizes of adult female *Ae. aegypti* mosquitoes in CHIKV infection status categories.

CHAPTER 4
LARVAL TEMPERATURE, COMPETITION, AND THE VECTOR COMPETENCE OF *Aedes aegypti* AND *Aedes albopictus* FOR CHIKUNGUNYA VIRUS

Introduction

Competition is often an important biotic mechanism in the shaping of insect distributions and abundances (Craig et al. 1990, Settle and Wilson 1990, Human and Gordon 1996, Kaplan and Denno 2007). In nature, resources may be scarce in a limited area, and competing organisms often have a choice as to whether to stay and compete or disperse to another resource patch. However, in artificial and natural containers that house developing *Ae. aegypti* and *Ae. albopictus*, the immature competitors cannot leave the container environment, and competition can have considerable effects on population growth components such as, development time, size, fecundity, and survival to adulthood (Teng and Apperson 2000, Armistead et al. 2008, Reiskind and Lounibos 2009). Furthermore, larval competition can affect adult mosquito susceptibility to an arbovirus, which can result in changes in the distribution and transmission intensity of an arbovirus (Baqar el al 1980, Alto et al. 2005, Alto et al. 2008, Bevins 2008).

Aedes albopictus and *Ae. aegypti* are invasive vectors with geographic ranges that span large portions of the globe. *Aedes aegypti* is believed to have traveled to the New World from its native Africa in water storage jars aboard slave ships (Christophers 1960), while the spread of *Ae. albopictus* from its native Asian range has mostly been a more recent event in part due to the trade in used tires (Hawley et al. 1987). In many parts of the world larvae and pupae of both *Aedes* species may be found developing and feeding in the same container (MacDonald 1956, Fontenille and Rodhain 1989, O'Meara et al. 1992, Braks et al. 2003), and resource competition within and between these species is well documented (Juliano 1998, Braks et al. 2004).

In larval competition experiments using leaf detritus as a basal resource, *Ae. albopictus* exhibited a competitive advantage over *Ae. aegypti* (Juliano 1998, Braks et al. 2004), which might account for the observed decline of *Ae. aegypti* in areas of the United States now inhabited by *Ae. albopictus* (O'Meara et al. 1995, Lounibos 2007). Nevertheless, there are many regions of sympatry of these two species, including southern Florida (Rey et al. 2006), and condition-specific competition has been proposed as the process behind their sustained coexistence (Costanzo et al. 2005, Leisnham and Juliano 2009). *Aedes albopictus* is a better larval competitor, but superior desiccation resistance of the egg stage of *Ae. aegypti* allows greater numbers of this species to survive during the dry season (Sota and Mogi 1992, Juliano et al. 2002). Furthermore, *Ae. aegypti* presence or abundance is positively associated with lower humidity, higher temperature, and urbanization while *Ae. albopictus* is negatively associated with hot, dry climates and is more common in sites with shade and vegetation (Hawley 1988, Braks et al. 2003, Rey et al. 2006, Reiskind and Lounibos 2009). Thus, in nature the outcome of competitive interactions between the two mosquito species changes temporally with the dry and wet seasons and spatially with environmental features associated with humidity and temperature.

Both *Ae. aegypti* and *Ae. albopictus* are important epidemic vectors of chikungunya virus (CHIKV), a single stranded, positive sense enveloped RNA alphavirus. Chikungunya virus was first isolated in 1953 from a febrile patient in Tanzania (Ross 1956), and sporadic epidemics were recognized subsequently from Africa, Asia, and India, but a particularly explosive epidemic of CHIKV began in 2004 in coastal Kenya and spread throughout African nations in the Indian Ocean, infecting high

proportions of island inhabitants, and subsequently spreading to India, Southeast Asia, Italy and other countries (Powers and Logue 2007, Gould and Higgs 2009). In previous epidemics. *Ae. aegypti* was implicated as more important, with virtually all Asian vector isolates coming from this mosquito species (Powers and Logue 2007), but in recent CHIKV outbreaks in regions where *Ae. aegypti* and *Ae. albopictus* distributions overlap, both species have tested positive for the virus. In Singapore, in 2008, larval surveys identified *Ae. albopictus* as more common than *Ae. aegypti,* testing of wild-caught mosquitoes yielded both *Ae. albopictus* and *Ae. aegypti* adult females positive for CHIKV (Ng et al. 2009). Similar patterns were found in Thailand, where wild-caught adults of both species were positive for CHIKV (Thavara et al. 2009). Entomological surveys done during the 2006 CHIKV outbreak in the north eastern Indian state of Orissa, revealed the presence of both *Aedes* species with *Ae. albopictus* having a slightly higher abundance than *Ae. aegypti* (Dwibedi et al. 2009).

This study addresses whether larval rearing temperature modulates the competitive larval interactions between *Ae. albopictus* and *Ae. aegypti* and how that may in turn influence adult susceptibility to CHIKV. Most laboratory larval competition studies on *Ae. albopictus* and *Ae. aegypti* have been carried out at temperatures between 25° and 27°C (Black et al.1989, Barrera 1996, Daugherty et al. 2000, Alto et al. 2005, Alto et al. 2008), with the exception of Lounibos et al. (2002) which compared competition between these two species at 24° and 30°C. The container habitat of *Ae. albopictus* and *Ae. aegypti* larvae may be subjected to temperature lows of approximately 12° to 14°C and highs ranging from 30° to 35°C or above (Lounibos 1992, Reiskind unpublished data). Physical features of the environment, such as

temperature may act in concert with, or in opposition to, biotic factors like competition to cause changes in physical or physiological traits in mosquito vectors that alter their susceptibility to arbovirus. Previous studies in other insect systems have shown that a change in temperature can reverse the outcome of interspecific competition between insects that occupy the same environment (Birch 1953, Park 1954, Ayala 1970, Russell 1986). It is also possible that an increase or a decrease in temperature may increase the magnitude of competitive effects without changing the direction of the interspecific competitive outcomes between *Ae. aegypt* and *Ae. albopictus.*

Materials and Methods

Mosquitoes, Temperature, and Competition

Aedes aegypti and *Ae. albopictus* used in this experiment were second generation (F2) progeny of individuals collected as eggs, larvae, and pupae in St. Lucie and Palm Beach counties in southern Florida. Adults maintained in cages under constant environmental conditions (26 ±1˚C, 14:10 L:D photoperiod, >80% relative humidity) were provided with 20% sucrose *ad libitum,* and blood meals from restrained chickens (housed and maintained in accordance with federally mandated animal use and care policies as part of the University of Florida, IACUC Protocol VB-17). Oviposited F_2 eggs were collected on seed germination paper. Eggs from both species were simultaneously hatched in separate Erlenmeyer flasks with tap water under a vacuum for 30 minutes to approximately synchronize hatching time. Newly hatched (<16 hours after hatching) larvae were counted and added to respective competition and temperature treatments. Competition treatments consisted of *Ae. aegypti*:*Ae. albopictus* species abundance ratios of 200:0, 100:0, 100:100, 0:200, 0:100, with five replicates per competitive treatment. All five competitive treatments, replicated five times, were run in incubators at

temperatures of 22, 27 and 32°C, each with a 14:10 L:D photoperiod. Larvae in each temperature treatment were from the same cohort of eggs whose hatch was staggered to synchronize adult emergence among all treatments, which ensured that individuals of the same species were approximately the same adult age when blood fed.

Five-liter white plastic buckets partially filled with 2500ml of tap water, 500ml of oak leaf infusion water (O'Meara et al, 1989), and 0.15 grams (g) particulate food (1:1 lactoalbumin: Brewer's yeast) were used as larval rearing containers. Oak leaf infusion was made by collecting fallen oak (*Quercus virginiana*) leaves, oven drying them for 48 hours at 80°C, combining 35.5 g of leaves per liter of tap water and letting it incubate at 27°C for ten days. A total of 50 liter of infusion was prepared and then frozen so that all buckets, some of which were set up on different days because of staggered temperature treatments, would receive infusion that was derived from the same starting material and treated identically. Enough oak leaf infusion to set up all the competitive treatments and replicates was thawed for 12 hours before adding it to each 5 L bucket. The 0.15 g of particulate food allowed for competitive interactions between *Ae. aegypti* and *Ae. albopictus* in preliminary experiments with limited mortality so that enough adult females were produced for the CHIKV infection portion of the study, as accomplished in similar experiments using other arboviruses (Alto et al. 2005, 2008a).

Pupae were collected daily, sexed and identified to species in the mixed treatments, and the pupation date in days since hatching was recorded. Pupae were collected until all individuals emerged or died. All female pupae from a given replicate were placed in a water-filled 10 ml cup inside a 1-L cylindrical, waxed cardboard container (Dade Paper Co., Miami, FL) with fine mesh screening. Enough adult males

were retained so that there was approximately a 1:5 ratio of males to females for mating to take place in the cages; the remaining male pupae were discarded. All pupae inside adult 1-L cylindrical cages were maintained at 27°C and as adults emerged humidity was maintained at >90% rh and adults were given 20% sucrose *ad libitum*. The idea was that larvae completed development in one of the three different temperature treatments, but once pupation occurred and feeding in the aquatic stage ceased, pupae were moved to 27°C and as adults maintenance continued at 27°C though the blood feeding and the EIP.

