

DISSERTATION

INVASIVE MOSQUITOES, LARVAL COMPETITION, AND POTENTIAL  
IMPLICATIONS FOR VECTOR COMPETENCE

Submitted by

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Graduate Degree Program in Ecology

In partial fulfillment of the requirements

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Colorado State University

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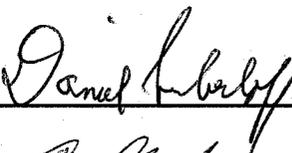
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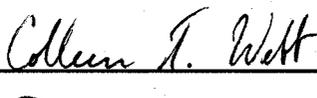
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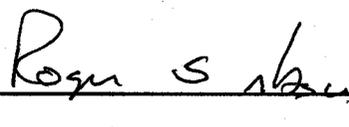
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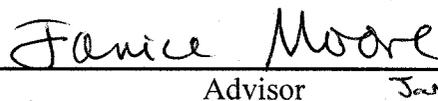
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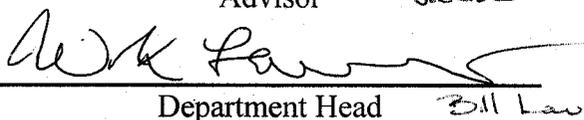
  
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## ABSTRACT OF DISSERTATION

### INVASIVE MOSQUITOES, LARVAL COMPETITION, AND POTENTIAL IMPLICATIONS FOR VECTOR COMPETENCE

*Aedes albopictus* is an invasive mosquito species which established rapidly in the US. It is characterized as an aggressive biter, prolific breeder, and dominant larval resource competitor. Declines in resident mosquito species have followed its arrival, and species that persist often emerge from shared larval habitats as small, nutritionally deprived adults. *Aedes albopictus* is a competent disease vector in both its native range and in areas of recent establishment. It therefore has the potential to alter vector-borne disease transmission either directly, by creating a new pathogen association, or indirectly, via interactions with native mosquito vectors.

*Ochlerotatus triseriatus* is a native mosquito species that shares larval habitat with *Ae. albopictus* in regions of sympatry. Interspecific competition has been documented between these two species. Replacement series experiments were carried out in the laboratory to determine the extent of intraspecific and interspecific larval competition between *Oc. triseriatus* and *Ae. albopictus*. Competitive consequences were only documented when nutrition was added to treatments in a single pulse, versus the same nutritional amount being gradually added over time. Resource amount and input appear to determine if the two species co-exist or if the native species is competitively excluded in the laboratory.

The invasive species was a dominant larval resource competitor, decreasing native species survival to adulthood. Decreased *Oc. triseriatus* survival, however, led to reduced intraspecific competition, with survivors from high mortality treatments being larger than survivors from low mortality treatments. These larger native females were more likely to host-feed, and more likely to develop both La Crosse virus midgut and disseminated infections. Results indicate that presence of the invasive species reduced native species survival, but the resulting reduction in intraspecific competition leads to fewer surviving females that have an enhanced ability to participate in La Crosse virus transmission. *Ochlerotatus triseriatus* fecundity was also reduced when *Ae. albopictus* was present, but vertical transmission rates of LACV were unaffected.

Field surveys revealed rapid spread and establishment of a newly introduced mosquito species, *Ochlerotatus japonicus*. Its pervasive presence highlights the need for increased understanding and monitoring of invasive vector species, as well as vigilance in preventing the arrival of others.

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## **Chapter 1**

### **Larval nutrition, adult size, and vector competence in medically important disease vectors: a review**

#### **Abstract**

Many factors influence the ability of an arthropod vector to transmit a pathogen. One factor that has been explored in medically important mosquitoes is the relationship among larval resource availability, adult vector size, and pathogen transmission; however, despite a great deal of research effort, the relationship between vector size and transmission efficiency remains unresolved. Here, I review the literature on how vector size interacts with ability to transmit a pathogen, as well as how larval environment influences vector size, immune function, and behavior.

#### **Introduction**

Arthropods are known transmitters of numerous medically important diseases throughout the world. Mosquitoes in particular have received attention for their ability to come into contact with, harbor, and transmit infectious pathogens to humans and livestock. Several factors play a role in the ability of a mosquito to be a competent vector and successfully transmit a disease. External factors such as larval habitat characteristics and host availability can play a role, as well as internal factors, such as genetics that can make a vector a permissive pathogen carrier, or refractory to infection entirely.

One area of research has addressed the effect vector size has on the efficiency with which mosquitoes transmit pathogens. Takahashi (1976) stimulated numerous studies which indicated smaller female mosquitoes to be more efficient at transmitting an arbovirus when compared to larger females (Baqar et al. 1980, Grimstad and Haramis 1984, Paulson and Hawley 1991, Alto et al. 2005, Anderson et al. 2005). Results from subsequent research have supported this pattern in some cases, and offered contradictory evidence in others. Still other studies have found adult mosquito size to have no effect on pathogen transmission. These inconstancies are perplexing, and comprehending the reasons behind these conflicting results is imperative if we are to understand the role adult mosquito size plays in pathogen transmission efficiency. Clarifying this relationship may also shed light on what variables impact mosquito size and the consequences for vector populations and their associated pathogens.

The following explores patterns of vector size and pathogen transmission, patterns that emerge from a complex interplay of several factors. (1) Adult vector size is influenced by the amount of nutrition available in the larval stage. (2) Enhanced pathogen transmission has been documented in small vectors, setting the stage for increased disease circulation in the presence of nutritionally deprived vectors, but discrepancies exist across the literature. (3) Some of these discrepancies may result from a limited understanding of the mechanisms underlying vector-borne transmission efficiency. (4) Additionally, nutrition impacts immune system function as well as vector behavior, but how these then impact pathogen transmission has yet to be fully integrated into research on vector size and pathogen transmission.

## **Size and Nutrition in Mosquitoes**

Adult mosquito size differences are at least partly due to the amount of nutrition available in the larval environment, the effects of which echo throughout the adult stage (Christophers 1960). While genetics are almost certainly influential in overall size attained in mosquitoes (Anderson et al. 2005), the larval environment has repeatedly been shown to affect mosquito body size, an extremely plastic trait (Takahashi 1976, Baqar 1980, Grimstad et al. 1980, Grimstad and Walker 1991, Paulson and Hawley 1991, Novak et al. 1993, Nasci and Mitchell 1994, Suwanchaichinda and Paskewitz 1998, Reiskind et al. 2004, Alto et al. 2005). For instance, mosquitoes reared in laboratory conditions are larger than their field counterparts, indicating that nutrient limited larval habitats occur naturally and are ubiquitous (Paulson and Hawley 1991). Additionally, laboratory reared progeny of field mosquitoes have been shown to be significantly larger than their parents (Klowden et al. 1988). Size differences among progeny disappear when reared under consistent lab conditions, even when parent size is highly variable and correlated with conditions of larval habitat in the field (Paulson and Hawley 1991).

Limited nutrient availability in the larval environment can often be traced back to one of two factors, low productivity environments with limited resource input, highly competitive conditions, or a combination of the two. Larval habitats with limited nutrients are common and yield adult mosquitoes that are smaller than counterparts from high productivity environments (Fish and Carpenter 1982, Walker et al. 1987, Mahmood et al. 1997, Sumanochitrapon et al. 1998, Suwanchaichinda and Paskewitz 1998, Gimnig et al. 2002). As for competitive interactions among mosquitoes, both intraspecific and

interspecific effects have been repeatedly demonstrated in the laboratory (Black et al. 1989, Ho et al. 1989, Novak et al. 1993, Tsuda and Takagi 2001, Alto et al. 2005) and in the field (Juliano 1998, Lounibos et al. 2001, Braks et al. 2004, Juliano et al. 2004b). High densities of mosquito larvae in resource poor habitats often produce smaller adult mosquitoes. Asymmetric competitive interactions, where one species routinely out-competes the other, also result in smaller adult body size in the less dominant species. Such reduced size has been documented in native mosquito species following the arrival of some invasive mosquito species. For example, the establishment of *Ae. albopictus*, an extremely efficient larval resource competitor, led to population declines in some mosquito species in the US, and body size reduction in other species that were emerging from shared larval habitats (Juliano 1998, Lounibos et al. 2001, Lounibos et al. 2003).

In summary, effects of larval environment are not transitory, and their continued impact on the adult stage is not in dispute: how these effects interact with vector-borne diseases is less clear.

### **Vector size and pathogen transmission:**

#### *Small adult size increases transmission efficiency*

There are several examples of links between adult female mosquito size and ability to transmit a disease. One data-rich system involves La Crosse virus (LACV) and its primary mosquito vector, *Ochlerotatus triseriatus* (Gerhardt et al. 2001). La Crosse virus is a Bunyavirus that circulates between mosquito vectors and rodent reservoirs, with occasional tangential transmission to other mammals, including humans. Grimstad et al. (1984) found that out of three size classes of mosquitoes (small, medium, and large)

small female *Oc. triseriatus* transmitted LACV to suckling mice at a much higher rate than mosquitoes from the medium or large size classes (82% versus 54% and 52% respectively). The three size classes of mosquitoes were the result of low, medium, and high larval diet amounts. Closer examination revealed that midgut infection rates did not differ among size groups, but disseminated infection and transmission rates did. This evidence suggests that transmission differences arise, not from the intake of virus (although smaller mosquitoes were shown to have ingested a larger viral dose), but from the ability of the virus to disseminate out of the midgut and into the salivary glands (Grimstad and Haramis 1984).

Several other studies of various species have described a similar pattern: smaller females are more efficient arboviruses transmitters (Table 1a). A trend of increased viral transmission from smaller *Culex tritaeniorhynchus* was described for both Japanese Encephalitis and West Nile Virus (Takahashi 1976, Baqar 1980). A recent study of *Oc. triseriatus* and *Oc. hendersoni* hybrids found that smaller than average adult females were more likely to transmit LACV (Anderson et al. 2005).

A majority of these studies used laboratory adapted mosquito strains reared under resource-limited conditions that induced intense intraspecific competition; however, a field study also revealed the same pattern, with mosquitoes collected as pupae from container habitats and given an infectious bloodmeal in the lab displaying an inverse relationship between adult body size and LACV infection rates (Paulson and Hawley 1991). There has also been one study examining the effects interspecific competition has on mosquito size and ability to transmit a virus. Alto et al. (2005) demonstrated that competitive treatments produced smaller *Ae. albopictus* females compared to less

competitive treatments. A greater proportion of these smaller females were infected with Sindbis virus compared to larger females, signifying that competition (both intraspecific and interspecific) resulting from high densities increased the vector competence of emergent *Ae. albopictus*. There was no relationship between size and disseminated infections with the other competitor in the study, *Ae. aegypti*, possibly pointing to species-specific effects of size on pathogen transmission.

#### *Large adult size increases transmission efficiency*

Despite the many studies documenting an increased ability to transmit pathogens in smaller female mosquitoes (Table 1a), the opposite pattern has been found as well. Large *Ae. aegypti* mosquitoes were found to be more susceptible to infection with Ross River virus than smaller mosquitoes (Nasci and Mitchell 1994), and they were also more likely to develop disseminated infections (Sumanochitrapon et al. 1998). Additionally, field studies of *Anopheles gambiae* found that larger mosquitoes, on average, harbored more *Plasmodium falciparum* oocysts than smaller mosquitoes (Lyimo and Koella 1992). Simple infection with any stage of *P. falciparum*, however, was independent of mosquito size. This is one of the few studies to examine how vector size interacts with protozoan pathogens.

#### *No relationship between vector size and pathogen transmission*

Additional data accumulation does not necessarily clarify the picture. Several studies have also looked for, and not found, a relationship between vector size and pathogen transmission (Table 1b). No relationship was found between the size of *Aedes vigilax* and disseminated infection or transmission rates of Ross River virus (Jennings and Kay 1999), nor was a relationship found in the ability of *Culex annulirostris* to transmit

Murray Valley encephalitis (Kay et al. 1989). Once again this raises the possibility of system specificity when examining vector size pathogen transmission efficiency; however, even within the same system, studies have not always agreed. Additional investigations of the LACV system found that interspecific competition with *Ae. albopictus* led to lower survival of *Oc. triseriatus*, but there was no relationship between *Oc. triseriatus* size and LACV disseminated infections. A majority of mosquitoes that fed on an infectious bloodmeal developed LACV infections, regardless of size (Bevins, unpublished).

#### *Vector size and quantitative measure of pathogens*

A handful of these studies have quantitatively measured the amount of virus taken in by different size classes of mosquitoes. It is possible that larger mosquitoes simply consume larger blood-meals, and therefore more virus, when feeding. This turns out to be the case; however, several analyses have shown the smallest mosquitoes to have the highest viral titers per unit body weight, regardless of blood-meal size (Grimstad and Haramis 1984, Nasci and Mitchell 1994, Alto et al. 2005). Hypotheses put forward to explain relationships between vector size and viral titers include the idea that larger mosquitoes simply have larger gut surface-areas which in turn could lead to greater receptor-site availability for virions (Nasci and Mitchell 1994); however, higher viral titers found in some smaller mosquitoes contradicts this idea. The extreme variability seen in laboratory blood-meal titers across studies clouds the issue and indicates a need for consistent titers, comparable to field levels, to be used throughout vector ecology studies.

An examination of field *An. gambiae* infected with *P. falciparum* found the opposite pattern, with larger mosquitoes, on average, harboring more oocysts than smaller mosquitoes, even though a majority of mosquitoes harboring the later-occurring infectious stage of malarial parasites were intermediate sized (Lyimo and Koella 1992). This may point to high mortality of large mosquitoes burdened with a heavy parasite load (oocysts).

*Vector size, mortality, and behavior*

The potential asymmetric mortality of larger mosquitoes due to parasite load brings in the questions of disparities in longevity, survival, and behavior in mosquitoes of different sizes. Previous studies have shown that smaller female mosquitoes emerging from resource-limited habitats were less likely to engage in host-seeking behavior, nor did they feed as successfully, compared to larger mosquitoes (Klowden et al. 1988, Klowden 1995, Sumanochitrapon et al. 1998). Larger female mosquitoes have more protein, glycogen, and lipid at emergence than smaller mosquitoes (Takken et al. 1998); these constitute energy reserves that can be crucial when it comes to something as energetically costly as host-seeking. Larger mosquitoes also demonstrate greater biting persistence when attempting to feed and are known to live longer than small mosquitoes; vector longevity is crucial when a pathogen does not become infectious immediately (Nasci 1991, Sumanochitrapon et al. 1998).

It is possible that if nutrient deprived mosquitoes behave differently and are less likely to successfully host feed compared to their well-fed counterparts, then they will have limited opportunity to become infected in the first place, regardless of how efficiently they transmit post-infection. This trait would remove them from disease

transmission cycles. This altered behavior in adult vectors from nutrient-deficient larval habitats is well documented (Klowden et al. 1988, Klowden 1995, Takken et al. 1998), yet it has not been considered in studies of vector size and pathogen transmission. Relatedly, vector behavior can also be altered by the pathogens they carry (Moore 1993, 2002). Sand flies (*Phlebotomus dubsoi*) increase probing attempts when infected with the trypanosome *Leishmania major* (Beach et al. 1985) and behaviors of several other vector species are modified by parasites (Moore 1993). If we are to discern patterns relating vector size and ability to transmit pathogens, and if we are to apply those results to field transmission, future studies must incorporate vector behavior as well.

Even without incorporating behavior, a consistent, general pattern behind mosquito size and ability to transmit a pathogen has not emerged from these studies (Table 1a; Table 1b). All but one were carried out on naturally occurring vector/pathogen systems, arguably giving a good base of knowledge for what could be expected in the field. While it is possible that the confounded relationship between vector size and pathogen transmission arises from system specific differences, it also possible that the mechanism for this pattern has not been fully elucidated, and that more work needs to be done on what the mechanisms are, what systems they function in, and how they link to larval nutrition, viral dissemination, and transmission.