Virus and Mosquito Infection

The LR2006-OPY1 CHIKV strain, (GenBank accession number DQ443544) was isolated in France from a febrile patient who had been infected on the island of Reunion in 2006 (Parola et al. 2006). *Aedes aegypti* and *Ae albopictus* females from Palm Beach County were shown previously to be highly susceptible to this recently emergent CHIKV strain (Reiskind et al. 2008), which contains the alanine to valine substitution at the 226 position of the E1 envelope structural protein (E1 A226V) that has been identified as a feature in many recent CHIKV epidemics (Rezza et al. 2007). Virus for infectious blood meals was produced by inoculating a confluent monolayer of Vero cells in a T-75 cm^2 flask with 250 µL of previously frozen stock virus, and incubating them in a 5% CO_2 atmosphere (atm) and 35°C for 36 h. After 36 h, infectious blood was prepared by combining freshly recovered media-viral suspension with defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) in a 1 to 10 ratio. Concentration of virus in blood meals offered to mosquitoes was measured at 7.4 Log_{10} plaque-forming units (PFU)/mL by one-step quantitative RT-PCR.

Each 1-L cylindrical, waxed cardboard containers contained all the emerged females of the same species from each replicate. *Aedes albopictus* females from all treatments were approximately 6-10 days old and *Ae. aegypti* were approximately 10-14 days old at blood feeding. Because intense larval competition increased development time, very young mosquitoes that had recently emerged from highly competitive treatments were not offered a blood meal, and only those mosquitoes within the previously stated age range were brought into the biosafety level-3 laboratory for blood feeding. Mosquitoes were sucrose-starved for 24 hours, but water was available to them, before blood was offered. Adult mosquitoes were initially offered an infectious blood meal using water-jacketed glass membrane feeders (Rutledge et al. 1964) covered with 38-42 mm hog casings (SausageMaker, Buffalo, NY) with an additional layer of Parafilm M™ (American National Can, Chicago, IL) as a barrier between the infectious blood and hog casing. Membrane feeders were attached to each other and to a Haake Series F water circulator (Thermo Haake, Paramus, NJ) which maintained the blood meal at 37°C. No mosquitoes fed from the Parafilm M plus hog casing membranes, and 24 hours later all cages of adult females were offered an infectious blood meal from cotton pledgets, each containing 3 ml of infectious blood pre-heated to 35°C for 20 min.

Mosquitoes were given 30 minutes to feed. Immediately afterwards, mosquitoes were cold anesthetized, and engorged mosquitoes were separated from unfed, which were removed from the experiment. Engorged mosquitoes were held for a 10 day extrinsic incubation period (EIP) at 27°C and provide with 20% sucrose *ad libitum*, after which surviving mosquitoes were killed by freezing. Mosquitoes were stored individually

in 1.5 ml microcentrifuge tubes at -80°C and, after thawing, wings were removed for measurements, bodies were assayed to determine infection status and titer, and legs were tested to check for disseminated infections. An assayed mosquito could be, (1) uninfected, have an (2) isolated infection, which specified a CHIKV positive body, but legs negative for the presence of the virus or have a (3) disseminated infection, which meant virus was found in the legs, indicating that the infection had spread beyond the midgut and on to other organs. A disseminated infection signifies that a mosquito is capable of transmitting the virus (Turell et al. 1984). Wings and legs were removed from each mosquito using forceps that were sterilized with 100% ethanol followed by intense flaming with a portable one-touch burner (Daigger®). Wing length was measured in millimeters as an indicator of body size (Blackmore and Lord 2000) from the alula to wing tip, excluding wing fringe. Photographic images of the wing were captured with a digital camera mounted on a dissecting microscope and measured with a computer imaging and measurement program (i-Solution lite®, AIC Inc., Princeton, NJ).

Mosquito bodies were homogenized at 25 Hz for 3 min using a Tissuelyzer® tissue homogenizer (Qiagen Inc., Valencia, CA). RNA was extracted separately from bodies and legs. Mosquito bodies were homogenized in TRI Reagent® (Molecular Research Center, Inc., Cincinatti, OH), and then RNA was extracted according to the manufacturer's protocol using 50 µl of DEPC treated water as the final elution volume. Mosquito legs were homogenized in 250 µl BA-1 media (Lanciotti et al. 2000) with two zinc-plated BBs (Daisy®), whereafter the homogenate was added to 750 µl of TRI Reagent-LS® (Molecular Research Center, Inc., Cincinatti, OH) for RNA extraction, also with a final elution volume of 50 µl in DEPC treated water.

One-step quantitative RT-PCR was used to determine infection status and body titer of samples. Primers were designed from the E1 gene and had the following sequences: forward: 5'-ACC CGG TAA GAG CGA TGA ACT-3'; reverse: 5'AGG CCG CAT CCG GTA TGT-3'; and probe: 5'-/5cy5/CCG TAG GGA ACA TGC CCA TCT CCA /3BHQ_2/-3' (IDT DNA, Coralville, IA). Reactions were performed in a 96-well reaction plate, with each reaction containing: 0.4µl SuperScript III RT/Platinum Taq mix (Invitrogen, Carlsbad, CA), 10µl 2x reaction mix (a buffer system, $MgSO_4$, dNTPs and stabilizers), 1µl forward primer (10 µmol/L), 1µl reverse primer (10 µmol/L), 0.4µl fluorogenic probe (10 µmol/L), 2.2µl DEPC-treated H_2O, and 5µl of the test sample RNA. Viral RNA was quantified using a Roche LC480 light-cycler (Roche Applied Sciences, Indianapolis, IN) with the following thermal conditions: 20 minutes at 48º C and 2 minutes at 95º C, followed by 40 cycles of PCR, 10 seconds at 95º C and 15 seconds at 60º C followed by a cool down for 30 seconds at 50º C. A negative control (DEPC-treated water in place of sample) and a positive control (CHIKV stock virus, 10^{-2} dilution) were included in each reaction run. A standard curve was generated by assaying a full range of ten-fold serial dilutions of CHIKV virus stock (7.8 Log_{10} PFU/ml) as previously described in Chapter 3.

Statistical Analysis

All statistical analyses were conducted using SAS 9.2 (SAS Institute, Cary, NC). Only data from female mosquitoes were included in the analysis. Each 5 L bucket containing a developing cohort of larvae was considered a replicate, and survivorship to adulthood per replicate was calculated as the proportion of adults that emerged from the initial cohort of first-instar larvae. Female survivorship was estimated by assuming each original cohort contained 50% of each sex. Using individual time to pupation, the mean

female time to pupation was calculated for each replicate. Female wing-length was only measured for females assayed for CHIKV infection. Two-way multivariate analysis of Variance (MANOVA) (PROC GLM) was used to analyze the effects of temperature and competitive treatment on *Ae. albopictus* female time to pupation, wing length, and survival to emergence simultaneously. Proportional data for survival to emergence were transformed using an arcsine transformation, which is recommended when percentages are outside the range 30% to 70% (Sokal and Rohlf 1995). Because of poor blood feeding by female *Ae. aegypti* very few wing-length measurements were taken of this species, therefore, this variable was removed from the female *Ae. aegypti* MANOVA analysis. MANOVAs were done separately for each mosquito species.

MANOVA creates a composite index of all measured response variables, which provides a distinct advantage over separate ANOVAs because the correlations among the variables are a factor in the model (Bray and Maxwell 1985). It is valuable to have several measures of group differences, and using multivariate methods to assess the influence of treatment on groups provide a more valid assessment of effects. All dependent variables had multivariate normal distributions within each group (temperature and competitive treatments). Pillai's trace was used to assess significance because this test statistic is robust to violations of assumptions concerning homogeneity of the covariance matrix and provides maximum protection against finding a statistical significance when there is none, with small samples (Bray and Maxwell 1985). Significant temperature and competitive treatment effects were further analyzed by multivariate pairwise contrasts of main effect multivariate means with a Bonferroni correction for experiment wise $\alpha = 0.05$ ($\alpha=0.05/3 = 0.017$). Standardized canonical

coefficients were used to determine the relative contribution of each response variable to significant multivariate effects as well as their relationship to each other (e.g., positive or negative).

Differences in CHIKV susceptibility in *Ae. albopictus* were evaluated by two-way MANOVA and standardized canonical coefficients on the response variables proportion infected (# with virus/# fed) and proportion with disseminated infection (# with virus in their legs/# with virus). Proportional data for infection and disseminated infection were transformed using an arcsine transformation. Differences in CHIKV infection and disseminated infection were also analyzed using a generalized linear mixed model (PROC GLMMIX), with temperature and competition as fixed effects and replicate as a random effect, specifying a logistic link function and a binomial error distribution. A multiplicative overdispersion component was added to the generalized linear mixed models using a simple R-side residual effect because of a higher than expected variance in the distribution of the data (Schabenberger 2007). Few numbers of blood fed *Ae. aegypti* precluded analyses of infection and disseminated infection data with MANOVA or a generalized linear mixed model (PROC GLMMIX). Overall, *Ae. aegypti* and *Ae. albopictus* mean infection and disseminated proportions and standard errors of the mean (SEM) were calculated from replicate means from 40 *Ae. albopictus* and 20 *Ae. aegypti* replicates pooled across all temperature and competitive treatments.

A generalized linear mixed model (PROC GLMMIX) was used to test for effects of temperature and competition (fixed) and replicate (random) on *Ae. albopictus* body titer, after the 10 day EIP. Body titer was a continuous variable and the model specified an identity link function and a gaussian error distribution. Titer data did not fit the model

assumptions of normality, but approximate normality was achieved through a log transformation of titer values. Differences between main effect means (temperature and competition) were further analyzed by pairwise comparisons using the LS means statement in PROC GLIMMIX.

To investigate the relationship between size and titer correlation analysis was performed on the mean size of *Ae. albopictus* females per replicate, pooled across all competitive and temperature treatments, and titer values. This analysis was also performed on the mean size of *Ae. aegypti* individuals, pooled across all competitive and temperature treatments, and titer values. In addition, to determine if there was a difference in size among uninfected, infected (non-disseminated), and disseminated females a one-way ANOVA (PROC GLM) was used. The one-way ANOVA was used to compare differences in mean wing length among the females of the three infection status categories, which were .categorical variables in the model. The females in the analysis were those that blood fed, completed the 10 day EIP, and were pooled across all larval treatments. Each species was analysed separately.

Results

Mosquitoes, Temperature, and Competition

Results from the MANOVAs showed that in both *Ae. aegypti* and *Ae. albopictus* female growth and development varied significantly as a result of temperature and competition, with no significant interaction between these variables in *Ae. albopictus* and a significant interaction term in *Ae. aegypti* (Table 4-1). An examination of the standardized canonical coefficients for temperature effects on growth parameters, shows that temperature had the greatest effect on time to pupation in both , *Ae. albopictus* and *Ae. aegypti* (Table 4-1). For *Ae. albopictus* time to pupation varied in the

same direction as wing length (Table 4-1), so lower temperature produced larger mosquitoes that took longer to pupate. Also in *Ae. albopictus* survival to emergence was negatively correlated with time to pupation and wing length, but the SCC value was small, so differences in survivorship contributed very little to the overall significance of temperature. For *Ae. aegypti* survival to emergence varied positively with time to pupation (Table 4-1), but similar to *Ae. albopictus* the smal SCC value indicated that differences in survivorship contributed only slightly to differences among temperatures.

Significant temperature effects of treatments on growth parameters of both *Ae. albopictus* and *Ae. aegypti* were further investigated in pairwise contrasts (Table 4-2). All pairwise temperature contrast for both *Ae. albopictus* and *Ae. aegypti* were significant. Associated SCC indicated that time to pupation was the primary source of differences between the pairs. Wing length contributed secondarily to pairwise differences in *Ae. albopictus* (Table 4-2). In examination of the SCC values for survivorship to adulthood in *Ae. albopictus,* survival was the highest at the middle temperature of 27°C with little difference between survival at 22°C and 32°C. In *Ae. aegypti* survivorship to adulthood was the lowest at 32°C with little difference between 22°C and 27°C (Table 4-2).

Significant competitive effects of treatments on growth parameters of both *Ae. albopictus* and *Ae. aegypti* were further investigated in pairwise contrasts (Table 4-3). All pairwise competitive contrast for both *Ae. albopictus* and *Ae. aegypti* were significant. As was found in examination of the SCC form temperature, SCC for competitive pairwise contrasts indicated that time to pupation was the primary source of differences between the pairs. *Aedes albopictus* from the 0:100 treatment replicates

were larger, took less time to emerge, and suffered less larval mortality than the two high competition treatments of 0:200 and 100:100 (Figures 4-1, 4-2, 4-3). There were also significant differences between the 0:200 and 100:100 competitive treatments for *Ae. albopictus.* The interspecific larval competition treatment produced *Ae. albopictus* that took longer to develop, had lower survivorship to emergence, and were smaller than *Ae. albopictus* produced under intraspecific competitive conditions of the same density (Table 4-3). In contrast, *Ae. aegypti* generally did better under the interspecific than intraspecific competitive conditions (Table 4-3). However, at 27°C *Ae. aegypti* survivorship to adulthood was higher in the intraspecific than the interspecific treatment (Figures 4-4, 4-5). This pattern accounts for the significant interaction between temperature and competition in this species (Table 4-1). The pairwise contrast between *Ae. aegypti* 100:100 and 0:100 was only marginally significant (P= 0.0487). Thus, the 100 *Ae. aegypti* larvae housed with 100 heterospecific *Ae. albopictus* larvae only slightly differed in survival to emergence or time to pupation from the 100:0 *Ae. aegypti* treatment.

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A total of 317 blood fed *Ae. albopictus* survived the 10 day EIP and were processed and assayed for CHIKV susceptibility. The 57 *Ae. aegypti* that survived through the 10 EIP were also processed and assayed for CHIKV, but replicate size and number were not large enough to include *Ae. aegypti* in any kind of treatment effect analysis. When pooled across treatments, the mean (± SEM) replicate infection and disseminated infection rates for *Ae. aegypti* were 0.69±0.085 and 0.72±0.093 and for *Ae. albopictus* 0.95±0.017 and 0.70±0.037 (Figure 4-6).

Results from the MANOVA show that temperature did not have a significant effect on the proportion of infected and the proportion with disseminated infections for *Aedes albopictus* (Pillai's trace = 0.112, $F_{4,62} = 0.92$, P = 0.4578) nor did competition (Pillai's trace = 0.009, $F_{4,62} = 0.07$, P = 0.9906) or their interaction (Pillai's trace = 0.107, $F_{8,62} = 0.44$, P = 0.3736) (Figure 4-7). Similarly, when *Ae. albopictus* infection data were analyzed with generalized linear mixed models (PROC GLMMIX) there were no significant effects from temperature (F=0.01, df = 2, P = 0.9940), competition (F=0.01, df = 2, P = 0.9912) or the interaction of these factors (F=0.58, df = 4, P = 0.6786) on the likelihood of infection, and on the probability of disseminated infection there were also no significant effects from temperature (F=0.67, df = 2, P = 0.5106), competition (F=2.05, df = 2, P = 0.1307) or their interaction (F=0.82, df = 4, P = 0.5150).

After the 10-day EIP, results from the generalized linear mixed model (PROC GLMMIX) showed titer of CHIKV in infected *Ae. albopictus* females was significantly affected by larval rearing temperature (F= 8.36; df = 2; P = 0.0003), but not by competition (F= 1.62; df = 2; P= 0.1999), nor the temperature x competition interaction (F= 1.33; df = 2; P<0.2580). There was no significant difference in body titer between the 22 and 27°C treatments, but both were signicantly different than the body titer of *Ae. albopictus* reared at 32°C (Figure 4-8). Correlation analysis showed no relationship for *Ae. albopictus* (*r* = 0.255; P= 0.1385, df=34) or *Ae. aegypti r* = 0.311; P= 0.1480, df=21) between wing length and log transformed-CHIKV body titer of infected mosquitoes (Figures 4-9,4-10). A one-way ANOVA showed that there were no significant wing length differences among CHIKV infection status categories for *Ae. albopictus* (F = 0.97, df = 2, P = 0.3795) or *Ae. aegypti* (F = 0.61, df = 2, P = 0.5457) (Figures 4-11, 4-12).

Discussion

In this experiment *Ae. albopictus* was the inferior larval competitor. An interspecific competive environment (100:100) produced the smallest sized *Ae. albopictus* females, with the lowest survival, and the longest pupation times. Intraspecific competition (0:200) also had an effect on *Ae. albopictus*, but the effect was not as severe on the three growth measurements as the interspecific competitive environment. In contrast, *Ae. aegypti* from the interspecific competitive environment were only slightly affected in survivorship to emergence and days to pupation (P = 0.0487), compared to conspecifics reared in the 100:0 treatment. Essentially, the presence of 100 *Ae. albopictus* had very little effect on the 100 *Ae. aegypti* developing in the same 5L bucket. Intraspecific competition affected *Ae. aegypti* and the 200:0 treatement was the poorest performer. This confirmed similar findings by Lounibos et al. (2002) in which *Ae. aegypti* female growth was uniquely retarded by a high density of its own species.