### **Underlying mechanisms in differential infection/transmission rates:**

#### *Vector Competence*

Only one mechanism to date has been offered as explanation for the pattern of smaller mosquitoes being more efficient vectors (Grimstad and Walker 1991). Research

on *Oc. triseriatus* and LACV examined the basal lamina surrounding the mosquito midgut of adult mosquitoes that developed in resource-limited environments. The basal lamina in these mosquitoes was found to be thinner, consisting of 3-6 lamina ( $\approx 0.14 \mu\text{m}$ ) versus 9-16 lamina ( $\approx 0.24 \mu\text{m}$ ) in well fed adults. Existing lamina also exhibited a tattered appearance in nutrient deprived mosquitoes, potentially allowing La Crosse virions an easier, more efficient passage from the mosquito midgut to the hemocoel and surrounding tissues (Grimstad and Walker 1991). Such a mechanism could lead to increased susceptibility to infection in smaller mosquitoes.

Vector competence is defined as the ability of an arthropod to acquire, maintain, and transmit a pathogen. In order for an arbovirus to be successfully transmitted by its vector (excluding mechanical transmission), it must navigate around several infection barriers. For a virus ingested with an infectious bloodmeal, the first barrier it encounters is the midgut infection barrier, where the virus must infect and replicate in the midgut epithelium. The second barrier is the midgut escape barrier, where the virus must disseminate from the midgut by passing through the basal lamina in order to successfully replicate in other tissues. The final barrier is the transmission barrier, where the virus must infect the salivary glands, and then penetrate the lumen of the salivary glands in order to be transmitted (Black IV and Severson 2005). The degree to which these barriers are present in an arthropod determine its vector competence for a particular pathogen.

### *Barriers to Infection*

Evidence of these barriers to infection has been found throughout disease vector systems. Midgut infection barriers have received much attention for mosquito strains that are actually refractory for infection (Gomez-Machorro et al. 2004). Transmission barriers

have also been identified. In the *Ochlerotatus hendersoni* and LACV system, the mosquito can develop a disseminated infection, but the virus is unable to escape from the salivary glands and therefore *Oc. hendersoni* is not a competent vector, even though *Oc. triseriatus*, a closely related mosquito species, is (Paulson et al. 1992).

The barrier described in the *Oc. triseriatus* and LACV system is the midgut escape barrier. Midgut escape barriers have been detected in numerous pathogen/vector systems. For example, only 5% of *Aedes taeniorhynchus* females transmitted Rift Valley fever virus (RVFV) after feeding on an infectious bloodmeal; however, ingestion of a bloodmeal concurrently infectious for RVFV and *Brugia malayi* resulted in RVFV transmission by 31% of mosquitoes. The microfilariae of *B. malayi* penetrate the mosquito midgut wall, potentially allowing the virus more efficient transit out of the midgut (Turell et al. 1984). Intrathoracic injections in a laboratory setting are also known to result in much higher vector infection rates than feeding on infectious blood-meals, once again most likely the result of punctures to, or bypassing of, the midgut escape barrier (Black IV and Severson 2005).

The midgut escape barrier has also received attention because the mechanism of viral escape from the midgut is still not fully understood. The basal lamina of mosquito midgut cells is a multilayered mesh framework that gives structural integrity to epithelia and acts as a barrier to macromolecules. It also may prevent access to viral receptors on the mosquito cell surface. Houk et al. (1981) demonstrated that the largest particles to permeate the basal lamina were 5-8 nm; therefore, how a virus is able to escape through this barrier, when a typical virion is approximately 50 nm in size, is unknown. An active transport mechanism has been suggested (Mellor 2000). Other possibilities include an

enzymatic interaction between the virus and basal lamina which allows passage of virions, or distortion of the basal lamina (Houk et al. 1981, Houk and Hardy 1982). It is also possible that arboviruses disseminate from the midgut through alternate pathways.

For example, in addition to horizontal transmission, LACV is vertically transmitted with high efficiency from female *Oc. triseriatus* mosquitoes to their offspring. La Crosse virus has been found in *Oc. triseriatus* ovaries before virus disseminated from the midgut. Replicating LACV was found in the ovaries 2 days after females fed on an infectious blood-meal, while virus did not disseminate from the midgut to mosquito heads until day 6, indicating an alternate route of infection from the midgut to the ovaries (Chandler et al. 1998).

Interestingly, recent work by Romoser et al. (2004) has implicated trachea and tracheoles, which appear to fully penetrate the basal lamina, as potential routes for virions to pass out of the midgut and into the hemocoel and surrounding tissues.

Electronmicroscopy revealed Rift Valley Fever virus in mosquito tracheal endocuticle (Romoser et al. 2004). Midgut tracheoles in mosquitoes are 0.2  $\mu\text{m}$  to 1  $\mu\text{m}$  in diameter, large enough to accommodate virions, and this offers a new possibility of explaining how large virions escape out of a vector midgut. Baculoviruses are known to use a similar tracheal dissemination route in their insect host (Engelhard et al. 1994), and Sindbis virus has been detected in *Ae. albopictus* tracheoles as well (Bowers et al. 1995).

It is possible that smaller mosquitoes are better transmitters only in specific systems. Perhaps larval nutrition only impacts pathogen transmission when a physical barrier, such as a midgut escape barrier, is present in the vector (Jennings and Kay 1999). There is also little known about mosquito midgut epithelial cell receptors and their role in

arbovirus infection and dissemination in the vector. Either way, definitive information on viral escape from mosquito midguts is lacking, and if transmission barriers are the mechanisms responsible for correlations between vector size and transmission efficiency, and possibly the reason why a trend has been detected in some vector/pathogen systems and not in others, then only continued research on putative mechanisms will allow us greater insight.

### **Nutrition and Immune Function**

Larval nutritional deprivation can also impact adult immune system function in mosquito species, potentially altering their ability to transmit a pathogen (Table 2). The mosquito immune system consists of both humoral and cellular immune responses. Taken together, these responses to foreign organisms include production of antibacterial factors, phagocytosis, RNA interference (RNAi), mucopolysaccharide responses, and encapsulation (Suwanchaichinda and Paskewitz 1998, Koella and Sorensen 2002, Higgs and Beaty 2005). Similar to vertebrate trade-offs between immune system function and life history traits (Nordling et al. 1998, Ilmonen et al. 2000), insect immune responses appear to carry with them costs as well (Rantala and Roff 2005, Lambrechts et al. 2006). Bumble bee survival is reduced when the immune system is activated (Moret and Schmid-Hempel 2000), and damselfly immune system function is significantly reduced as reproductive effort is increased (Siva-Jothy et al. 1998). In mosquitoes specifically, findings of Koella and Boete (2002) indicate that more effective immune responses to parasites can lead to slower development in the mosquito (Table 2). These studies and others demonstrate a physiological cost of immunity (Koella and Boete 2002). In a

resource-limited situation it would be expected that an organism may be unable to mount a costly immune response in the face of a pathogen.

Research on the beetle *Tenebrio molitor* showed that immune response as indicated by phenyloxidase activity was down-regulated during nutritional deprivation, indicating that immune function can indeed be context-dependent (Siva-Jothy and Thompson 2002). Access to a high protein diet led to higher survivorship in Lepidoptera (*Spodoptera littoralis*) when challenged with a virus (Lee et al. 2006). In the mosquito *Anopheles gambiae*, nutritional deprivation in the larval stage decreased the ability of adults to melanize Sephadex beads (Suwanchaichinda and Paskewitz 1998).

Thus, nutritional availability in the larval environment is expected to influence the vector competence of adult mosquitoes, and when those environments are limiting, the costs associated with immune system function could limit the immune response to a transmissible pathogen. If interspecific or intraspecific competition can lead to resource limitation, than it is plausible to expect decreased immune function in response to increased competition for nutrients. It is possible that increased vector competence in smaller vectors results from an inability to mount an immune response; however, no studies to date have addressed these intriguing interactions. If we hope to explain how vector size impacts vectorborne disease transmission in the field, the role of the mosquito immune system must be understood and incorporated as well.

## **Summary**

Does nutritional deprivation, and by extension, do competition and environmental resources, affect vector competence in mosquitoes? Is there a link between adult female

mosquito size and ability to transmit a pathogen? Many experimental findings support such a pattern, but several studies have also found the opposite pattern, and still other systems have failed to produce a correlation between vector size and pathogen transmission. The only mechanism offered to explain the connection among larval resource availability, adult vector size, and pathogen transmission is a compromised basal lamina leading to more efficient midgut escape; however, studies on *Ae. albopictus* found no relationship between basal lamina thickness and viral dissemination (Thomas et al. 1993). More recent research has suggested that arboviruses may disseminate from the midgut through an alternate route, the trachea. If this is the case, a more porous basal lamina may not greatly affect dissemination, and we are left without a mechanism to explain any relationship between vector size and efficiency of pathogen transmission. Our knowledge of viral dissemination in the vector is incomplete, and the mechanics must be elucidated before we can better understand how vector size influences transmission dynamics.

Sound evidence shows that nutritional status impacts immune system function, and we would expect to see immunocompromised mosquitoes emerging from resource-deficient habitats. If immune system function is depressed enough so that a vector can no longer effectively respond to a pathogen, then vector-borne disease transmission may be enhanced in nutritionally deprived mosquitoes (allowing for the fact that nutritionally deprived vectors may not efficiently find, and subsequently feed upon, hosts). Our knowledge is incomplete in this area as well and increased understanding of interactions among larval resources, immune responses, and vector competence will further our ability to predict how transmission dynamics are affected in the field.

More information is needed on specifics of the mosquito immune response, as well as the method by which pathogens move through vectors. What channels do they use and what events must take place in order for dissemination to be successful? A recent explosion in immunology research makes integration of immune function into vector ecology research feasible. Vector behavior, specifically how larval nutrition impacts behaviors in the field and the ramifications for pathogen transmission, must also be incorporated into any future studies.

In the end, resource availability in the larval habitat affects all aspects of an adult disease vector – behavior, immune function, morphology, fecundity, and longevity. Deciphering how these factors combine to affect vector competence is difficult to assess, and this may be the reason behind the current confluence of contradictory results. Nonetheless, understanding how the larval environment impacts vectors, and in turn, vector-borne diseases, is fundamental in a time when changing land-use practices can drastically alter the types of larval habitat available (Manoukis et al. 2006), and when the arrival of invasive vector species can bring about novel competitive interactions in larval habitats (Black et al. 1989, Novak et al. 1993, Lounibos 2001, Lounibos 2002, Lounibos et al. 2003, Braks et al. 2004, Juliano et al. 2004b, Alto et al. 2005, Juliano and Philip Lounibos 2005). Increased understanding will determine if these interactions are relevant to the natural transmission setting.

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Table 1a: Smaller vectors found to be more efficient transmitters than larger vectors.

Vector	Pathogen	Effect	Citation
<i>Ochlerotatus triseriatus</i> and <i>Ochlerotatus herndersoni</i> hybrids	La Crosse virus	Mosquitoes smaller than mean more likely to transmit virus	Anderson et. al, 2005
<i>Ochlerotatus triseriatus</i>	La Crosse virus	Ability to develop disseminated infection and to orally transmit inversely proportional to body size	Paulson and Hawley, 1991
<i>Culex tritaeniorhynchus</i>	West Nile Virus	Trend of increasing susceptibility to midgut infections with a decrease in size	Baqar et al., 1980
<i>Ochlerotatus triseriatus</i>	La Crosse virus	Small, nutritionally deprived female mosquitoes ingested a larger viral dose and transmitted virus more often than large females	Grimstad et al., 1984
<i>Ochlerotatus triseriatus</i>	La Crosse virus	Basal lamina of small female mosquitoes thinner than larger female, potentially allowing more efficient viral escape	Grimstad et al., 1991
<i>Aedes albopictus</i> and <i>Aedes aegypti</i>	Sindbis virus	Smaller <i>Ae. albopictus</i> female mosquitoes from high competition treatments had higher infection rates than larger females	Alto et al., 2005
<i>Culex tritaeniorhynchus</i>	Japanese Encephalitis	Trend of increased viral transmission in smaller female mosquitoes	Takahashi, 1976

Table 1b: Smaller vectors not found to be more efficient transmitters.

Vector	Pathogen	Effect	Citation
<i>Anopheles gambiae</i>	<i>Plasmodium falciparum</i>	Infection independent of size, although larger mosquitoes harbored more oocysts and intermediate sized mosquitoes more likely to be infected with sporozoites	Lyimo and Koella, 1992
<i>Aedes aegypti</i>	Dengue-2 virus	Larger mosquitoes more likely to develop disseminated infections	Sumanochitraon et al., 1998
<i>Aedes vigilax</i>	Ross River Virus	Vector competence not related to adult female size or larval diet	Jennings and Kay, 1999
<i>Culex annulirostris</i>	Murray Valley Encephalitis	No differences in infection rates detected between differentially sized mosquitoes	Kay et al., 1989
<i>Aedes aegypti</i>	Ross River Virus	Large mosquitoes more susceptible to infection than smaller mosquitoes, although smaller females ingested more virus per unit body weight	Nasci and Mitchell, 1994

Vector	Pathogen (Immune System Stimulant)	Effect	Citation
<i>Tenebrio molitor</i>	Nylon monofilament	Immune effector system down-regulated in response to nutritional deprivation	Siva-Jothy and Thompson, 2002
<i>Anopheles gambiae</i>	Sephadex beads	Nutritional deprivation lessens ability to melanize Sephadex beads	Suwanchaichinda and Paskewitz, 1998
<i>Aedes aegypti</i>	Sephadex beads	Early pupators were smaller, and demonstrated a reduced ability to melanize beads	Koella and Boete, 2002
<i>Spodoptera littoralis</i>	Nucleopolyhedrovirus	Higher protein diet led to higher caterpillar survival when challenged with virus	Lee et al., 2006
<i>Gryllus bimaculatus</i>	Nylon monofilament	Encapsulation rate negatively correlated with body size and development time	Rantala and Roth, 2005
<i>Aedes aegypti</i>	Sephadex beads	Nutrition type and amount critical in melanization immune response in mosquitoes	Koella and Sorenson, 2003
<i>Anopheles stephensi</i>	<i>Plasmodium yoelli yoelli</i>	Environmental conditions and nutritional status impacted immune system response to pathogen	Lambrechts et al. 2006

Table 2: Effect of resources on arthropod immune system function.

## Chapter 2

### **Timing of resource input and larval competition between invasive and native container-inhabiting mosquitoes (Diptera: Culicidae)**

#### **Abstract**

Container-inhabiting mosquito species are subject to both intraspecific and interspecific competition during larval development in resource limited habitats. The arrival of an invasive species, *Aedes albopictus*, in the US has altered competitive interactions among container-inhabiting mosquito species and, in some cases, has led to displacement of these species. Resource enrichment of container habitats has been shown to alleviate competitive interactions and to promote species co-existence; however, the importance of the timing of enrichment has yet to be explored. Larval competition between *Ae. albopictus* and a native species, *Ochlerotatus triseriatus*, was explored when resources were added either gradually or in a single pulse. Replacement series experiments revealed that *Ae. albopictus* was able to outcompete and displace *Oc. triseriatus* via resource monopolization when all resources were made available simultaneously; however, when the same resource amount was added over time, survival was high for both species, leading to co-existence. Timing of resource input also had an effect in monospecific treatments, indicating that intraspecific competition impacts survival as well. Duration of larval development was influenced by both species presence and by timing of resource

input for *Oc. triseriatus*. These results indicate competitive outcome is condition-specific and that timing of resource input can determine whether a dominant invasive competitor displaces a native species, or if the two species are able to co-exist. Both intraspecific and interspecific competition occur at different temporal scales due to species-specific differences in larval developmental time. Timing of resource availability in container habitats can impact mosquito survival via competitive interactions, which can ultimately influence vector population size and behavior, possibly influencing vector-borne disease transmission.