In the water-filled containers that serve as habitat for *Ae. aegypti* and *Ae. albopictus* in Florida the primary food source is microorganisms such as bacteria and fungi that grow on decaying oak and other leaf detritus and parts of dead invertebrates (Fish and Carpenter 1982, Lounibos et al. 1992, Lounibos et al. 1993). In this environment, where the dominant resource is leaf litter, *Ae albopictus* is the superior competitor (Barrera 1996, Juliano 1998). To successfully execute an experiment on CHIKV vector competence it was essential to generate enough adult mosquitoes that also emerged within a reasonable time span, and leaf litter as the sole food source in preliminary studies did not produce enough adults in an acceptable age range. A combination of oak leaf infusion and an artificial food source of a one-to-one mixture of brewer's yeasts and lactoalbumin, was used as a compromise between the need for a

reliable number of mosquitoes within a narrow age range and the maintenance of ecological relevance for the experiment. Because competitive interactions betweenthese two mosquitoes are context dependent, and the relative effects of competition may change under different ecological conditions (Juliano 2009) it was not surprising that *Ae. aegypti* was the superion larval competitor. In previous studies the type of food resource affected the outcome of competiton between these two *Aedes* species, with the addition of a high protein food (i.e. yeast, lactoalbumin, liver powder) favoring *Ae. aegypti* (Black et al. 1989, Barrera 1996, Daugherty et al. 2000).

Differences in temperature did not change the outcome of competition, which confirmed previous results of Lounibos et al. (2002), when competition between these two species was investigated at 24° and 30°C. As expected, based on results in Chapters 2 and 3, temperature did have an effect on pupation time and size in both *Aedes* species, with low temperature increasing development time and leading to larger sized adults. Temperature also had a small effect on survivorship to adulthood in *Ae. albopictus,* survival was the highest at the middle temperature of 27°C and in *Ae. aegypti* survivorship to adulthood was high at both 22°C and 27°C. Therefore, the low temperature only seemed to have a negative effect on survival in *Ae. albopictus*. For *Ae. aegypti* there was a significant interaction between temperature and competition and how temperature modified the effect of competition is seen in Figure 4-4, where the 200:0 treatment had greater survival than the 100:100 at 27°C, while at the other temperatures and for the other growth measurement, time to pupation, the 100:100 treatment out performed the 200:0 for *Ae. aegypti.*

Neither competitive interactions nor temperature treatment had an effect on CHIKV infection or disseminated infection for *Ae. albopictus.* However, temperature did have a significant effect on CHIKV body titer in *Ae. albopictus*. The lack of a temperature effect on infection and disseminated infection in *Ae. albopictus* was unexpected because Chapters Two and Three demonstrated that aquatic larval temperature had an effect on CHIKV infection in both *Ae. aegypti* and *Ae. albopictus* and larval food level and temperature had an effect on disseminated infections in *Ae. aegypti*. In addition, larval rearing temperature has been shown to affect mosquito competence for viruses of Rift Valley fever (RVFV), Venezuelan equine encephalitis (VEEV) (Turell 1993), Murray Valley encephalitis (MVEV) (Kay et al. 1989a), Japanese encephalitis (JEV) (Takahashi 1976), and western equine encephalitis (WEEV) (Hardy et al. 1990). Larval competition has been shown to affect *Ae. triseriatus* vector competence for LaCrosse virus (LACV) (Bevins 2008) and *Ae. albopictus* vector competence for viruses of Sindbis (SINV) (Alto et al. 2005) and dengue (Alto et al. 2008a).

Body titer of CHIKV infected *Ae. albopictus* females was significantly affected by larval rearing temperature, with females reared at the two lower temperatures of 22° and 27°C having higher meanCHIKV body titers than counterparts from 32°C. This effect is in agreement with results from Chapter Two, in which CHIKV susceptibility was greater in *Ae. albopictus* adults reared at lower temperatures compared to individuals from higher rearing temperature treatments.

The lack of a significant effect due to larval competition and temperature on CHIKV infection and disseminated infection in this study is most likely due to low

sample size and the general logistical difficulties of bringing an ecological experiment into a laboratory setting. The mosquitoes used were first generation progeny of field collected individuals. It was very difficult to get recently derived field colonies of *Ae. albopictus* and *Ae. egypti* to feed on an artificial blood sources. The original experimental design used water-jacketed glass feeders with hog casing membranes, with parafilm added to prevent the membranes from leaking. In preliminary experiments sufficient feeding took place on the hog casing leading to the belief that is would be a good feeding method. However, the parafilm was a last minute required addition in order to comply with BL-3 protocols established at FMEL, and the added barrier seems to have inhibited feeding on the parafilm-hog casing combination. Subsequently, an alternative protocol using blood-soaked, heated cotton pledgets was attempted. Because previous studies showed that only when *Ae. aegypti* fed on pledgets soaked in a very high titer of CHIKV infectious blood, is this species able to establish a midgut infection (Pesko et al. 2009), it was decided that a high ratio of 1:10 virus supernantant to defibrinated bovine blood would be used. This produced a blood meal titer of 7.4 Log_{10} plaque-forming units (PFU)/mL, which was markedly higher than blood meal titers used in prior experiments and historic human viremias, which have generally not circulated above 7.0 $log_{10}TCID_{50}$/ml (Jupp and McIntosh 1988).

Unfortunately, a combination of low feeding on the pledgets, combined with a large amount of mortality during the 10 day EIP led to entire missing replicates from the different temperature and competitive treatments for *Ae. aegypti*, and it was necessary to remove this species from the infection and dissemination analyses. *Aedes aegypti* developed faster than *Ae. albopictus* maintained in an identical growth environment, but

both species in interspecific larval treatements needed to be reared simultaneously leading to adult *Ae. aegypti* that were older than *Ae. albopictus*. The older age of *Ae. aegypti* combined with the somewhat stressful rearing conditions may have caused the higher adult mortality that was noted for this species over the 10 day EIP.

For *Ae. albopictus* the high blood meal titer led to a mean infection rate of approximately 95% among replicates pooled across temperature and competitive treatments, which likely prevented any observed treatment effect on infection rates. Previous laboratory studies with historic epidemic CHIKV isolates have indicated that, in the laboratory *Ae. albopictus* is a significantly more competent vector than *Ae. aegypti* (Mangiafico 1971; Turell et al. 1992) and this superior laboratory competence of *Ae. albopictus* is even more exaggerated with the emergent E1-A226V CHIKV isolate (Tsetsarkin et al. 2007, Pesko et al. 2009). Titers of between 4.0 and 5.0 $\log_{10}TCID_{50}$/ml resulted in about a 90% infection rate in *Ae. albopictus* and a <10% infection rate in *Ae. aegypti* and at titers above 6.0 $\log_{10}TCID_{50}$/ml infection approached 100% in *Ae. albopictus* and 30% in *Ae. aegypti* (Tsetsarkin et al. 2007). In this study, replicate means and SEMs for the overall proportion with infections and with disseminated infections pooled across treatments. suggest lower infection rates in *Ae. aegypti* compared with *Ae. albopictus* for the emergent E1-A226V CHIKV isolate (Figure 4-6).

With such high infection rates in *Ae. albopictus* absence of a treatement effect on infection was partly expected. Among all nine temperature-competitive treatments average infection rates were between 90 and 100% (Figure 4-7). The lowest proportions of infected individuals were among the three competitive treatements run at 22°C. The overall disseminated infection rate among infected individuals for *Ae. albopictus* was

70% (Figure 4-6). Among the nine temperature-competitive treatments proportions of disseminated infections varied between approximately 50 and 80% (Figure 4-7), however low sample sizes and large standard errors decreased the ability to find statistically significant differences between treatements.

Body size for neither *Ae. albopictus* nor *Ae. aegypti* was correlated with CHIKV body titer, and body size had no significant effect on whether either species established an isolated or disseminated infection. In prior experiments, size was shown to influence susceptibility of *Ae. aegypti* for DENV (Sumanochitrapon et al. 1998, Alto et al. 2008b) and Ross River virus (RRV) (Nasci and Mitchell 1994). In this experiment the absence of significant correlations was somewhat expected because of the mostly non-significant temperature and competitive treatment effects on CHIKV susceptibility, since both temperature and competition have asignificant influence on size. However, temperature did have an effect on CHIKV body titer in *Ae. albopictus*, but size was not found to be significantly correlated with body viral titer in either species of mosquitoes. This may be because CHIKV body titers were very high overall, which possibly resulted from the high CHIKV titers in the blood meal these mosquitoes were fed. In both figures 4-9 and 4-10 the majority of the CHIKV body titer values are clustered in a fairly narrow range of titers for *Ae. albopictus* between 6.0-6.5 Log_{10} pfu/0.1ml and *Ae. aegypti* between 6.3-7.0 Log_{10} pfu/0.1ml. These high ranges of titers may represent biological maximum titers for these species, which may have led to the lack of significant correlations.

In summary, inter- and intra-specific competitive interactions between larval *Ae. aegypti* and *Ae. albopictus* influenced immature survival, the length of the larval period, and body size. Under these specific experimental conditions, in which a protein-rich

artificial food source was provided to developing larvae, *Ae. aegypti* was the superior competitor across all three temperature treatments. Larval temperature and competition did not influence the likelihood of CHIKV infection or disseminated infection, but CHIKV body titers were significantly greater in female *Ae. albopictus* from the lower larval temperatures. The larval environment strongly influences adult size, but in this study there was no significant relationship between mosquito size and measures of CHIKV susceptibility. Future studies will be aimed at exploring what other physical or physiological traits may play roles in predicting vector susceptibility.

Table 4-1. MANOVA for temperature and competitive treatment effects and their interaction on population growth parameters of female *Aedes albopictus* and *Aedes aegypti* : time to pupation, juvenile survivorship, and wing length

Comparison	Pillai's trace (num df, den df)	P	Standardized canonical coefficients		
			Time to Pupation	Survivorship	Wing
Aedes albopictus					
Temperature	1.12 (6, 58)	<0.0001	3.398	-0.153	0.978
Competitive treatment	0.96 (6, 58)	<0.0001	-2.633	1.049	0.821
Temperature x competitive treatment	0.50 (12, 90)	0.1351			
Error d.f.	30				
Aedes aegypti					
Temperature	1.30 (4, 72)	<0.0001	4.854	0.245	-
Competitive treatment	0.88 (4, 72)	<0.0001	4.671	-0.222	-
Temperature x competitive treatment	0.52 (8, 72)	0.0040	2.821	1.231	
Error d.f.	36				

Table 4-2. Multivariate pairwise contrasts of temperature treatment effects on female *Aedes albopictus* and *Aedes aegypti* for growth measurements time to pupation, juvenile survivorship, and wing length.

Competitive treatment pairwise comparisons	Pillai's trace (num df, den df)	P	Standardized canonical coefficients		
			Time to Pupation	Survivorship	Wing
Aedes albopictus					
22°C vs. 27°C	0.86 (3, 28)	<0.0001	3.411	-0.435	0.954
22°C vs. 32°C	0.91 (3, 28)	<0.0001	3.374	-0.029	0.983
27°C vs. 32°C	0.47 (3, 28)	0.0004	2.740	0.985	0.897
Aedes aegypti					
22°C vs. 27°C	0.94 (2, 35)	<0.0001	4.849	0.072	
22°C vs. 32°C	0.96 (2, 35)	<0.0001	4.834	0.313	
27°C vs. 32°C	0.59 (2, 35)	<0.0001	3.757	1.013	

Table 4-3. Multivariate pairwise contrasts of competitive treatment effects on female *Aedes albopictus* and *Aedes aegypti* for growth measurements time to pupation, juvenile survivorship, and wing length.

Competitive treatment pairwise comparisons	Pillai's trace (num df, den df)	P	Standardized canonical coefficients		
			Time to Pupation	Survivorship	Wing
Aedes albopictus					
0:100 vs. 0:200	0.83 (3, 28)	<0.0001	-2.409	1.281	0.691
0:100 vs. 100:100	0.92 (3, 28)	<0.0001	-2.676	0.998	0.848
0:200 vs. 100:100	0.54 (3, 28)	<0.0001	-3.046	0.406	1.101
Aedes aegypti					
100:0 vs. 200:0	0.72 (2, 35)	<0.0001	4.518	-0.356	
100:0 vs. 100:100	0.16 (2, 35)	0.0487	-0.883	1.337	
200:0 vs. 100:100	0.67(2, 35)	<0.0001	4.688	-0.243	

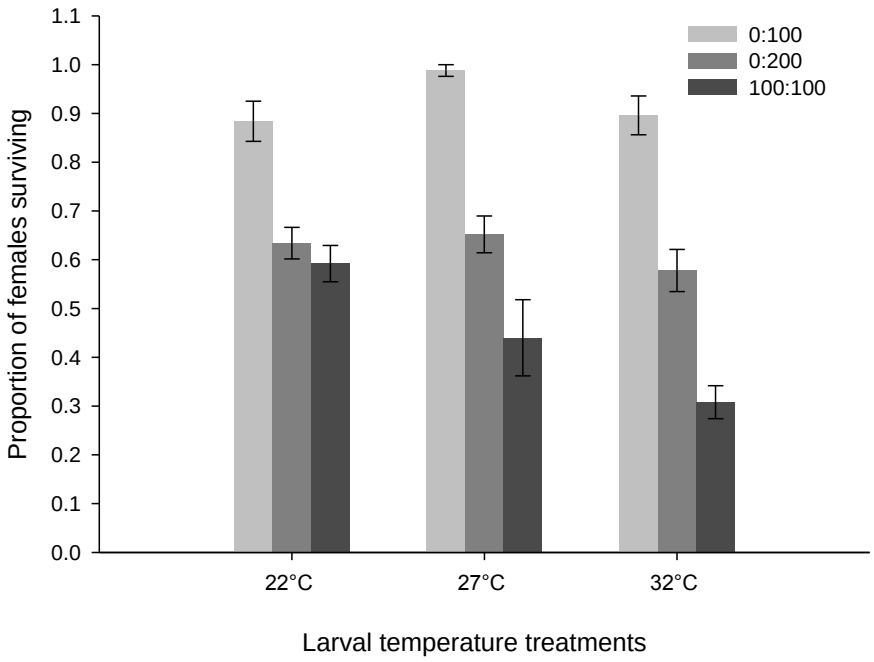

Figure 4-1. Proportion of female *Ae. albopictus* (±SEM) surviving to adult emergence.

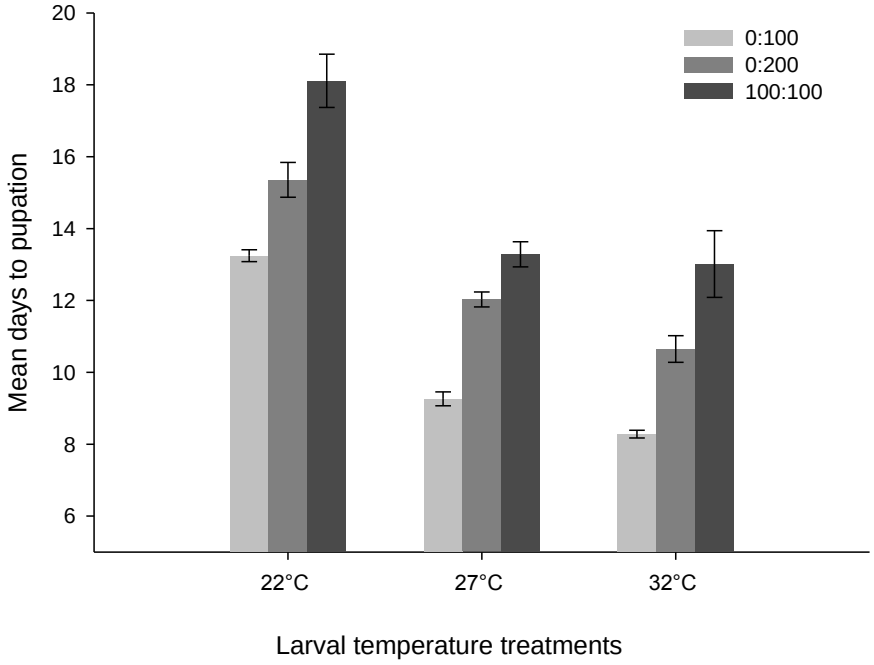

Figure 4-2. Female *Ae. albopictus* mean (±SEM) days to pupation.

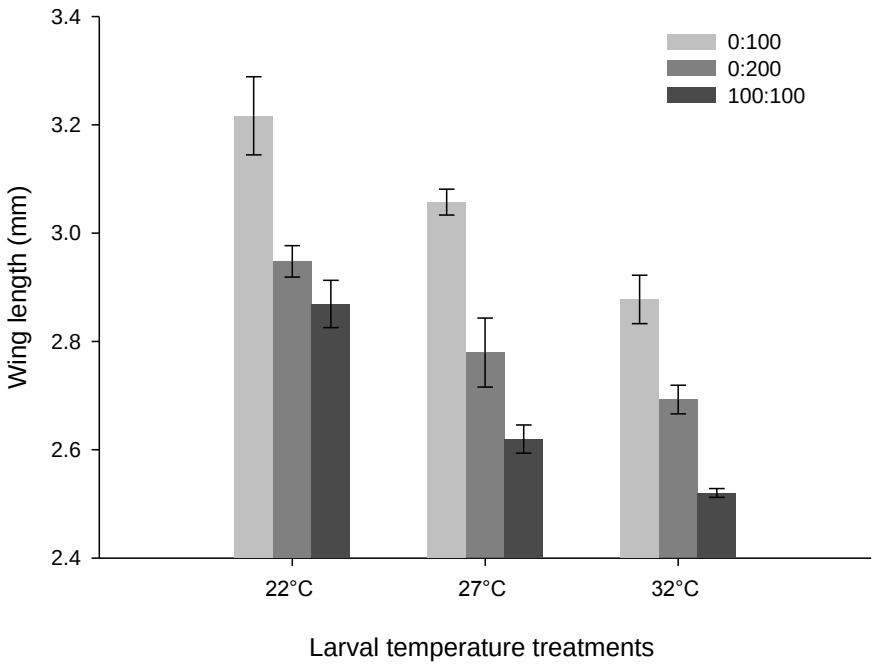

Figure 4-3. Female *Ae. albopictus* mean (±SEM) wing length.