## **Introduction**

Invasive insects have been shown to substantially alter community dynamics in the area of introduction (Elton 1958, Simberloff 1986). This is indeed the case with the Asian tiger mosquito, *Aedes albopictus*, which was first found in the United States in 1984 (Sprenger and Wuithiranyagool 1986, Hawley et al. 1987). Since its introduction, *Ae. albopictus* has become the most prevalent container-inhabiting mosquito in the United States (Moore 1999). It is an aggressive biter, a competent vector for disease both in its home range and now in the US as well (Moncayo et al. 2000, Gerhardt et al. 2001, Turell et al. 2001a) and a strong competitor for larval resources (Ho et al. 1989, Novak et al. 1993, Teng and Apperson 2000, Lounibos 2001, Braks et al. 2004, Juliano et al. 2004b).

Container-inhabiting mosquito species oviposit in both natural (tree holes) and artificial containers (tires, birdbaths, etc.), which are often described as being small, transitory bodies of water with low primary productivity (Fish and Carpenter 1982),

although conditions can vary widely and bacterial production can reach high levels (Kaufman et al. 2002). The possibility of limited nutrients in some habitats sets the stage for resource competition among mosquito larvae found in the same habitat. Indeed, the arrival of *Ae. albopictus* in the US coincided with declines in other container-inhabiting mosquito species; the leading hypothesis behind these declines is competitive exclusion via resource competition (Juliano 1998, Lounibos 2001, Lounibos et al. 2002, Juliano et al. 2004a). While competition on its own is seldom the primary driver of community composition and dynamics (Simberloff 1982), competitive effects would be expected in the face of an extremely aggressive invasive species (Duyck et al. 2006).

Several studies have examined competitive interactions between larval *Ae. albopictus* and other mosquito species, and in many instances, *Ae. albopictus* has been shown to be a superior resource competitor in the larval stage (Ho et al. 1989, Novak et al. 1993, Teng and Apperson 2000, Lounibos 2001, Braks et al. 2004, Juliano et al. 2004b); however, numerous factors influence food availability and competitive interactions among mosquitoes in container habitats, and some studies have shown no competitive advantage for *Ae. albopictus* (Black et al. 1989). Larval density, water temperature, food type, and predation have been shown to affect competitive interactions, and in some cases, alter the outcome. Overall, however, field caught mosquitoes are generally smaller than laboratory counterparts (Grimstad and Walker 1991), indicating that larval nutrition may be a limiting factor in the field since adult mosquito size partially reflects available resources (Grimstad and Haramis 1984, Nasci and Mitchell 1994).

The low productivity found in many containers has led to studies of resource input during larval development. Resource input into container habitats, in the form of fallen leaf litter, invertebrate carcasses, and stemflow, has been shown to provide a significant nutritional resource for container-inhabiting mosquito larvae in both the laboratory and field (Fish and Carpenter 1982, Lounibos et al. 1993, Leonard and Juliano 1995). Daugherty et al. (2000) demonstrated that additions of invertebrate carcasses affected both larval development and population growth, and they postulated that nutrient addition may limit competition between larval mosquito species, allowing for coexistence where exclusion would have been expected.

The pivotal role of resource input into container habitats leads to the question of how temporal variation in resource addition affects competitive interactions between larval container-inhabiting mosquitoes. In resource-limited containers, mosquito larvae engage in exploitative competition (Smith et al. 1995), with the superior competitor being the species that uses the resource with greater speed and efficiency. This, combined with interspecific differences in developmental times, indicates that timing of resource addition could play a significant role in competitive interactions.

*Ochlerotatus triseriatus* is a container-inhabiting mosquito species native to the US, whose range and oviposition preferences overlap those of *Ae. albopictus* (Barker et al. 2003, Joy et al. 2003). Larvae of both species can be found in the same container in the field, but *Oc. triseriatus* has a longer development time than *Ae. albopictus* and has been shown to be a poor competitor in interspecific competition experiments in both the laboratory and field (Teng and Apperson 2000); however, field populations have not been displaced by *Ae. albopictus* establishment. This study investigates how the timing of

resource input affects interspecific and intraspecific competition in larvae of container-inhabiting mosquito species by analyzing developmental time, adult wing lengths, larval survival, and differential mortality of the sexes. These variables are known to affect both population parameters and mosquito behavior. The ramifications of temporal variation in resource input for mosquito communities and vector-borne disease transmission are explored.

## Methods

This experiment employed a replacement series design (de Wit 1960, Novak et al. 1993) to study competitive interactions between larval *Oc. triseriatus* and *Ae. albopictus*. Mosquito eggs from laboratory adapted colonies were supplied by the Arthropod-borne Infectious Disease Lab (AIDL) in Fort Collins, CO. Eggs from both species were hatched simultaneously in separate 1 L plastic containers containing 300 mL of tap water and hatching solution (1:100; Difco [Detroit, MI] brain-heart infusion). Larvae were counted and added to respective treatments two days after hatching commenced to ensure sufficient larval numbers. All larvae were similarly sized when added to the respective treatments, ensuring that they were a similar age. The replacement series design employed five treatments (Table 1), with differing ratios of *Oc. triseriatus* and *Ae. albopictus* for each treatment (48:0, 36:12, 24:24, 12:36, 0:48), while overall density of mosquito larvae per treatment remained constant at 48 individuals (de Wit 1960, Novak et al. 1993). Each treatment contained three replicates.

Mosquitoes were raised in clear, 2 L plastic containers containing 800 ml of tap water and housed at 75 degrees F, with a 16:8 (L:D) photoperiod. Container locations on

insectary shelves were randomized, and randomization was repeated every 2 days. Diet consisted of 4 grams of beef liver powder dissolved in 1 L of water. Beef liver powder was ground with a mortar and pestle and suspended in solution. The beef liver solution was heated and mixed for one hour, allowed to cool and then measured and pipetted into each treatment in order to ensure homogeneous addition of food across treatments. This artificial food source was used to enable comparison with previously published literature (Novak et al. 1993) and because it allowed precise, consistent measurement of food. For diet 1, food was incrementally added every other day until 24 mLs had been added (Table 2). For diet 2, 24 mLs of the beef liver solution was added in one pulse on the first day of the experiment (Table 2). The food amount added to each treatment was a modification of methods in previous studies (Grimstad and Haramis 1984, Novak et al. 1993). *Ochlerotatus triseriatus* mosquitoes reared on this amount of food attained the size of field caught *Oc. triseriatus* (Grimstad and Haramis 1984).

Pupae were collected every other day, counted, identified to species and sex using a dissecting microscope, and placed in mesh-covered paperboard containers. Larval mortality was also recorded during this period. Adult mosquitoes were sustained on water, sugar cubes, and raisins until wings were removed and wing lengths recorded. Mosquito wing length is an acceptable criterion for intraspecific comparisons of overall body size (Blackmore and Lord 2000). Wings were measured from the alular notch to the distal margin, excluding the fringe scales.

Indices are a common way to evaluate competitive interactions in replacement series experiments (Black et al. 1989, Jolliffe 2000). In this experiment, interspecific competition between *Ae. albopictus* and *Oc. triseriatus* was measured using a relative

crowding coefficient (RCC) index for each diet (de Wit 1960, Harper 1977, Novak et al. 1993). Using the number of survivors from each species in individual treatments as a proxy for competition, the following RCC formula, modified from Novak et al. (1993), was calculated:

$$\frac{(\text{Survival}_{12:36}^a / \text{Survival}_{36:12}^t) + (\text{Survival}_{24:24}^a / \text{Survival}_{24:24}^t) + (\text{Survival}_{36:12}^a / \text{Survival}_{12:36}^t) / 3}{(\text{Survival}_{0:48}^a / \text{Survival}_{48:0}^t)}$$

In this index, “survival” is the number of individual larvae that successfully emerged as adults, the ratio subscript is the treatment in question and the species is indicated by either an ‘a’ for *Ae. albopictus* or a ‘t’ *Oc. triseriatus* (superscripts). Relative crowding coefficients > 1 indicate that *Ae. albopictus* is outcompeting *Oc. triseriatus*; RCC = 0 indicates that neither species is a superior competitor. Greater competitive asymmetry is indicated by increasing distance from 0.

All data were examined for normality and homogeneity of variance prior to analysis. Pupation data for each species and sex were arcsine square root transformed and analyzed with a two-way analysis of variance using diet and treatment as fixed variables, with an interaction term, using a generalized linear model (SAS® software, 9.1). For each species, sex was assumed to make up 50% of the total number of mosquitoes initially added to each treatment. This is a commonly used assumption since it is not feasible to sex first-instar larvae; however, the potential for sampling bias must be kept in mind. Time to pupation, as well as wing lengths, for each species were analyzed using a two-way analysis of variance with diet and treatment designated as fixed variables, and an interaction term. Replications within each treatment were set as random effects in all analyses of variance. Any significant treatment effects were further analyzed between individual treatments using Tukey-Kramer adjusted least squares

means for multiple comparisons in an unbalanced design. Degrees of freedom were calculated using a Satterthwaite correction for unequal sample sizes.

## Results

**Survival to adult stage:** Diet had a significant effect on proportion of mosquitoes that survived to the adult stage in both *Oc. triseriatus* ( $F=101.32$ ,  $p<0.0001$ ) and *Ae. albopictus* ( $F=8.76$ ,  $p=0.0009$ ). In both mosquito species, percent survival was lower in almost all treatments on diet 2 then on diet 1 (Figures 1a and 1b).

There was a significant diet x treatment interaction for *Oc. triseriatus* ( $F = 7.380$   $p = 0.003$ ) indicating a complex relationship among food input, the presence of *Ae. albopictus*, and the percentage of *Oc. triseriatus* that emerged as adults (Table 3). Tuke-Kramer pairwise least square means comparisons revealed that for *Oc. triseriatus*, survival was significantly different among more treatments on diet 2 than on diet 1 (Table 4). This indicates that competition with *Ae. albopictus* was not detected when food was gradually added over time (diet 1), but that on diet 2, *Ae. albopictus* presence had an impact upon the number of *Oc. triseriatus* able to emerge. Conversely, for *Ae. albopictus*, there were no significant pairwise differences among treatments on either diet 1 or 2 (Table 5), indicating that the presence of *Oc. triseriatus* does not influence *Ae. albopictus* survival. There was not a significant diet by treatment interaction for *Ae. albopictus* and none of the treatment effects were significant for the number of *Ae. albopictus* that emerged on diet 2, signifying that the presence of *Oc. triseriatus* had no influence on *Ae. albopictus* metamorphic success. This held true for both diet 1 and diet 2.

Survival of both species was also compared using a relative crowding coefficient index (RCC), with the results being consistent with previous findings (Figure 2). On diet 1, when food was added over time, the RCC value was 0.92, indicating little to no competition. The RCC value for diet 2 was 3.75, which indicates that *Ae. albopictus* survival was more than 3 times higher than *Oc. triseriatus*.

**Sex:** For *Oc. triseriatus* females, diet had a significant effect on survival ( $F=54.05$ ,  $p<0.0001$ ), with very few females eclosing on diet 2 ( $\bar{x} = 13.5\%$ ,  $S.E = 5.5\%$ ) compared with diet 1 ( $\bar{x} = 70\%$ ,  $S.E. = 5.4\%$ ). There was not a significant treatment effect for female *Oc. triseriatus* survival, nor was there a diet by treatment interaction (Table 3). Low sample sizes resulting from high female mortality on diet 2 may have influenced this result. Survival of male *Oc. triseriatus* was significantly effected by a diet x treatment interaction ( $F=4.93$ ,  $p=0.013$ ). There were no differential effects for *Ae. albopictus* males versus females for diet or treatment. Examining the male:female ratio for *Oc. triseriatus* control treatments (48:0) on diet 1 does indicate some variability around the assumption that all treatments contained a 50 percent representation of each sex (50:50, 34: 66, 66.6:33.3); however, the failure of female *Oc. triseriatus* to emerge as adults on diet 2 was consistent throughout.

**Time to pupation:** For those larvae that did survive, the time it took to reach the pupal stage was influenced by experimental conditions. For *Ae. albopictus*, diet had a significant influence on time to pupation ( $F=63.96$ ,  $p<0.0001$ , Table 3) and there was a significant diet by treatment interaction ( $F=7.41$ ,  $P=0.0022$ , Table 3); however, treatment alone did not significantly influence time to pupation. For *Oc. triseriatus*, pupation times were also significantly affected by a diet by treatment interaction ( $F=8.96$ ,  $p=0.0042$ ,

Table 3), but in this case, treatment had a significant effect on its own while diet did not. This indicates that *Ae. albopictus* presence influences *Oc. triseriatus* developmental time. For both species, time to pupation was shorter on diet 2 (*Oc. triseriatus*  $\bar{x}$  = 15 days, S.E. = 0.97, *Ae. albopictus*  $\bar{x}$  = 13 days, S.E. = 0.19) when compared to diet 1 (*Oc. triseriatus*  $\bar{x}$  = 17 days, S.E. = 0.54, *Ae. albopictus*  $\bar{x}$  = 14 days, S.E. = 0.15), possibly reflecting the high mortality rate of larvae that did not pupate quickly. *Aedes albopictus* pupated more quickly than *Oc. triseriatus* on both diets and in all treatments.

**Wing length:** Diet by treatment interactions significantly influenced both female *Oc. triseriatus* wing lengths ( $F=75.09$ ,  $p=0.0002$ ) and female *Ae. albopictus* wing lengths ( $F=8.77$ ,  $p<0.0001$ ). Females of both species were smaller on diet 2 compared to diet 1 (Figure 3). Treatment did not have a significant effect on *Oc. triseriatus* female wing length on diet 1, but treatment did significantly effect wing length on diet 2 ( $F=21.00$ ,  $p=0.0002$ ); however, treatment differences on diet 2 did not indicate any clear pattern, possibly due to the low number of female *Oc. triseriatus* survival on diet 2. *Aedes albopictus* demonstrated the opposite pattern. Treatment had a significant effect on female wing length for diet 1 ( $F=6.78$ ,  $p=0.0003$ ), with female wing length increasing as the number of conspecifics per treatment increased. In contrast, there was not a significant treatment effect for *Ae. albopictus* female wing length on diet 2.

## Discussion

Competition for nutritional resources can lead to decreased survival in larval mosquitoes. This study indicates that when food is added gradually over time during larval mosquito development, competitive interactions between *Ae. albopictus* and *Oc.*

*triseriatus* are not intense enough to impact survival to adulthood for either species; however, *Oc. triseriatus* survival is significantly reduced in the presence of *Ae. albopictus* when the same amount of food is added to the experimental environment in a single pulse when larval development begins. Thus, the timing of food input can alter competitive interactions between *Oc. triseriatus* and *Ae. albopictus*; when provided with a limited diet in this laboratory study, *Oc. triseriatus* was able to persist in a mixed-species treatment only when nutrition was added gradually. The replacement series design also revealed that as the proportion of *Ae. albopictus* increased, the survival of *Oc. triseriatus* decreased. The presence of *Oc. triseriatus* in mixed treatments did not significantly affect *Ae. albopictus* survival, signifying that the presence of *Oc. triseriatus* had no influence on *Ae. albopictus* metamorphic success. These findings are consistent with previous work that has documented the competitive advantage of larval *Ae. albopictus* over other mosquito species in a resource-limited environment (Black et al. 1989, Ho et al. 1989, Novak et al. 1993, Juliano 1998, Teng and Apperson 2000, Lounibos 2001, Tsuda and Takagi 2001, Lounibos et al. 2002, Lounibos et al. 2003, Braks et al. 2004, Juliano et al. 2004a); however, this study is the first to show that competitive outcome depends on when resources become available.