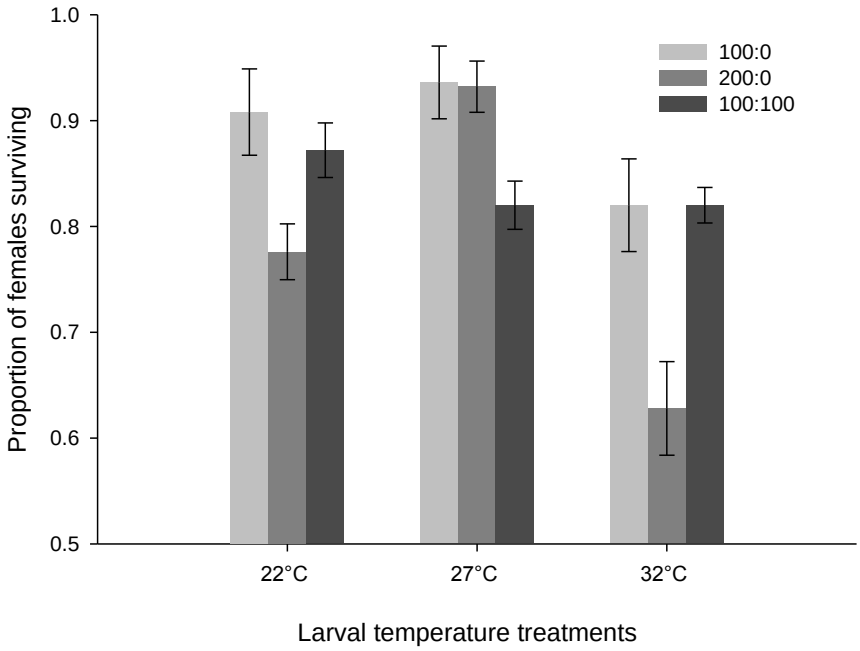

Figure 4-4. Proportion of female *Ae. aegypti* (±SEM) surviving to adult emergence.

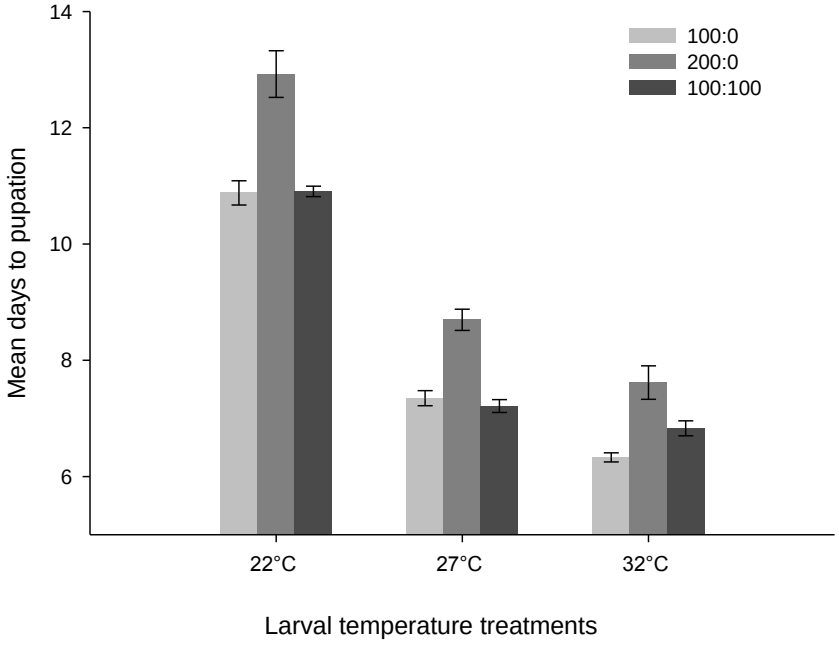

Figure 4-5. Female *Ae. aegypti* mean (±SEM) days to pupation.

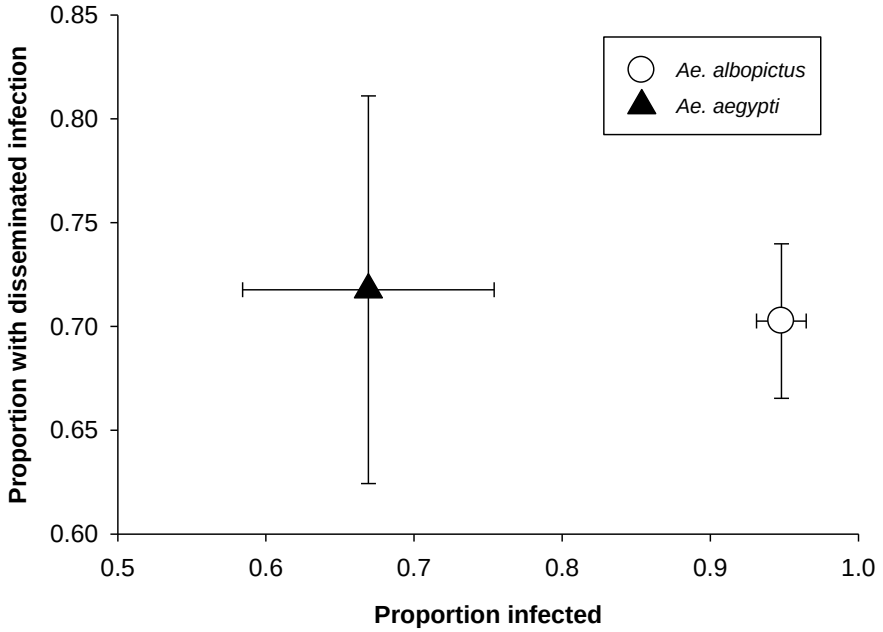

Figure 4-6. Bivariate means (±SEM) of replicates for proportions of *Ae. aegypti* and *Ae. albopictus* with infections and disseminated infections.

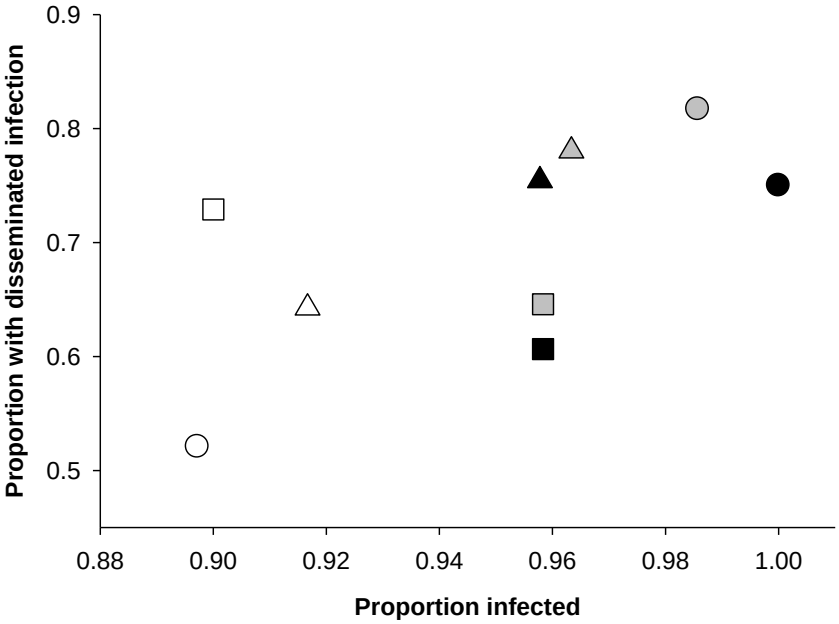

Figure 4-7. Bivariate plot of least squares means for proportion of *Ae. albopictus* with infections and disseminated infections. Open symbols specify 22°C, grey symbols 27°C, and black symbols 32°C. Triangles represent 0:100, circles 0:200, and squares 100:100 treatment.

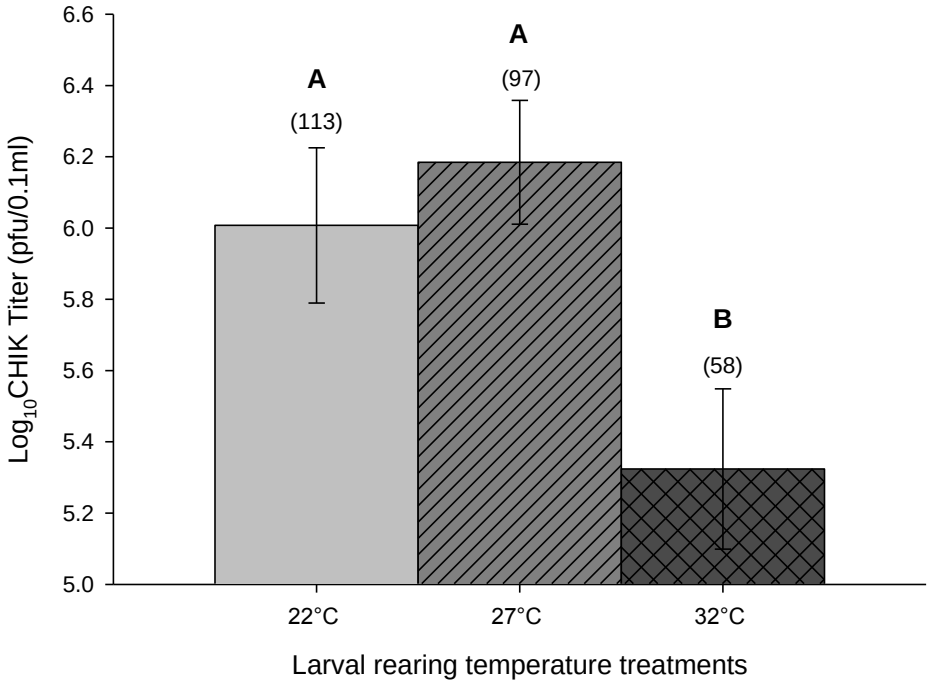

Figure 4-8. LS means (±SEM) of *Aedes albopictus* CHIKV body titer for temperature treatments, LS means with different letters are significant at a per comparison P-level of 0.017.