Resource availability also impacted mosquito size. Wing length in mosquitoes has been shown to correlate with overall mosquito body size (Nasci 1990), and adult mosquito body size is indicative of the amount of food available in the larval habitat (Christophers 1960). Both mosquito species in this experiment attained greater adult size on diet 1, even though the same amount of food was present in both diets. This size discrepancy probably results from monopolization of resources by early instar stages on

diet 2, many of which would not successfully complete development. The use of resources by early stages left little for later stages, resulting in smaller mosquitoes overall.

Prior field and laboratory experiments have demonstrated that resource input into habitats used by container-inhabiting mosquito species can have significant impacts on larval development (Fish and Carpenter 1982, Daugherty et al. 2000). On average, a tree hole in Indiana received 1.1 grams of leaf litter input a year (Fish and Carpenter 1982) and Daugherty et al. (2000) demonstrated that input of invertebrate carcasses can alter developmental success. Nutrient input can also stimulate bacterial production, giving larvae an additional food source (Kaufman et al. 2002). While these studies have documented the role of nutrient input in containers found in the field, there is also evidence of containers receiving no additional food input during larval development (Bevins, unpublished). Resource availability in containers will obviously vary over time and space under field conditions; however, results reported here demonstrate that the timing of resource input into larval mosquito habitat can lead to different outcomes between competing mosquito species. Different food types (leaves, leaf species, liver powder, yeast, etc.) have been shown to influence results in studies on larval mosquitoes (Fish and Carpenter 1982, Black et al. 1989, Livdahl and Willey 1991, Leonard and Juliano 1995, Lounibos 2001). While the use of a liver powder resource may differ from field conditions, these results are nonetheless in agreement with other studies, demonstrating that *Ae. albopictus* is a superior larval competitor over *Oc. triseriatus* on numerous food substrate types (Livdahl and Willey 1991).

While the ability of immature *Ae. albopictus* to outcompete other mosquito species is well established, the proximate mechanisms behind the competitive superiority have not been conclusively demonstrated. In this set of experiments, the rapid developmental time of *Ae. albopictus* appears to give it an advantage. On average, *Ae. albopictus* completed larval development 3 days before *Oc. triseriatus*, potentially allowing it to capture resources before its competitor. This would also explain why the cohort with the longest developmental time, female *Oc. triseriatus*, was most significantly affected by *Ae. albopictus* presence. Because females generally take longer to pupate than males, female *Oc. triseriatus* would be at a distinct disadvantage since they pupate long after *Ae. albopictus*, as well as males of their own species, have used up any available nutrition. This cohort also attains the largest size. It is also possible that *Ae. albopictus*, a much smaller mosquito than *Oc. triseriatus*, simply has lower nutritional requirements. Previous studies, however, have shown that *Ae. albopictus* has a similar competitive advantage over *Ae. aegypti* (Ho et al. 1989, Tsuda and Takagi 2001, Juliano et al. 2004b), a small-bodied mosquito species with an equally short developmental time. Thus, while developmental time may influence competition between *Ae. albopictus* and *Oc. triseriatus*, other factors must be involved as well.

Studies have shown that *Ae. albopictus* larvae spend more time engaging in feeding activities than do *Ae. aegypti* larvae and that *Ae. albopictus* has superior resource harvesting abilities compared to its competitors (Yee et al. 2004a). Competition between *Ae. albopictus* and other mosquito species appears to be a classic case of exploitative competition, where two consumers are competing for the same resource; in this case *Ae. albopictus* can exploit the resource before *Oc. triseriatus*. *Aedes albopictus* has been

shown to persist much longer than other mosquito species when reared on an extremely limited diet (Barrera 1996), possibly indicating an ability to consume and efficiently store nutrients. In field experiments examining larval competition between *Ae. albopictus* and *Ae. aegypti*, Braks et al. (2004) found that *Ae. albopictus* maintained positive population growth at lower *per capita* resource availability than *Ae. aegypti*. The abilities of *Ae. albopictus* both to monopolize resources via exploitative competition and to maintain population growth on lower *per capita* nutritional requirements are consistent with findings of the experiments reported here, and these abilities likely contribute to its success as an invasive species (Moore 1999) and to its competitive advantage.

While the dominant competitive ability of larval *Ae. albopictus* highlights the importance of interspecific competition and the relative crowding coefficient index demonstrates that *Ae. albopictus* negatively impacts *Oc. triseriatus* survival, intraspecific competition is also evident in these experiments and must be kept in mind. Intraspecific competition is often expected to be stronger than interspecific competition due to the similar nature of the competitors (Connell 1983). Survival decreased for both species when resources were added in a single pulse not only in mixed species treatments but in treatments that contained only conspecifics. In resource limited environments, competition will occur among conspecifics as well as among species, but in this system, interspecific competition has a more dramatic impact on *Oc. triseriatus* survival than intraspecific interactions.

Gradual addition of resources alleviates competitive interactions between immature *Oc. triseriatus* and *Ae. albopictus*; however, if there is limited resource input into already depauperate larval container habitats, fast developmental time, small adult

size, enhanced resource gathering efficiency, and ability to withstand starvation allow *Ae. albopictus* to outcompete *Oc. triseriatus*, thus limiting *Oc. triseriatus* survival. This pattern differs from research that predicts increased species diversity in response to temporal variability in resource availability, *i.e.* resource pulses (Grover 1988). The results from this laboratory-based experiment suggest that *Ae. albopictus* actually limits species diversity if time between nutrient pulses into resource-limited containers is too long to sustain *Oc. triseriatus*. Similar habitat requirements (Barker et al. 2003), feeding behaviors (Yee et al. 2004b, a), and overlapping field distributions in the US (Moore 1999) all indicate that *Ae. albopictus* could displace *Oc. triseriatus* in resource-limited larval environments; however, differences in habitat preference as well as non-resource limiting environments would allow persistence of both species, a pattern observed in the US since the arrival of *Ae. albopictus* (Lounibos 2001). This condition-specific competition has also been demonstrated, whereby abiotic conditions determined competitive outcomes between native and invasive larval mosquitoes (Costanzo et al. 2005). These results also reinforce the notion competitive interactions across taxa are not static.

Knowledge of larval mosquito dynamics is crucial to understanding vector-borne disease transmission, and both of these species are known vectors of several pathogens (Moncayo et al. 2000, Gerhardt et al. 2001, Turell et al. 2001a, Turell et al. 2001b, Endy and Nisalak 2002). Pathogen transmission models (Macdonald 1961) are sensitive to both number and size of adult mosquitoes; the latter can affect mosquito feeding behavior (Klowden et al. 1988, Klowden 1995, Takken et al. 1998) and, in some cases, vector competency (Grimstad and Haramis 1984, Grimstad and Walker 1991, Paulson and

Hawley 1991, Suwanchaichinda and Paskewitz 1998). With the continued introduction of non-native mosquito species around the world, changes in these competitive interactions warrant further study, as larval interactions can reverberate through the adult stage, ultimately impacting disease transmission dynamics.

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## Tables

Table 1: Replacement series design used to examine competitive interactions between *Ochlerotatus triseriatus* and *Aedes albopictus*.

	Treatment				
	1	2	3	4	5
	# of larvae				
<i>Oc. triseriatus</i>	48	36	24	12	0
<i>Ae. albopictus</i>	0	12	24	36	48
Total	48	48	48	48	48

Table 2: Comparison of beef liver solution addition schedules.

	Diet	
	1	2
Day		
0	4 mLs	24 mLs
2	4 mLs	0 mLs
4	4 mLs	0 mLs
6	4 mLs	0 mLs
8	4 mLs	0 mLs
10	4 mLs	0 mLs
Total	24 mLs	24 mLs

Table 3: Analysis of variance results for overall survival, female survival, time to pupation, and wing lengths for *Ochlerotatus triseriatus* and *Aedes albopictus*.

Source	DF	Survival		Female Survival		Time to Pupation		Wing Length	
		F	p	F	p	F	p	F	p
<i>Ae. albopictus</i>									
Treatment	3	0.28	0.839	0.24	0.864	0.44	0.727	10.51	<0.0001
Diet	1	8.76	<b>0.0091</b>	3.39	0.084	63.96	<b>&lt;0.0001</b>	112.74	<b>&lt;0.0001</b>
Treatment*Diet	3	6.07	0.389	1.41	0.275	7.41	<b>0.0022</b>	8.77	<b>&lt;0.0001</b>
<i>Oc. triseriatus</i>									
Treatment	3	0.19	0.9006	1.27	0.319	7.88	<b>0.0036</b>	12.78	<b>0.0009</b>
Diet	1	101.32	<b>&lt;0.0001</b>	54.05	<b>&lt;0.0001</b>	0.93	0.3535	149.82	<b>&lt;0.0001</b>
Treatment*Diet	3	7.38	<b>0.0025</b>	7.38	0.2483	8.96	<b>0.0042</b>	9.29	<b>0.0002</b>

Table 4: Tukey-Kramer adjusted pairwise comparisons of *Ochlerotatus triseriatus* survival between individual treatments both within and across diet regimes.

	Treatment	Treatment	t-value	p-value
Diet 1	1	2	-0.59	0.561
	1	3	-1.7	0.109
	1	4	-3.04	<b>0.007</b>
	2	3	-1.1	0.286
	2	4	-2.45	<b>0.028</b>
	3	4	-1.35	0.196
Diet 1 vs. 2	1	1	2.12	<b>0.049</b>
	2	2	3.51	<b>0.002</b>
	3	3	6.39	<b>&lt;.0001</b>
	4	4	8.11	<b>&lt;.0001</b>
Diet 2	1	2	0.79	0.44
	1	3	2.57	<b>0.02</b>
	1	4	2.95	<b>0.009</b>
	2	3	1.78	0.094
	2	4	2.16	<b>0.046</b>
	3	4	0.38	0.71

Table 5: Tukey-Kramer adjusted pairwise comparisons of *Aedes albopictus* survival between individual treatments both within and across diet regimes.

	Treatment	Treatment	t-value	p-value
Diet 1	2	3	-1	0.33
	2	4	-0.31	0.757
	2	5	-0.82	0.424
	3	4	0.95	0.356
	3	5	0.36	0.722
	4	5	-0.73	0.478
Diet 1 vs. 2	2	2	-0.23	0.817
	3	3	2.45	<b>0.026</b>
	4	4	2	0.062
	5	5	3.25	<b>0.005</b>
Diet 2	2	3	1.26	0.224
	2	4	1.39	0.184
	2	5	1.54	0.144
	3	4	0.06	0.95
	3	5	0.19	0.848
	4	5	0.14	0.886

## Figure Legends

Figure 1. Replacement diagram for mean proportion of surviving *Ochlerotatus triseriatus* and *Aedes albopictus* in competitive treatments under both a gradual food addition schedule (diet 1) and under a single pulse of food addition (diet 2).

Figure 2. Calculated relative crowding coefficients for *Aedes albopictus/Ochlerotatus triseriatus* survival for both gradual nutrient enrichment over time (diet 1) and for a single pulse of enrichment (diet 2). A greater RCC value indicates competitive asymmetry in favor of *Aedes albopictus*.

Figure 3. Overall mean wing lengths for both *Ochlerotatus triseriatus* and *Aedes albopictus* on a diet consisting of gradual enrichment (diet 1) and on a diet consisting of a single enrichment pulse (diet 2).

## Figures

Figure 1a

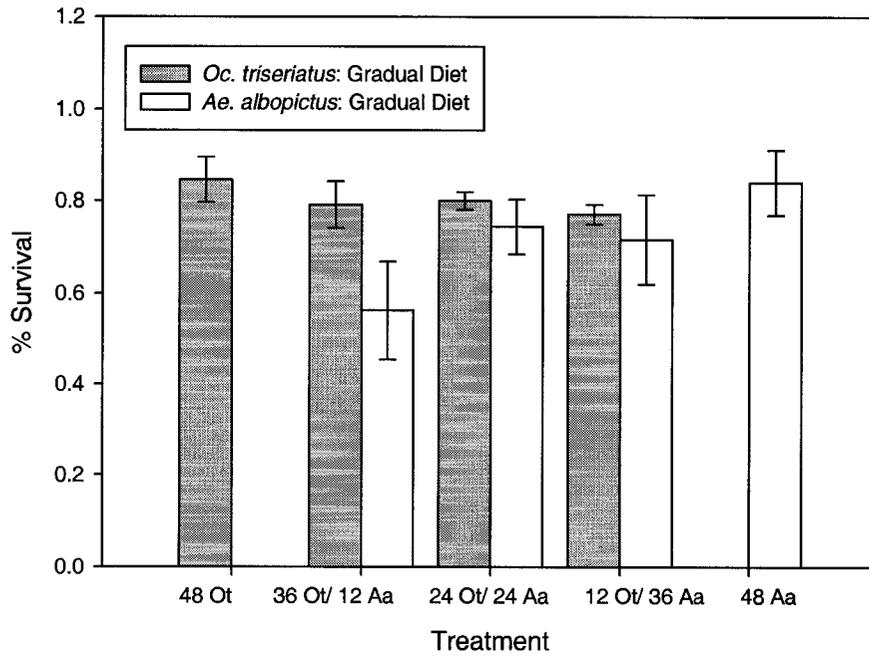


Figure 1b

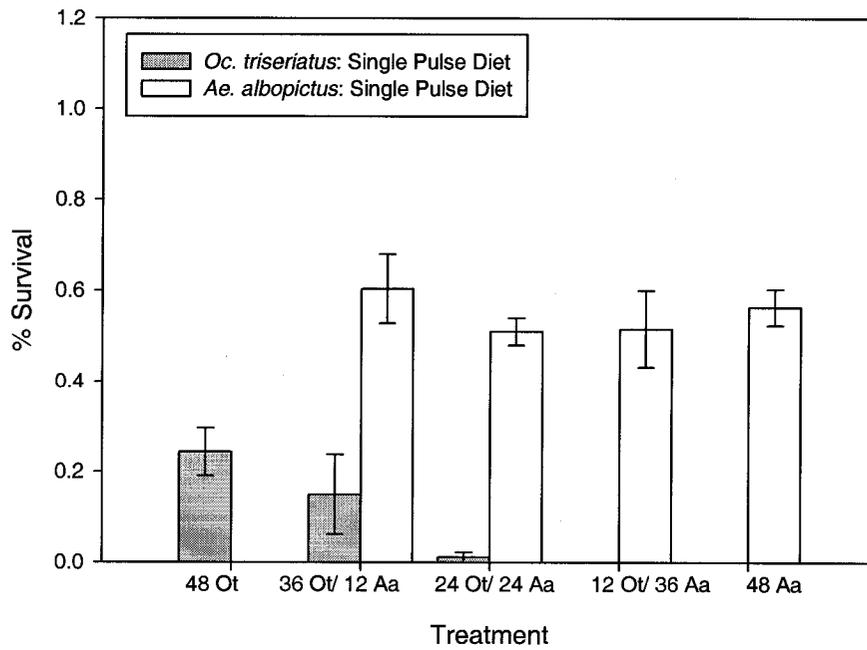


Figure 2

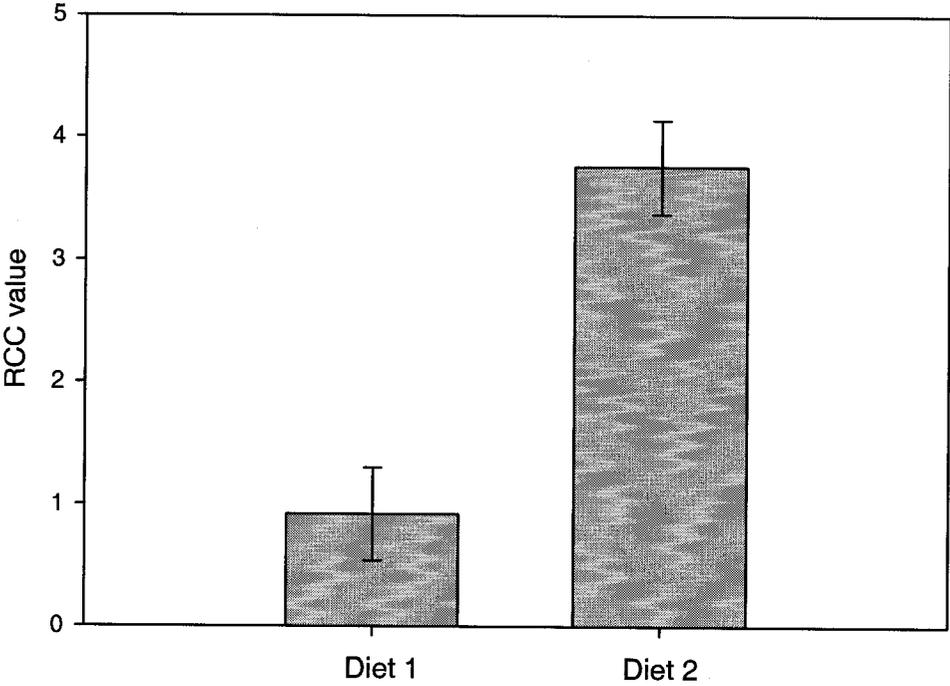
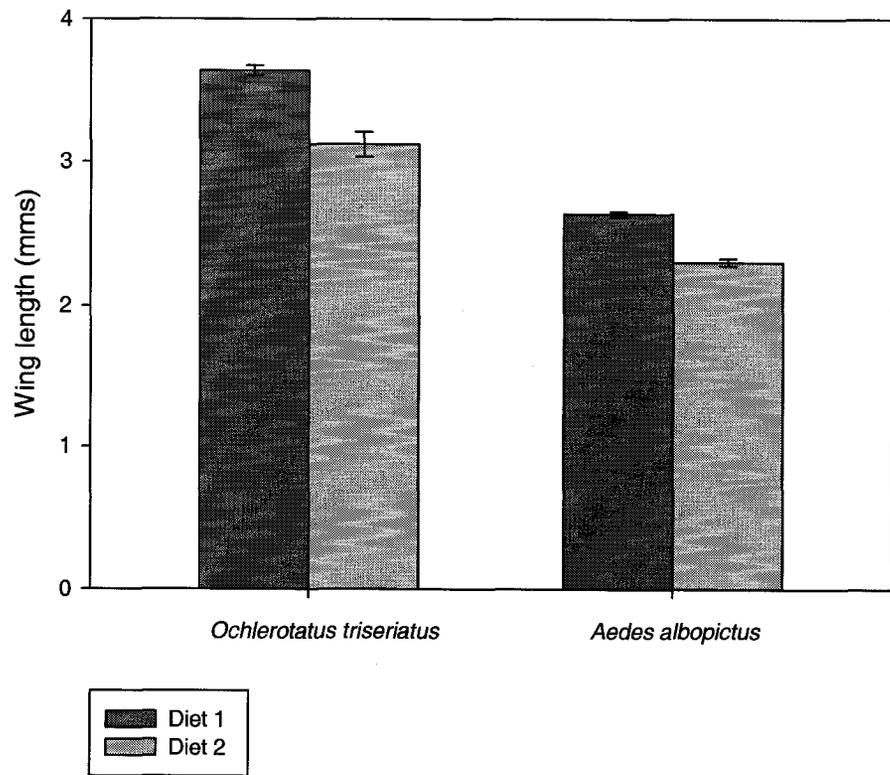


Figure 3



## Chapter 3

### **Invasive mosquitoes, larval competition, and indirect effects on native mosquito species vector competence (Diptera: Culicidae)**

#### **Abstract**

Invasive species can alter ecological interactions in their area of introduction. Invasive arthropods that vector pathogens have the potential to influence pathogen transmission both directly, by becoming a novel pathogen vector, or indirectly, by interacting with native vectors. This is the first study to examine the indirect impacts that larval competition between *Aedes albopictus*, an invasive mosquito species, and *Ochlerotatus triseriatus*, a native mosquito species and the primary vector for La Crosse virus in the US, has on native mosquito larval survival and adult size. Adult size in mosquitoes influences viral transmission efficiency. In this study, the presence of *Ae. albopictus* decreased *Oc. triseriatus* larval survival, but surviving *Oc. triseriatus* females were larger, potentially due to a release from intraspecific competition. These larger, native females were more likely to develop both midgut and disseminated infections than females emerging from monospecific treatments. Potential indirect effects of larval competition between invasive and native vectors on pathogen transmission are discussed.

## Introduction

Vector-borne diseases are ecologically complex, involving interactions among pathogen, vector, host, and environment. The introduction of a new vector could profoundly alter the transmission dynamics of a vector-borne disease, both directly, by vectoring the pathogen itself, and indirectly, via interactions with the natural vector. *Aedes albopictus* is an invasive mosquito species in the US. Established populations were first detected in 1984 in Houston, Texas, and it quickly spread throughout the Southeastern US and up the eastern seaboard (Hawley et al. 1987, Moore and Mitchell 1997, Moore 1999). *Aedes albopictus* is a container-inhabiting mosquito species, ovipositing in small water-filled containers (both natural and artificial) and is often found to co-occur with larvae of several other mosquito species. While general attributes remain elusive, many invasive species are characterized as being dominant competitors (Duyck et al. 2006) and *Ae. albopictus* is no different. Both laboratory and field experiments have documented the competitive superiority of larval *Ae. albopictus* over other mosquito species (Ho et al. 1989, Novak et al. 1993, Juliano 1998, Teng and Apperson 2000, Lounibos 2001, Braks et al. 2004, Juliano et al. 2004).

*Ochlerotatus triseriatus* is a container-inhabiting mosquito species native to the US, whose geographic range overlaps *Ae. albopictus* distribution. *Ochlerotatus triseriatus* is negatively impacted by *Ae. albopictus* presence in the larval habitat (Ho et al. 1989, Novak et al. 1993, Teng and Apperson 2000), with emergent adults being smaller and nutritionally deprived; however, unlike other mosquito species, it has not been displaced when *Ae. albopictus* becomes locally established (Lounibos 2001, Lounibos et al. 2002). An added complication arises in that both of these species can

transmit arboviruses. *Aedes albopictus* is a known arbovirus vector in its Asian home range and is a competent laboratory and field vector for pathogens in the US as well (Moncayo et al. 2000, Gerhardt et al. 2001, Turell et al. 2001). *Ochlerotatus triseriatus* is the primary vector for La Crosse virus (LACV) in the US (Woodring et al. 1998). La Crosse virus is an arbovirus endemic to the US that primarily circulates between its mosquito vector and small mammals. Tangential infections occur in other mammal species, including humans, where clinical symptoms in young adults occasionally result in severe encephalitis and rarely in death. Historically, human cases were primarily documented in the upper Midwestern US where both the vector and primary small mammal hosts were abundant, but the last 10-15 years now see a majority of human LACV infections originating from West Virginia, Tennessee, and North Carolina (Jones et al. 1999, Erwin et al. 2002). Possible explanations for this geographical shift encompass better medical monitoring, increased vector habitats near houses, and the introduction and establishment of *Aedes albopictus* (Gerhardt et al. 2001). Another factor potentially influencing LACV transmission is indirect effects stemming from larval competition between *Ae. albopictus* and *Oc. triseriatus*.

It is possible that competition between larval mosquitoes may directly influence *Oc. triseriatus* vector competence for LACV. In both the field and laboratory, *Oc. triseriatus* vector competence is inversely correlated with body size (Grimstad and Haramis 1984, Paulson and Hawley 1991); smaller females develop and transmit the virus more readily than do large females. Adult size in mosquitoes is extremely plastic and corresponds to larval resource availability (Christophers 1960, Fish and Carpenter 1982, Nasci and Mitchell 1994). Grimstad and Haramis (1984) found that smaller

females with a history of nutritional deprivation were at least twice as likely to transmit LACV to mice as larger females were (90% vs. 42%). The mechanism behind the increase in infections and transmission was later linked to a breakdown of a midgut escape barrier (basal lamina surrounding the mosquito midgut), allowing the virus an efficient escape from the midgut, followed by dissemination to the salivary glands where it is then transmissible (Grimstad and Walker 1991). Smaller *Oc. triseriatus* collected in the field were also found to be more efficient transmitters than larger individuals (Paulson and Hawley 1991).

Given these findings, it is possible the continued persistence of *Oc. triseriatus* in the wake of *Ae. albopictus* establishment, combined with the competitive superiority of *Ae. albopictus*, has the potential to lead to nutritionally deprived *Oc. triseriatus* adults with an enhanced ability to develop disseminated LACV infections. At the very least, the introduction of a competitively dominant invasive vector species into an area of endemic vector-borne disease would be expected to influence the complex dynamics involved in such disease transmission. The aim of this study was to determine the effects of larval competition on survival and adult size of *Oc. triseriatus* and *Ae. albopictus* mosquitoes. Adult *Oc. triseriatus* females were then offered an infectious LACV bloodmeal to examine the indirect effects larval competition has on blood-feeding success, viral infection, and viral dissemination. The role of *Ae. albopictus* as a widespread vector of LACV in the field has not been conclusively demonstrated (Gerhardt et al. 2001), and therefore, LACV infections were not studied in this species.

## Methods

This experiment employed a replacement series design (de Wit 1960, Novak et al. 1993) to study competitive interactions between larval *Oc. triseriatus* and *Ae. albopictus*. Mosquito eggs from laboratory-adapted colonies were supplied by the Arthropod-borne Infectious Disease Lab (AIDL) in Fort Collins, CO. Eggs from both species were hatched simultaneously in separate 1 L plastic containers containing 300 milliliters of tap water and hatching solution (1:100; Difco [Detroit, MI] brain-heart infusion). Larvae were counted and added to respective treatments two days after hatching to ensure sufficient larval numbers (Table 1). All larvae were similarly sized when added to the respective treatments, ensuring that they were a similar age. The replacement series design employed five treatments with differing ratios of larval *Ae. albopictus* and *Oc. triseriatus* but constant overall density (de Wit 1960, Novak et al. 1993). Each treatment contained five replicates (Table 1). This experiment was conducted twice for a total of 10 replicates of each treatment.

Larvae were raised in 2 L plastic containers containing 800 ml of tap water and were housed at 75 degrees F, at 75% humidity with a 16:8 photoperiod. Container locations on insectary shelves were randomized, and this randomization was repeated every 2 days resulting in a completely randomized design. Nutritional resources consisted of 0.07 grams of beef liver powder which was ground with a mortar and pestle, weighed to the nearest thousandth of a gram, and added to each respective treatment on the first day of experimentation. This artificial food source was used in place of leaf-litter because it allowed comparison with previously published literature (Novak et al. 1993) and permitted precise, consistent food measurements. The food amount added to each

treatment was a modification of previous study methods (Grimstad and Haramis 1984, Novak et al. 1993). A modified version of this diet was previously shown to generate competitive interactions between *Oc. triseriatus* and *Ae. albopictus*, while limiting severe mortality and producing adequate sample sizes (Bevins, Chapter 1).

Pupae were collected every other day, counted, identified to species and sex using a dissecting microscope, and placed in mesh-covered paperboard containers; larval mortality was also recorded. Adult mosquitoes were sustained on water, sugar cubes, and raisins.

In order to test *Oc. triseriatus* vector competence for LACV, adult females were held for seven days after eclosion and then starved for 24 hours before receiving a blood-meal. A LACV isolate, originally obtained from the Yale Arbovirus Research Unit in New Haven CT, was prepared according to protocol (Gabitzsch et al. 2006). Virus infected Vero cells were collected 48 hours post-infection, diluted in a fresh sheep blood preparation and placed both in an artificial membrane feeder and as droplets on mosquito netting (Grimstad et al. 1977, Gabitzsch et al. 2006). The artificial membrane feeder warmed the bloodmeal to approximately 37°C to encourage feeding. Female *Oc. triseriatus* were allowed to feed for one to two hours. Bloodmeals were analyzed immediately before and after feedings with viral titers  $\approx 8.51 \times 10^5$  plaque forming units/ml. Mosquitoes were sorted on a chill-table and females that failed to fully engorge (determined visually by distention) were removed and frozen at -70 degrees Celsius. Remaining mosquitoes were held for 14 days for viral incubation and then given a second non-infectious bloodmeal following the same protocols.

Mosquitoes were analyzed for infection and measured after an incubation period ( $\approx$  21 days). The presence or absence of infection was determined through indirect immunofluorescence assay using a fluorescein labeled anti-LACV labeled antibody (Beaty and Thompson 1978). Both heads and midguts from females that fed on an infectious bloodmeal were assayed for both viral infection and dissemination from the midgut. Wing length is an accepted proxy for mosquito body size when making intraspecific size comparisons (Grimstad and Haramis 1984, Nasci 1990). To measure size in this study, wings were mounted on a glass slide and measured from the alular notch to the distal margin (excluding fringe scales) using a calibrated eyepiece micrometer.

All statistical analyses were carried out on SAS® software, version 9.1, using individual general linearized mixed models (Proc Glimmix) to account for unbalanced design and non-homogeneous variances. *Aedes albopictus* and *Oc. triseriatus* treatments were set as ordinal fixed effect to determine the impact on *Oc. triseriatus* survival, adult size, blood-feeding propensity, LACV infection, and disseminated LACV infection. *Aedes albopictus* survival and wing lengths were also analyzed using competitive treatment as a fixed effect. Experiment and container were set as random effects in the model. Significant effects underwent additional analysis using Tukey adjusted pairwise comparisons to tease out contributions to significant effects. Degrees of freedom were calculated using a Ken-Ward-Roger adjustment to account for sample size differences and control for Type I error. Wing length comparisons between binomial variables -- fed versus failed to feed, infected versus uninfected, disseminated infections versus failed to disseminate -- were analyzed with t-tests.

## Results

**Survival:** *Aedes albopictus* survival, on average, was higher ( $\bar{x} = 0.84$ ,  $SD=0.15$ ,  $SE=0.02$ ; Figure 1) than average survival of *Oc. triseriatus* across all treatments and experiments ( $\bar{x} = 0.70$ ,  $SD=0.14$ ,  $SE=0.02$ ; Figure 1). *Ochlerotatus triseriatus* survival, measured as successful adult emergence, was significantly affected by treatment ( $F=3.83$ ,  $p=0.0164$ ; Table 2; Table 3). This indicates that the presence of *Ae. albopictus* influences *Oc. triseriatus* survival, and least square means pairwise comparisons reveal that *Oc. triseriatus* survival is significantly higher in the treatment containing only conspecifics than in two of the three other treatments (Treatment 1 vs. Treatment 3  $t=-3.23$ ,  $p=0.002$ , Treatment 1 vs. Treatment 3  $t=-1.96$ ,  $p=0.05$ ; Table 3; Figure 1). All other pairwise differences were not significant. *Aedes albopictus* survival was not significantly affected by treatment, indicating that *Oc. triseriatus* presence did not have an effect on the survival of the invasive species.