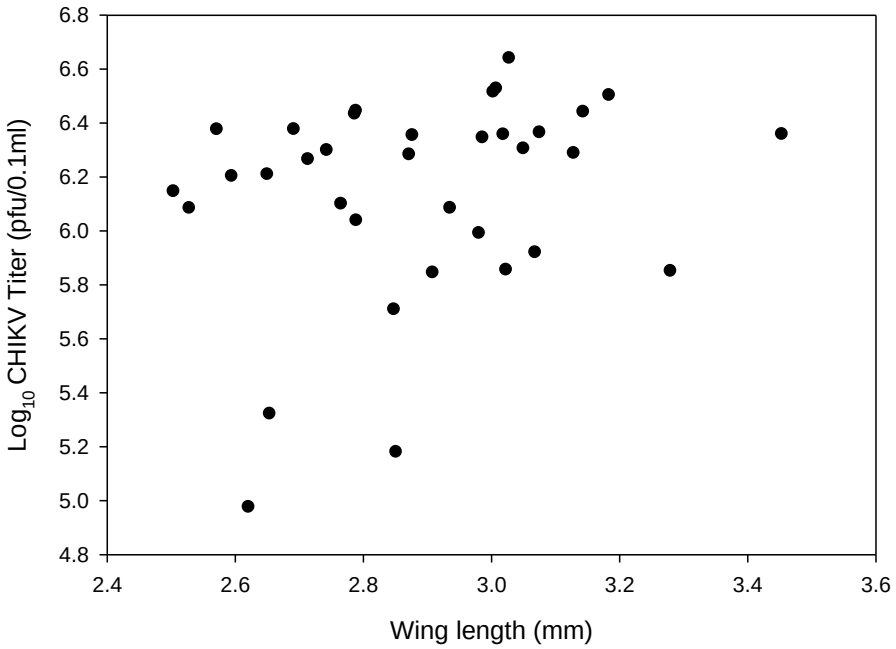

Figure 4-9. Correlation analysis of log transformed of CHIKV whole mosquito body titer and wing length replicate means for *Aedes albopictus* pooled across temperature and competitive treatments (r = 0.255; P = 0.1385).

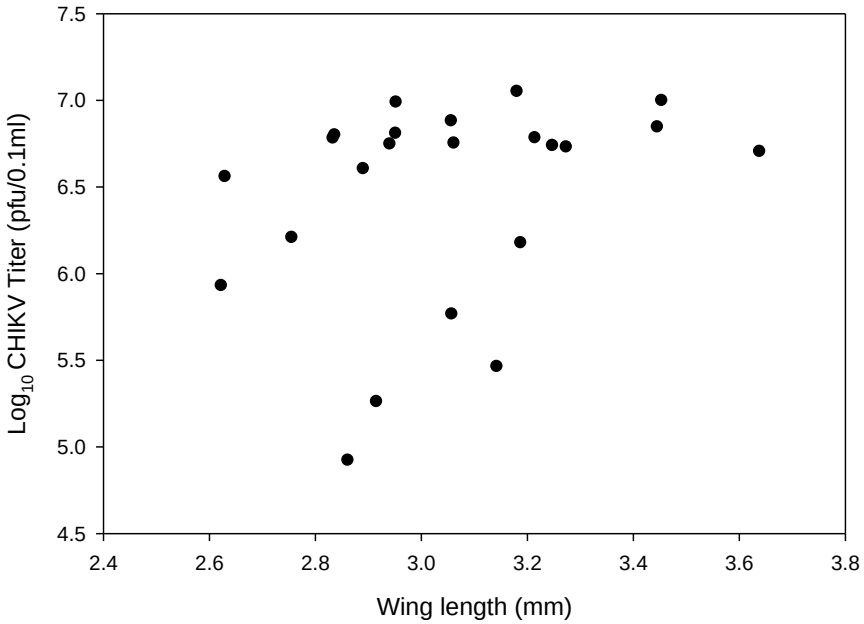

Figure 4-10. Correlation analysis of log transformed of CHIKV whole mosquito body titers and wing lengths for *Aedes aegypti* infected individuals pooled across temperature and competitive treatments (r = 0.311; P = 0.1480).

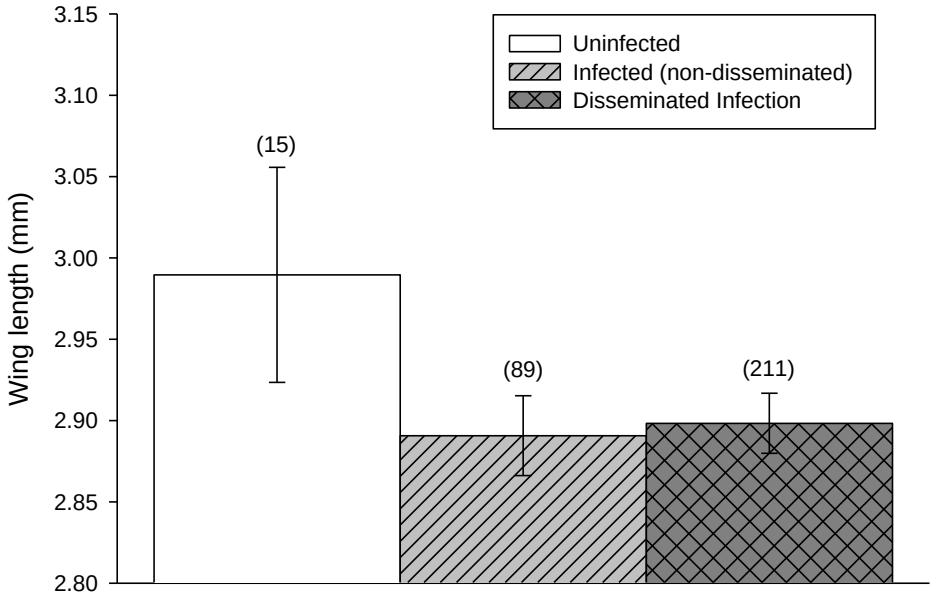

Figure 4-11. Least squared means (±SE) for sizes of adult female *Ae. albopictus* mosquitoes in CHIKV infection status categories.

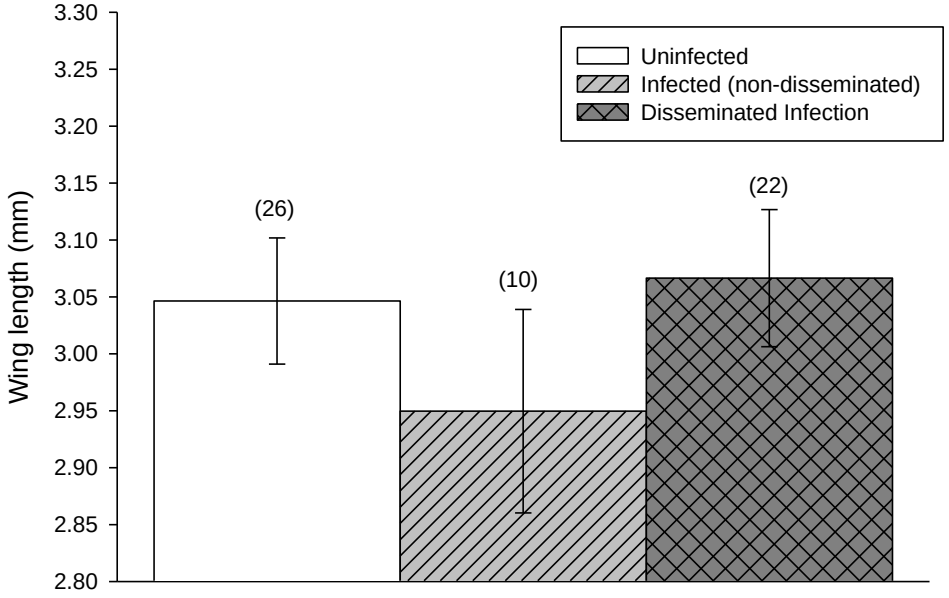

Figure 4-12. Least squared means (±SE) for sizes of adult female *Ae. aegypti* mosquitoes in CHIKV infection status categories.