**Size:** Competitive treatment had a significant effect on wing lengths of emergent *Oc. triseriatus* females ( $F=22.79$ ,  $p<0.0001$ ; Table 2). A positive relationship between increasing female wing length and increasing proportion of *Ae. albopictus* per treatment held through both experiments (Table 3), although differences were not always significant (Figure 2). Pairwise comparisons reveal that *Oc. triseriatus* females from the monospecific treatment were significantly smaller than females from all other treatments (Treatment 1 vs. Treatment 2,  $t=9.87$ ,  $p<0.0001$ ; Treatment 1 vs. Treatment 3,  $t=9.19$ ,  $p<0.0001$ ; Treatment 1 vs. Treatment 4  $t=7.41$ ,  $p<0.0001$ ). All other pairwise comparisons were non-significant. Under these experimental conditions, intraspecific

interactions contributed significantly to competition and smaller size; however, overall intraspecific competitive effects in treatments with a greater proportion of *Ae. albopictus* may have decreased due to greater *Oc. triseriatus* mortality (Figure 1 and Figure 2).

Treatment also significantly influenced *Ae. albopictus* size ( $F=27.97$ ,  $p<0.0001$ ; Table 3); however, there was not an overall pattern of wing length across treatments for this species (Table 3). Pairwise comparisons reveal that the largest *Ae. albopictus* emerged from the treatment containing 12 *Oc. triseriatus* and 36 *Ae. albopictus* (Figure 2). This treatment differed significantly from all other treatments (Treatment 4 vs. Treatment 2,  $t=2.80$ ,  $p=0.008$ ; Treatment 4 vs. Treatment 3,  $t=4.58$ ,  $p<0.0001$ ; Treatment 4 vs. Treatment 5,  $t=3.43$ ,  $p=0.001$ ).

**Blood Feeding:** *Ochlerotatus triseriatus* females that successfully fed were significantly larger than those females that did not, regardless of treatment or experiment ( $t=-7.82$ ,  $p<0.0001$ ; Figure 3). Competitive treatment also significantly affected successful blood-feeding by *Oc. triseriatus* ( $F=16.89$ ,  $p=0.009$ ; Table 3). The monospecific treatment, on average, produced the smallest mosquitoes and was also distinguished as having the lowest proportion of successful feedings (Table 2; Figure 4A). Pairwise comparisons revealed that it was the only treatment that differed significantly from other treatments (Treatment 1 vs. Treatment 2,  $t=4.21$ ,  $p=0.013$ ; Treatment 1 vs. Treatment 3,  $t=3.95$ ,  $p=0.016$ ).

**La Crosse virus infections:** There was a significant treatment effect on the proportion of females that developed LACV infections after successfully taking a blood meal ( $F=9.95$ ,  $p=0.025$ ; Table 2; Figure 4B). Treatment also significantly affected proportions of females that developed disseminated infections ( $F=7.85$ ,  $p=0.0376$ ; Table 2; Figure 4C).

Pairwise comparisons reveal, once again, that females from the monospecific treatment were less likely to develop infections when compared to all other treatments (Treatment 1 vs. Treatment 2,  $t=3.35$ ,  $p=0.028$ ; Treatment 1 vs. Treatment 3,  $t=2.81$ ,  $p=0.048$ ). The same pattern held for disseminated infections as well (Treatment 1 vs. Treatment 2,  $t=2.55$ ,  $p=0.063$ ; Treatment 1 vs. Treatment 3,  $t=2.80$ ,  $p=0.049$ ). There was not a significant difference in wing length of mosquitoes that successfully developed either midgut or disseminated LACV infections versus those that did not. Eighty-five percent of blood-fed females went on to develop midgut infections, and 92% of those mosquitoes later developed disseminated infections (Table 3).

## **Discussion**

This study was designed to examine competitive interactions between a native and an invasive mosquito species and to determine if those competitive interactions influenced the ability of the native species to transmit a mosquito-borne virus. The direct effects of competition were examined in terms of larval survival and adult wing length, while the indirect effects of competition were examined using propensity to blood-feed, infection rates, and disseminated infection rates. Four overall findings are highlighted: (1) native mosquito species survival decreased as the proportion of invasives per treatment increased; however, (2) there was a positive relationship between surviving native mosquito size and presence of the invasive competitor. In turn, (3) these larger females were more likely to blood-feed compared to their counterparts emerging from monospecific treatments where survival was higher, and (4) these larger females were also more likely to develop midgut and disseminate LACV infections.

These overall findings lead us to a conclusion that has some counter-intuitive elements. In essence, the dominant competitor, *Ae. albopictus*, does indeed influence the role of *Oc. triseriatus* as a vector of LACV in the laboratory, but it does so in two opposing ways. *Aedes albopictus* diminishes the number of *Oc. triseriatus*, therefore diminishing the number of individuals that can be LACV vectors; however, the *Oc. triseriatus* individuals that survive interspecific competition are relatively large, feed readily, and routinely develop LACV infections. I will discuss the findings in more detail.

In replacement series experiments, interspecific competition reduced *Oc. triseriatus* larval survival. This is consistent with previous findings (Ho et al. 1989, Novak et al. 1993, Teng and Apperson 2000, Braks et al. 2004); however, when examining wing lengths of those *Oc. triseriatus* females that did survive, a pattern opposite to what previous literature has reported was found. On average, monospecific treatments produced the smallest *Oc. triseriatus* mosquitoes, with wing lengths increasing across treatments as the proportion of *Ae. albopictus* per treatment increased. These results indicate that size of *Oc. triseriatus* adults was reduced by intraspecific competition. This pattern differs from previously published literature (Black et al. 1989), where *Ae. albopictus* is typically found to be a wholly dominant competitor, negatively influencing all aspects of other mosquito species found in the same larval habitats.

Why should an inferior competitor increase in size as interactions with a dominant competitor intensify? In this case, an increased proportion of the superior competitor led to increased *Oc. triseriatus* mortality, resulting in fewer mosquitoes overall, and more resources per surviving mosquito. Thus, these remaining mosquitoes were larger than

their counterparts in treatments with fewer dominant competitors and higher survivorship.

By contrast, *Ae. albopictus* survival did not significantly differ across treatments, indicating that the proportion of *Oc. triseriatus* per treatment had little impact on available resources. Wing lengths of emergent *Ae. albopictus* females differed significantly across treatments, but there was no consistent trend (Table 3). Therefore, interpretation of how interspecific and intraspecific competition influence *Ae. albopictus* size is not straightforward in this case. *Aedes albopictus* is, however, known to have a shorter developmental time than *Oc. triseriatus* (Teng and Apperson 2000), and this may interfere with consistency in the effects of interspecific competition (*i.e.* some *Ae. albopictus* may complete development before competitive effects become apparent). Rapid development and quick resource uptake on the part of *Ae. albopictus* would limit resource availability for the slower developing *Oc. triseriatus*.

While several studies have examined how larval nutritional deprivation increases the ability of an adult mosquito to transmit a pathogen once infected (Takahashi 1976, Baqar 1980, Grimstad and Haramis 1984, Kay et al. 1989, Grimstad and Walker 1991, Paulson and Hawley 1991, Jennings and Kay 1999, Alto et al. 2005), few of these studies have taken into account how nutritional deprivation impacts the ability of the mosquito to become infected in the first place. In a nutshell, smaller female mosquitoes are less likely to host- feed (Klowden 1995, Sumanochitraon et al. 1998). Many factors, including failure to seek hosts, contribute to an inability of smaller females to feed successfully (Klowden et al. 1988). Larger female mosquitoes have more protein, glycogen, and lipid at emergence than smaller mosquitoes (Takken et al. 1998), energy reserves that can be

crucial when it comes to energetically costly host-seeking. Larger mosquitoes also demonstrate greater biting persistence and are known to live longer than small mosquitoes; vector longevity is crucial for a pathogen with a significant patency period (Nasci 1991, Sumanochitrapon et al. 1998).

My experimental findings reinforce the importance of larval nutrition on the ability of adult female mosquitoes to blood-feed successfully. *Ochlerotatus triseriatus* females that fed successfully were significantly larger than those that did not, and in consequence, these larger females would be more likely to play a role in disease transmission in the field. Treatment significantly affected successful blood-feeding as well in this study and this effect was mediated by size differences among treatments. Females from the monospecific treatment that produced smaller females across the board were less likely to feed successfully than larger females from other treatments.

The likelihood of LACV infection appeared to be ecologically mediated in this laboratory study. *Ochlerotatus triseriatus* females that developed in the presence of *Aedes albopictus* were more likely to develop both midgut and disseminated LACV infections when compared to females from the monospecific treatment. La Crosse virus infections were analyzed only on mosquitoes that successfully blood-fed. Among the mosquitoes that did feed (that is, the larger survivors) there was not a significant difference in size of mosquitoes that developed infections versus those that did not. Of the females that successfully blood-fed, very few failed to develop LACV midgut infections, and almost all females with a midgut infection went on to develop a disseminated infection. The failure to detect a difference between midgut infections and disseminated infections indicates that there was not a significant midgut escape barrier

impeding viral dissemination. A compromised midgut escape barrier has often been cited as a mechanism behind the more efficient development of disseminated infections in small field and laboratory mosquitoes (Grimstad and Walker 1991). The lack of such a barrier may be related to the failure to detect a relationship between mosquito size and viral infection and dissemination in this study; mosquito size may be important to pathogen dissemination and transmission only when a physical barrier to viral dissemination exists. Nasci and Mitchell (1994) also found that differences in infectivity among mosquitoes could be masked by high viral titers in blood-meals; this should be kept in mind when examining infectivity in vectorborne disease studies.

The impacts of larval nutrition and competition on mosquito-virus interactions and pathogen transmission cannot be disputed. Intense competitive interactions or severely resource-limited environments can lead to fewer emergent mosquitoes or emergence of small, nutrient-deprived adults. Vector-borne disease models are sensitive to local vector population numbers, so a change in the number of emergent mosquitoes would influence disease transmission dynamics (Macdonald 1955, 1961). Smaller mosquitoes are known to behave differently in comparison to their larger counterparts, and many of these behaviors – host-seeking, biting persistence, longevity, and flight distance - have direct bearing on pathogen transmission (Klowden et al. 1988, Takken et al. 1998). The results of this study suggest that both intraspecific and interspecific competition between *Oc. triseriatus* and *Ae. albopictus* influence *Oc. triseriatus* larval survival, adult size, and propensity to blood-feed. Competition with *Ae. albopictus* should therefore remove many *Oc. triseriatus* females from the viral transmission cycle,

but a majority of remaining females will be larger and more likely to feed successfully on a host due to release from intraspecific competition.

Other studies have found that smaller mosquitoes develop disseminated infections more efficiently, and only one other study to date (Alto et al. 2005) has examined the indirect impacts of interspecific larval mosquito competition on the ability of adult mosquitoes to transmit a virus. Alto et al. (2005) found that for one species, *Ae. albopictus*, intense larval competition resulted in smaller mosquitoes with greater Sindbis virus infection rates when compared to mosquitoes from low competition treatments. In contrast, findings reported here found a positive relationship between mosquito size and infection rates. Questions remain on why these two studies found opposing patterns, and why there is little consensus on the relationship between vector size and pathogen transmission (Takahashi 1976, Baqar 1980, Grimstad and Haramis 1984, Nasci and Mitchell 1994, Jennings and Kay 1999, Kay and Jennings 2002, Anderson et al. 2005). Such inconsistencies may be the result of system and species-specific differences and may derive from how little we know about many vector-pathogen systems. Nonetheless, results presented here suggest that even if smaller mosquitoes are more efficient viral transmitters, their reduced propensity to blood-feed makes them less likely to acquire an infection from an infectious host.

How these vector population dynamics would influence LACV transmission in the field is unpredictable. Patterns found with laboratory manipulation may disintegrate in the field, as the outcomes of these competitive interactions are extremely dependent upon resource amount and timing of resource input during mosquito development (Bevins, unpublished). Many factors influence larval mosquito development including

resource amount, temperature, larval density, and presence of predators and competitors (Teng and Apperson 2000). It is unlikely that these indirect cascades are solely driving the altered distribution of LACV cases in the US; however, *Ae. albopictus* presence does have the ability to affect native vector populations, and these effects could cascade throughout the complex transmission cycle of a vector-borne disease. *Aedes albopictus* could also directly influence LACV transmission by vectoring the disease itself thus altering LACV distribution. Reinterpretation of vector-borne disease transmission dynamics may be required if an invasive species whose presence could profoundly impact native vectors establishes in regions with vector-borne diseases. In the case of LACV, areas of *Ae. albopictus* and *Oc. triseriatus* sympatry are likely to differ from allopatric areas in their transmission cycles. The impacts of invasive vectors on pathogen transmission extend beyond the direct influence of a new vector to indirect effects on pathogen transmission via interactions with native vectors, and one hopes continued research will elucidate consequences associated with invasive vectors.

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## Tables

	Treatment				
	1	2	3	4	5
	# of larvae				
<i>Oc. triseriatus</i>	48	36	24	12	0
<i>Ae. albopictus</i>	0	12	24	36	48
Total	48	48	48	48	48

Table 1: Replacement series design used to examine competitive interactions between *Ochlerotatus triseriatus* and *Aedes albopictus*.

Source	df	F	p
Survival	3	3.83	0.0164*
Size	3	50.6	<0.0001**
Feeding	3	16.89	0.0098**
Infection	3	9.95	0.0251*
Disseminated Infection	3	7.85	0.0376*

Table 2: Effects of treatment on survival, size, feeding propensity, infection, and disseminated infection in *Oc. triseriatus*.

Treatment	48 Oc. triseriatus	36 Oc. triseriatus/ 12 Ae. albopictus	24 Oc. triseriatus/ 24 Ae. albopictus	12 Oc. triseriatus/ 36 Ae. albopictus	48 Ae. albopictus
<i>Ae. albopictus</i>					
% Surviving, Mean	N/A	87.7	88.2	81.4	86.4
Wing length (mm), Mean $\pm$ SD	N/A	2.55 $\pm$ 0.052	2.61 $\pm$ 0.053	2.72 $\pm$ 0.054	2.60 $\pm$ 0.050
<i>Oc. triseriatus</i>					
% Surviving, Mean	65.1	54.1	44.5	50.7	N/A
Wing length (mm), Mean $\pm$ SD	3.63 $\pm$ 0.064	4.03 $\pm$ 0.066	4.04 $\pm$ 0.069	4.10 $\pm$ 0.082	N/A
% Feeding, Mean	39.2	73.5	75.8	64.1	N/A
% Infected, Mean	35.5	62.7	61.2	61.2	N/A
% with Disseminated Infection, Mean	33.8	53.5	58.8	59.9	N/A

Table 3: Summary of all measured variables, for both *Ae. albopictus* and *Oc. triseriatus*, for all competitive treatments.

## Figure Legends

Figure 1: Mean larval survival, measured across competitive treatments as successful adult emergence, for both *Oc. triseriatus* and *Ae. albopictus* (n=2400).

Figure 2: Mean wing lengths, for both *Oc. triseriatus* and *Ae. albopictus*, across competitive treatments (n=646).

Figure 3: Comparisons of *Oc. triseriatus* female wing lengths between those that fed successfully and those that did not feed successfully (n=268).

Figure 4: Bivariate plots of LS means ( $\pm$  SE) for two dependent variables for *Oc. triseriatus* females that fed on a LACV infectious bloodmeal. (A) Proportion (logit transformed) of females from each treatment that successfully blood-fed, compared with mean wing lengths (n=268). (B) Proportion (logit transformed) of blood-fed females from each treatment that developed midgut infections, compared with mean wing lengths (n= 141). (C) Proportion (logit transformed) of blood-fed females from each treatment that developed disseminated infections, compared with mean wing lengths (n=131).

## Figures

Figure 1

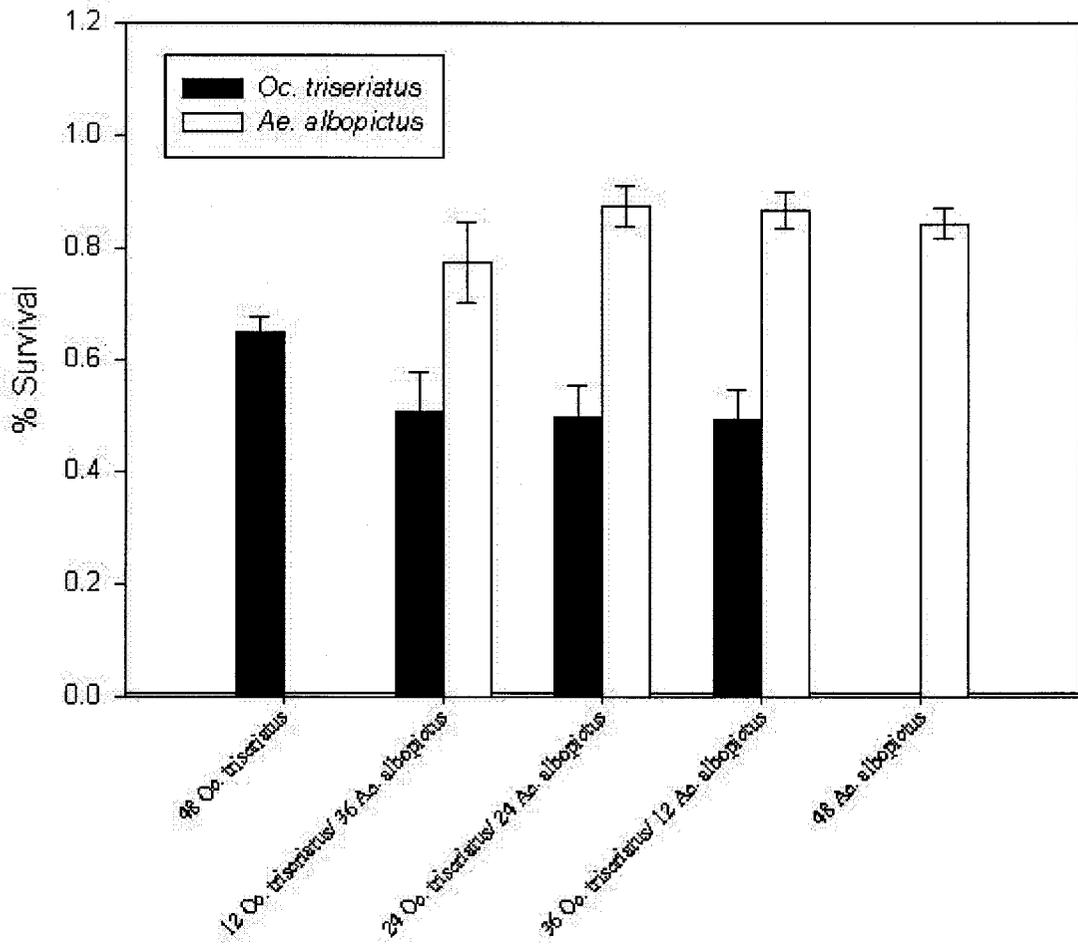


Figure 2

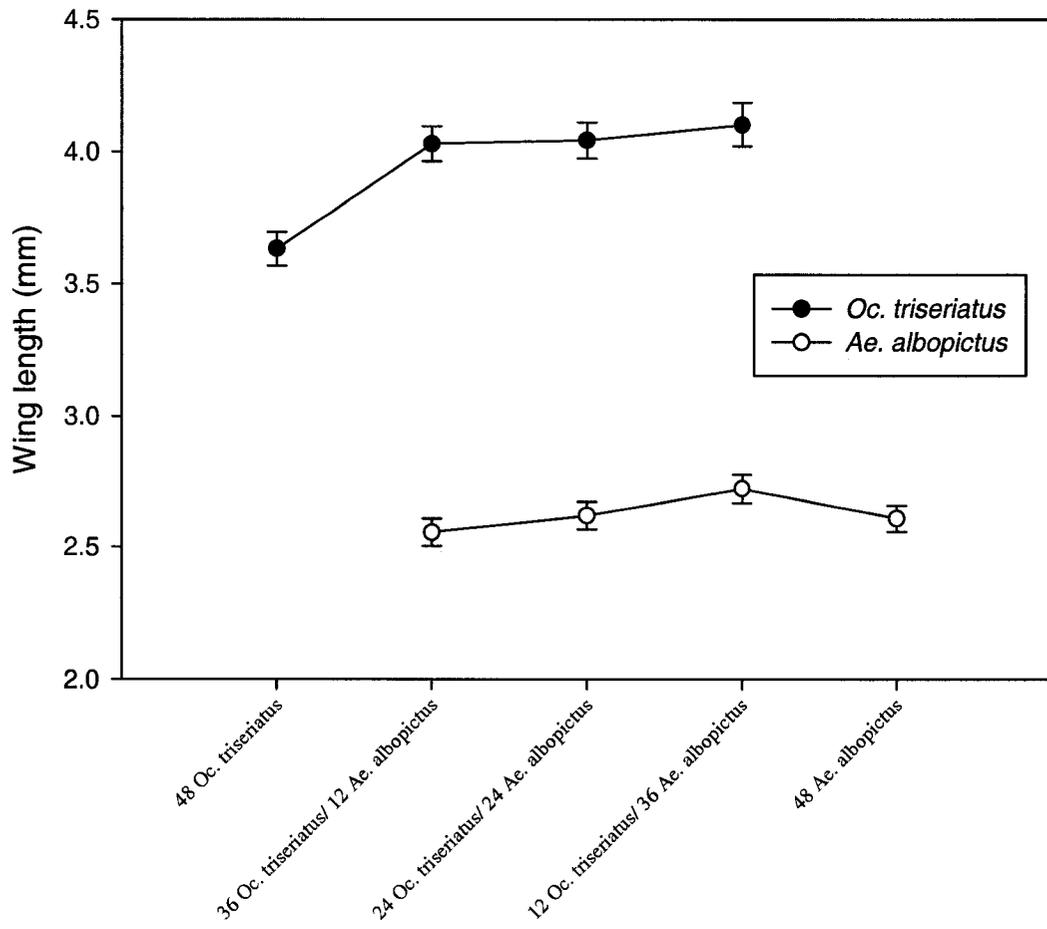


Figure 3

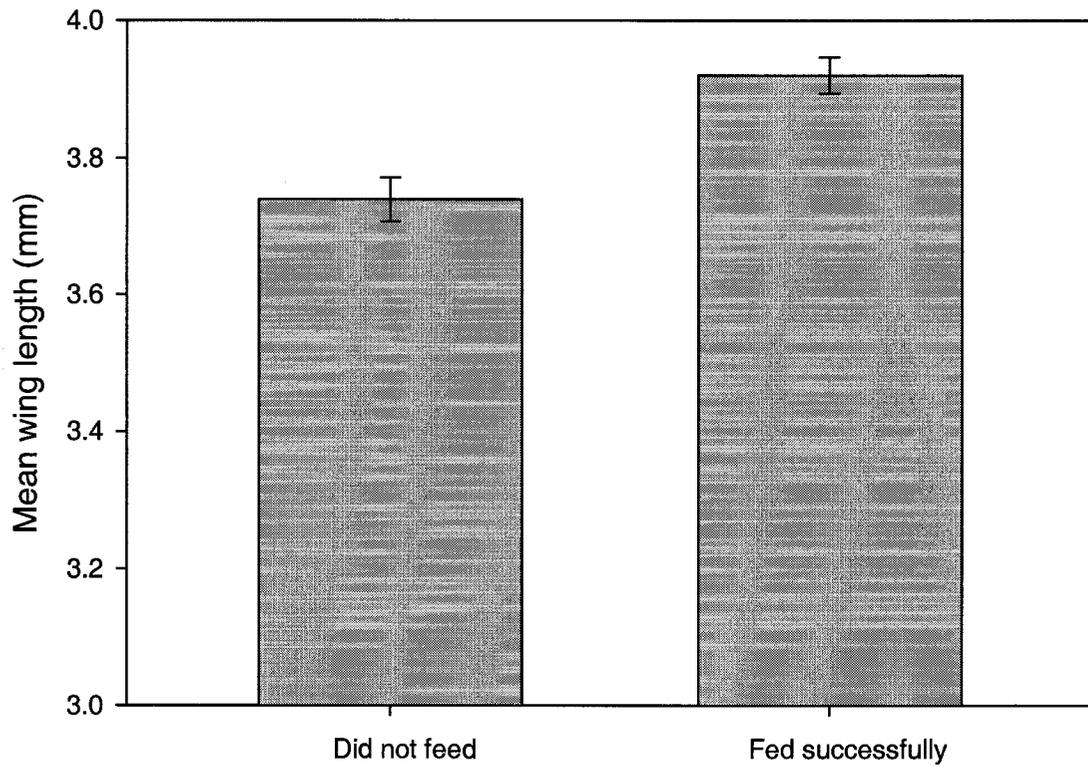


Figure 4(a)

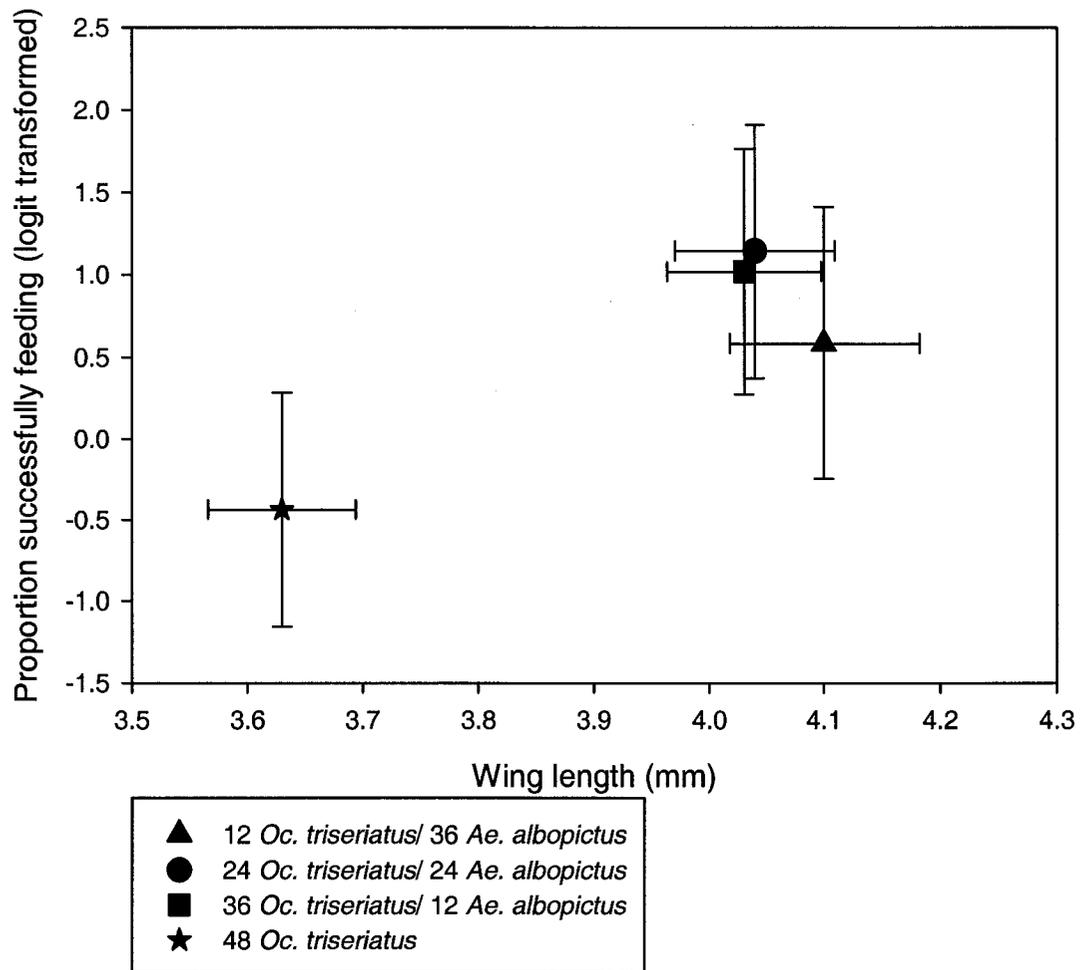


Figure 4(b)