CHAPTER 5
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The primary focus of this study was to explore the extent to which vector susceptibility is influenced by the larval environment within the *Ae. aegypti*, *Ae. albopictus* and chikungunya virus system. Previous studies with *Ae. aegypti* and DENV serotype-2 estimated that environmental variance accounted for up to 78-91% of the total phenotypic variance in vector competence (Bosio et al. 1998). Clearly, environmental effects are an important component of vector susceptibility and overall disease epidemiology (Tabachnick 2009). Many studies have looked at the effect of temperature, humidity, and other environmental effects on adult mosquito competence, but little attention has been paid as to how the larval environment may shape traits involved in adult vector competence.

Varying temperature was a common treatment in the three experiments described in this dissertation. Larval rearing temperature influenced *Ae. albopictus* and *Ae. aegypti* growth and susceptibility to CHIKV. In *Ae. albopictus,* CHIKV infection rates decreased with increasing larval rearing temperature and decreasing adult size. Also in *Ae. albopictus*, decreased larval temperature was associated with higher CHIKV body titers among infected individuals. In *Ae. aegypti,* larval temperature also influenced the likelihood of infection and disseminated infection, with the intermediate temperature of 27°C (22-32 degree range) resulting in the highest rates of infection and dissemination.

The larval habitat in nature is composed of multiple biotic and abiotic features that interact. To simulate some of the complexity that exists in the natural larval environment, differing levels of nutritional resources and intra- and interspecific competitive treatments were added to temperature treatments in experiments described

in Chapters 3 and 4, respectively. For *Ae. aegypti* higher levels of nutritional resources significantly increased the likelihood of having a disseminated infection. On the other hand, larval competition had no effect on susceptibility to CHIKV infection in either species of mosquito, but it did have a significant effect on growth parameters in both *Ae. aegypti* and *Ae. albopictus.*

It is unclear why *Ae. aegypti* and *Ae. albopictus* reared at different temperatures and different food levels were more susceptible to CHIKV. When the mesenteronal tissues from large and small *Ae. triseriatus* females reared at differing food levels, were examined with electron microscopy physical differences in the basement membrane (basal laminae) were found. Small *Ae. triseriatus* exhibited higher rates of transmission and disseminated infections for LACV and their basement membranes had fewer laminae resulting in a reduced mean thickness when compared with larger individuals (Grimstad and Walker 1991). It was suggested that the thinner basement membrane might allow for the more rapid release of LACV in to the hemocoel (Grimstad and Walker 1991). With the use of microscopy, a rapid release of EEEV into hemocoel due to disruptions in the posterior midgut was observed in *Culiseta melanura* immediately following an infectious blood meal (Weaver et al. 1991) and the concept of a “leaky midgut” has been supported by the rapid appearance of arbovirus in the hemocoel in other vector-viral systems (Boorman 1960, Miles et al. 1973, Hardy et al. 1983, Weaver 1986).

In Chapter 3, *Ae. aegypti* reared at two different food levels crossed with three different temperatures had significantly different dissemination rates, with the proportion of *Ae. aegypti* from the higher food level having a greater odds of developing a

disseminated infection. This contrasts with results for small *Ae. triseriatus* from the low food level (Grimstad and Walker 1991), although there was no significant relationship between size and susceptibility in the experiment with *Ae. aegypti* described in Chapter 3. It is possible that the mechanism responsible for differences in CHIKV susceptibility in *Ae. aegypti* from different food levels also involves anatomical features of the midgut cells and electron microscopy studies could help elucidate a mechanism.

Large *Ae. albopictus* generated, in Chapter 2, through low temperatures were more susceptible to CHIKV, which is in agreement with increased infection in *Ae. taeniorhynchus* for RVF and VEEV (Turell 1993), but contradicts previously described patterns between small *Ae. triseriatus* and LACV (Grimstad and Walker 1991). In other studies, large *Ae. aegypti* mosquitoes generated by increasing larval food quantities were significantly more susceptible to RRV (Nasci and Mitchell 1994) and DENV (Sumanochitrapon et al. 1989) than small mosquitoes. It is likely that virus enters midgut cells through receptor mediated endocytosis (Hardy el al. 1983) and viral determinants for mosquito midgut infection have been studies for a number of arboviruses (Ludwig et al 1989, Mourya et al. 1989, Houk et al. 1990, Mertens et al. 1996, Xu et al. 1997, Pletnev et al. 2001, Molina-Cruz et al. 2005, Smith et al. 2008). It is possible that the expression of viral determinants or receptors in the midgut is influenced by rearing temperature or larger mosquitoes generated through different larval conditions have a greater number of receptors or enhanced binding (Turell 1993). This is certainly a mechanism worth exploring and identification of viral binding sites or receptors would greatlt increase our understanding of vector-viral interactions.

Because all growth of mosquitoes is accomplished during the aquatic larval period, which may be long at colder temperatures and under stressful conditions of limited food and high competition, it seems likely that these environmental features are shaping insect immune pathways, and other physiological and anatomical features of the adult mosquito that may be more strongly correlated than body size to measures of arbovirus susceptibility. It is also possible that how body size is achieved can completely change the susceptibility of a mosquito for an arbovirus. Future experiments should investigate the underlying physiological and/or molecular mechanisms that are influenced by the larval environment and lead to differential vector competence.

Because laboratory colonization can cause significant changes in phenotype and genotype of organisms, first or second generation *Aedes*, the progeny of field collected parents or grandparents, were used in this study to more realistically represent the types of interaction that would take place in natural populations. Colonization often leads to a decrease in heterozygosity and a shift in allele frequencies due to selection, drift, non-random mating, and founder effect (Munstermann 1980, Lorenz et al. 1984, Mason et al. 1987). Frequencies of genes involved in vector competence may be influenced by evolutionary forces that accompany colonization, and the use of colonies freshly derived from field collected mosquitoes may limit the effect of selection. However, it was a challenge to get newly established colonies of *Ae. aegypti* and *Ae. albopictus* to feed on an artificial blood source. In the first two experiments Edicoll® collagen film was used as membrane for the mosquitoes to feed through in combination with water jacket glass feeders connected to a water circulator. Edicoll® collagen film is a manufactured product used as a casing for sausages and hotdogs. Low to moderate

feeding was accomplished with the film, and for the third experiment hog casing was used after a preliminary experiment established that its use increased blood feeding success. As described in Chapter 4, the hog casing and water jacketed feeder system was replaced by blood-soaked pledgets because the addition of parafilm as a secondary membrane over the hog casing inhibited feeding. The unanticipated switch to a different feeding method is one example of difficulties encountered while trying to get a substantial number of F_1 *Ae. aegypti* and *Ae. albopictus* to feed in the laboratory during this study.

This dissertation is a general exploration as to whether variations in temperature, food, and competition in the larval environment affect adult CHIKV susceptibility. An effect from temperature and food was established, and now the more difficult task of modeling mosquito viral susceptibility response patterns (infection, disseminated infection, and viral titer) to changes in individual and combined larval environmental factors may be a future goal. Some of the other factors in the study that most likely influenced mosquito susceptibility to CHIKV, but were held constant among treatments, were blood meal titer, adult holding temperature and the length of EIP. These factors are known to influence absolute values of infection, disseminated infection, and body titer, and variation in response to changing values of titer, adult temperature, and EIP would need to be included in future work in which predictability is a goal.

If relationships between viral susceptibility and larval environment can be elucidated and, in addition, a mosquito trait or traits are identified that are a product of the environment and are correlated to viral susceptibility, this could contribute to the predictability and risk assessment of epidemics. Pupal productivity surveys are common

in areas with container inhabiting mosquitoes and endemic disease such as dengue, yellow fever, chikungunya and filariasis. Surveys of immature mosquitoes are normally used as a method to assess vector population densities in a given area. More specifically, estimates of pupal abundance of *Aedes* vectors of DENV, have been promoted as a more accurate index of potential female vectors than traditional larval surveillance (Strickman and Kittayapong 2003). If pupal surveys could incorporate measurements of habitat quality and pupal or adult mosquito physical attributes related to susceptibility, the additional information obtained could result in a much more powerful and directed approach of vector and disease control.

Lastly, a clear understanding of how ecological factors in the larval environment influence vector competence, will be an important element in the use of genetically modified mosquitoes to control vector-borne disease. Genetically modified mosquitoes are not completely refractory to the pathogens they transmit. Vector competence may be significantly limited, as is the case with *Anopheles stephensi* that express the bee venom phospholipase A2 (PLA2) gene leading to a reduction in *Plasmodium berghei* oocyst formation by 87% (Moreira et al. 2002) or that express the C-type lectin CEL-III from the sea cucumber, *Cucumaria echinata*, resulting in only a moderate inhibition against *P. falciparum* (Yoshida et al. 2007). Because vector competence is heavily influenced by the environment it seems likely that the expression of the inserted genes may also be influenced by how the mosquito responds and develops to a changing environment.

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BIOGRAPHICAL SKETCH

Catherine Jane Westbrook was born in Washington, D.C. in 1972. She graduated from the University of California, Berkeley in 1996 with a Bachelor of Arts degree in integrative biology. In 2003, she received her Master of Science degree from Cornell University in entomology. In 2007, she began her doctoral work at the University of Florida under the guidance of Dr. L. Philip Lounibos at the Florida Medical Entomology laboratory in Vero Beach, FL.