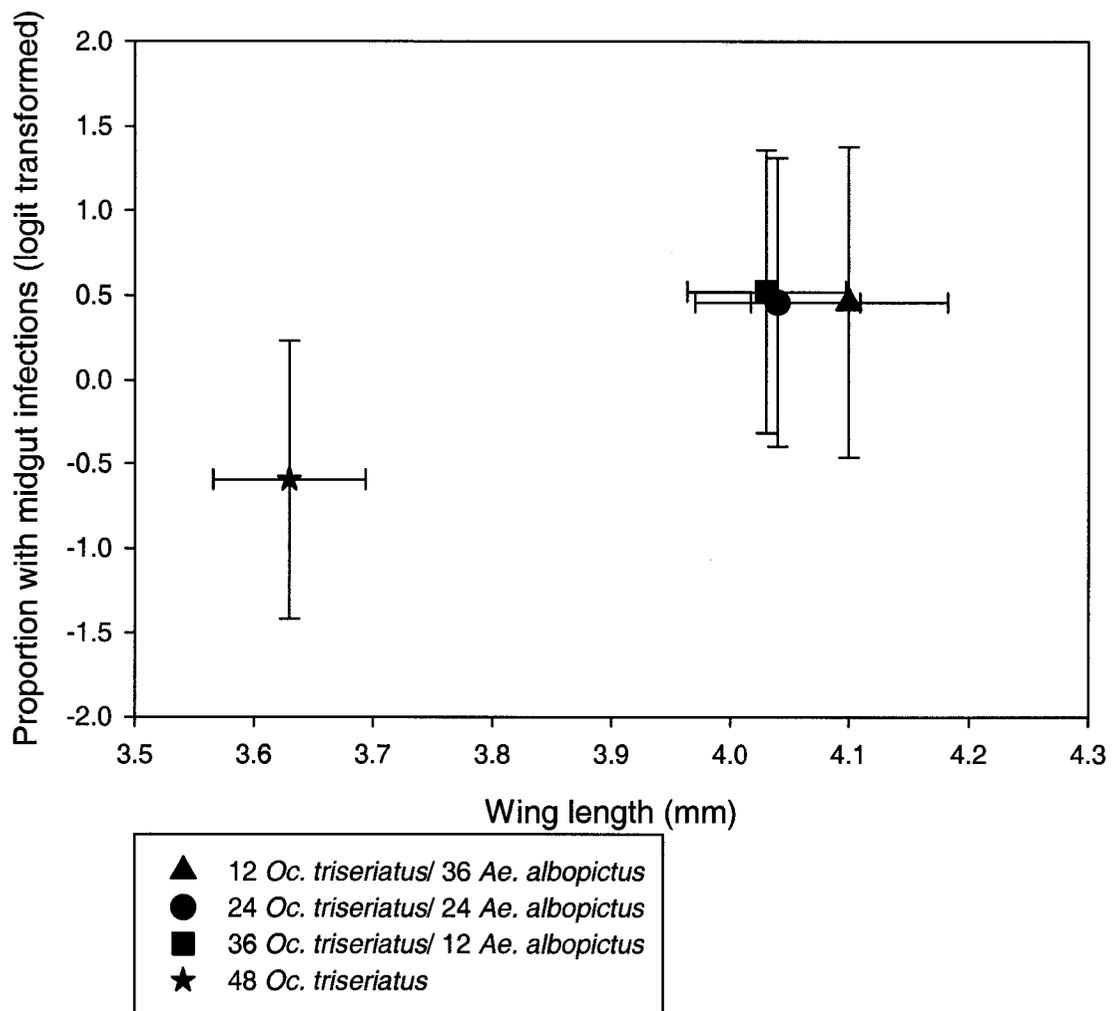
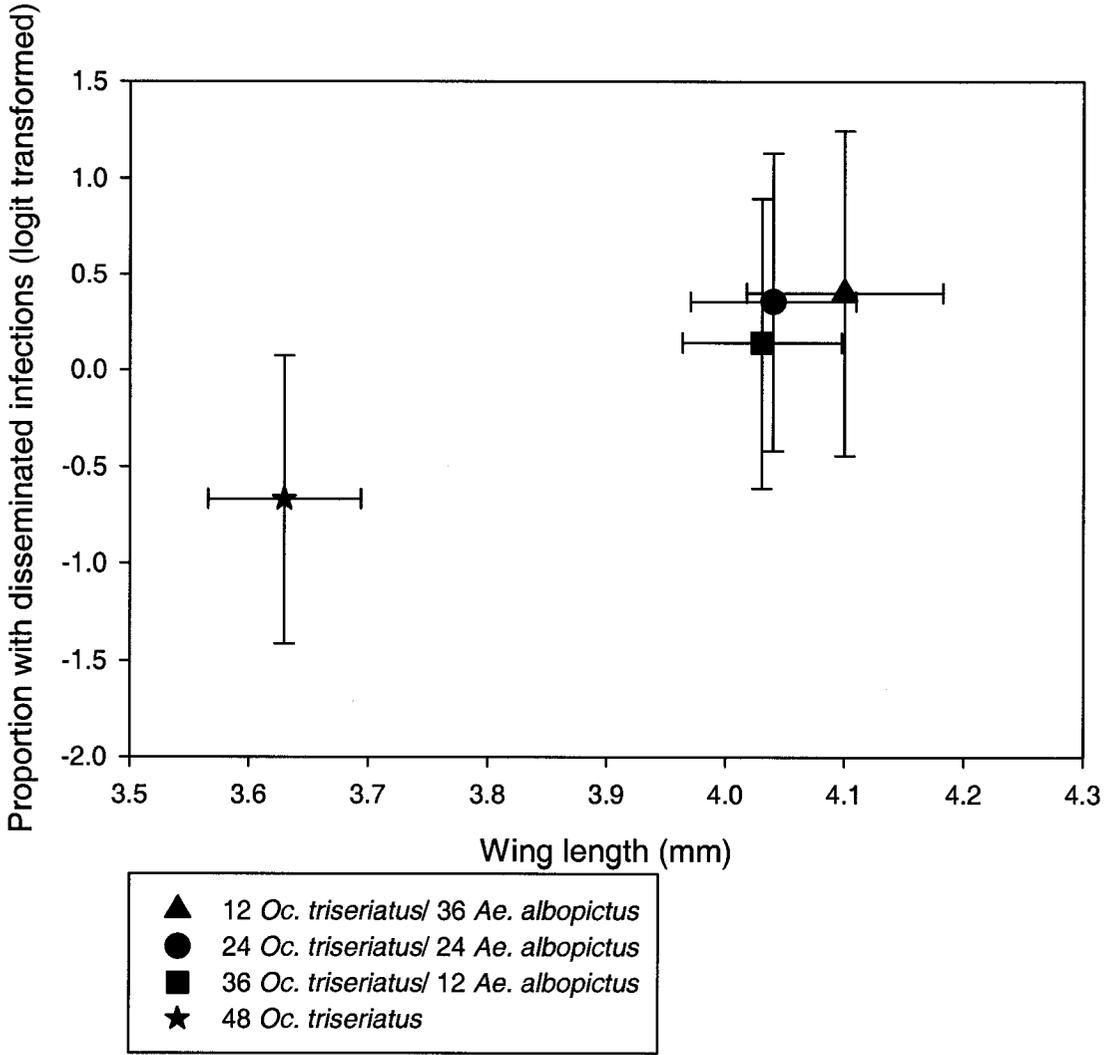


Figure 4(c)



## Chapter 4

### Establishment and abundance of a recently introduced mosquito species *Ochlerotatus japonicus* (Diptera: Culicidae) in the Southern Appalachians, USA

#### **Abstract**

*Ochlerotatus (Finlaya) japonicus japonicus* (Theobald; = *Aedes japonicus*, see Reinert 2000), is an introduced Asian mosquito species first detected in the Northeastern US in 1998. Since its initial discovery, this species has spread to many neighboring states and Canada. It was first identified in North Carolina, South Carolina, and Georgia in 2003. Larval surveys were conducted during 2005 and 2006 in the Southern Appalachians on a variety of natural and artificial containers to determine extent of *Oc. japonicus* establishment, as well as larval habitat requirements. Detritus amounts were measured in each container habitat, and co-occurring larval mosquito species were examined as indicators of container dynamics and potential interspecific competition. Data reveal that *Oc. japonicus* is now the most abundant container-inhabiting mosquito species in the study area. It exhibits flexibility in its use of container oviposition habitats, can survive in nutrient-limited conditions, and co-occurs with a wide range of larval mosquito species. Its rapid and successful establishment warrants continued monitoring, as its potential role as a nuisance species or arbovirus vector remains unknown.

## Introduction

Nonindigenous species introduction occurs commonly throughout the globe. Such introductions are not novel; recent increases in human movement and global commerce have likely increased the introduction of species as well (Di Castri 1989, Mack et al. 2000, Juliano et al. 2004). While species are continually being introduced to new areas, very few establish successfully, and very few of those earn the distinction of being invasive; that is, a species that proliferates, spreads, and persists in a new locale (Mack et al. 2000). A mosquito species that recently arrived in the US, *Ochlerotatus (Finlaya) japonicus* (Theobald), has successfully established in many areas and has the potential to become invasive.

*Ochlerotatus japonicus* is native to Japan, Korea, China, and Taiwan (Tanaka 1979, Sota et al. 1994) and was first identified in the US, in New York, New Jersey, and Connecticut in 1998 (Peyton et al. 1999, Andreadis et al. 2001). It has since been detected in several other areas within the continental US outside of its initial discovery site, including Maine (Holman et al. 2006), Pennsylvania (Fonseca et al. 2001), West Virginia (Joy and Sullivan 2005), Virginia (Harrison et al. 2002), Missouri (Gallitano et al. 2005), North Carolina, South Carolina, Georgia (Reeves and Korecki 2004, Gray et al. 2005), and Washington (Roppo et al. 2004). It has also recently been found outside the continental US in Ontario (Thielman and Hunter 2006), Hawaii (Larish and Savage 2005), New Zealand (Laird et al. 1994), and France (Schaffner et al. 2003). While there is evidence of multiple introduction sites (Fonseca et al. 2001), *Oc. japonicus* is clearly establishing and naturalizing.

*Ochlerotatus japonicus* is a container-inhabiting mosquito species, with larvae developing in a variety of artificial and natural containers (Tanaka 1979). Records from both the native home range and recently colonized regions indicate that it thrives in a range of habitats, including rural, suburban, and urban settings (Takashima and Rosen 1989, Andreadis et al. 2001). Adult females are known to feed primarily on mammals in the field (Apperson et al. 2004), including humans (pers. obs.), but they feed readily on avian hosts as well in the lab (Sardelis et al. 2002a, Sardelis et al. 2003). It is a suspected vector of Japanese Encephalitis in Asia (Takashima and Rosen 1989) and laboratory work has also shown it to be a competent vector of several viruses in the US, including West Nile virus (Sardelis and Turell 2001), eastern equine encephalitis (Sardelis et al. 2002a), St. Louis encephalitis (Sardelis et al. 2003), and Lacrosse virus (Sardelis et al. 2002b). The role *Oc. japonicus* may play as a nuisance species or disease vector in the US is unknown; however, its current distribution overlaps with several vector-borne diseases that are public health concerns, and for that reason alone, increased understanding of its distribution and abundance is needed.

This larval survey took place in regions of North Carolina, Georgia, and South Carolina. *Ochlerotatus japonicus* was first detected in this area in 2003 (Gray et al. 2005). While previous research has documented *Oc. japonicus* arrival and spread in the US, the primary goal of this field survey was to sample intensively a small region to determine the extent to which *Oc. japonicus* was establishing in the area, to qualitatively assess preferred habitat types and restrictions, to ascertain the types of oviposition habitat used for larval development, and to examine what other larval

mosquito species co-occur with *Oc. japonicus*. In addition, the amount of detritus in individual containers was quantified to estimate resource availability, and wing lengths of emergent *Oc. japonicus* females were recorded in order to examine potential interspecific competitive interactions between *Oc. japonicus* larvae and other immature mosquito species.

## **Methods**

Nearly 5,000 larval mosquitoes were collected from 75 sample sites in July and August of 2005 and 2006. Collection sites were located in Macon, Transylvania, and Jackson counties in North Carolina, Oconee County in South Carolina, and Rabun and Habersham counties in Georgia (Fig.1). This mountainous and heavily forested region ranges from 425 meters to nearly 1500 meters in elevation with mean winter low temperatures approaching -4.5 degrees C. Mean January temperature is a variable known to influence mosquito species distributions (Nawrocki and Hawley 1987). Annual precipitation often exceeds 2,000 millimeters, making this one of the wettest regions in the eastern US. Climatic conditions, combined with geologically old granite strata that readily collect water in rock pools, make this region similar to areas *Oc. japonicus* is known to inhabit in its Asian home range. While there is limited published information on specific native habitat requirements, *Oc. japonicus* has a known distribution that stretches from areas in Northern Japan southward to Korea, South China, and Taiwan (Tanaka 1979, Takashima and Rosen 1989).

Larval surveys were centered in both rural and suburban areas, with many sites sampled in consecutive years. Surveys were conducted by walking 100 step

wandering grids, with target habitats consisting of artificial and natural containers (< 2 meters above ground). When a container was encountered the location was marked using a Garmin™ GPS unit. Water temperatures of container habitats, as well as time of day, were recorded and the size of the container was measured to estimate water volume. Container contents, including detritus, water, and all mosquito larvae present, were collected with a 100 mL bulb pipette and placed in either 1 Liter Nalgene® containers (United States) or Nasco® whirlpak bags (United States) for transport to Highlands Biological Station. Large containers (>2500cm<sup>3</sup>) were encountered as habitats as well. The large size of these containers precluded collection of all larvae; therefore, a dipper was used to sample approximately 100 larvae from each of these containers. Larvae from each container were counted and identified to species (Darsie and Ward 2005). If instars were too small for identification, they were allowed to mature to later instars and were then identified. Voucher specimens for both adult and larval stages were deposited in the Gillette Museum at Colorado State University.

Pupae collected from sample sites were separated from larvae and allowed to emerge as adults. Adult mosquitoes were sustained on water and honey until they were identified to species. Of these adults, female *Oc. japonicus* also had one wing removed to estimate overall body size. Wings were mounted on a glass slide and measured from the alular notch to the distal margin (excluding fringe scales) using a calibrated eyepiece micrometer. Wing length in female mosquitoes correlates with body size (Christophers 1960), and adult body size in mosquitoes can indicate

resource availability in the larval habitat (Fish and Carpenter 1982, Nasci and Mitchell 1994).

In order to approximate the amount of nutritional resources available in larval habitats, detritus from each container was strained through a mesh sieve (1 mm aperture) and placed it in a drying oven at 80 degrees Celsius for 24 h. Samples were then weighed to the nearest 0.01 gram. All statistical analyses were run on SAS® software (version 9.1) using Spearman correlation coefficients (Proc Corr).

## Results

*Ochlerotatus japonicus* larvae and pupae were detected at the onset of field collection on both 15 July 2005 and on 21 July 2006, indicating that *Oc. japonicus* activity in this region had begun prior to field collection. Of the 4,961 mosquitoes collected during 2005 and 2006, *Oc. japonicus* accounted for 48% (2,381 mosquitoes) of the individuals collected, making it the most abundant container-inhabiting mosquito species in the region (Fig. 2). *Ochlerotatus japonicus* was still the most abundant mosquito species collected after rock pool samples (a highly-specific container habitat) were removed from data analysis. This invasive mosquito species was found in 55 of 75 sample sites. *Ochlerotatus japonicus* was collected from every sampled county (Fig.1) and at all sampled elevations (425 meters to 1,500 meters), indicating that the range of climatic and altitudinal factors encountered in this heterogeneous landscape does not constrain its distribution. It was the only container-inhabiting mosquito species collected at a sample site located above 1,400

meters. *Ochlerotatus japonicus* larvae were also detected in rock pools associated with all major rivers (Chatooga, Tallula, Nantahala, and Cullasaja) in the region.

In conjunction with an extensive regional distribution (Figures 3 and 4), *Oc. japonicus* also demonstrated flexibility in its use of larval habitat (Figures 3 and 4). When broken down into habitat types, 46% of all artificial containers, and 40% of all natural containers, contained *Oc. japonicus*. Artificial habitats included scrap tires, discarded buckets, drainage pipes, tarps, garden-based water features, drinking fountains, and a range of cans and bottles (Table 1). Natural habitats (rock pools and tree holes) also supported *Oc. japonicus* throughout the region (Table 1). Average container size supported 1.8 L of water (SD = 4.85 L, range 0.008 L – 27.5 L); however, *Oc. japonicus* larvae were found across a wide range of container sizes, including small containers with very high larval density, as well as extremely large containers. *Ochlerotatus japonicus* was found both in shaded sites and in containers located in full sun. Water temperatures of container habitats ranged from 16.8-28.6°C (S.D. = 1.58).

The diversity of containers used by *Oc. japonicus* as oviposition habitat resulted in co-occurrence with many other mosquito species. Other mosquito species collected from *Oc. japonicus* positive containers included *Ochlerotatus triseriatus* (= *Aedes triseriatus*, see Reinert 2000), *Ochlerotatus atropalpus* (= *Aedes atropalpus*, see Reinert 2000), *Aedes albopictus*, *Culex restuans*, *Culex territans*, *Culex quinquefasciatus*, and *Anopheles punctipennis* (Table 1). In artificial container habitats, *Oc. japonicus* was most often found with *Cx. restuans*, the second most common mosquito larvae collected at sample sites (32%; Table 1). In natural

containers, specifically rock pools, *Oc. japonicus* was most often found in the presence of *Cx. territans* (Table 1).

Larger containers held up to 1,000 or more individual larvae, but there was an inverse relationship between container volume and larval density ( $r = -0.44$ ,  $p > 0.0001$ ,  $n = 157$ ). Detritus amounts in these container habitats ranged from 0.1 grams to 21.1 grams (SD = 8.02), and all of these containers supported *Oc. japonicus*. There was a significant relationship between container volume and amount of detritus ( $r = 0.36$ ,  $p < 0.0001$ ,  $n = 113$ ); larger containers tended to have more detritus. There was also a positive correlation between female *Oc. japonicus* wing length and the amount of detritus in a container ( $r = 0.40$ ,  $p > 0.0001$ ,  $n = 113$ ), with one gram of detritus, on average, supporting 100 mosquito larvae (S.D. = 167; Range = 2 - 750). Emergent adult female wing lengths ranged in size from 2.88 mm – 4.26 mm, with a mean wing length of 3.67 mm (SD = 0.28). The presence or absence of other larval mosquito species was also analyzed to examine any effect on female *Oc. japonicus* wing lengths; however, there were no significant effects. Greater species diversity occurred primarily in larger containers and since larger containers, on average, also contained more detritus, the *Oc. japonicus* females measured in this study were not nutrient-limited when in the presence of other mosquito species.

## **Discussion**

*Ochlerotatus japonicus* was first detected in western North Carolina and northern Georgia in 2003 (Gray et al. 2005). Summer field data from the same region in 2005 and 2006 now show it to be the most abundant container-inhabiting mosquito species in the region. The rapid colonization and establishment of this recently

introduced mosquito species, combined with flexibility in container use for oviposition sites and a reported tolerance for a wide range of climatic conditions, suggest that this species may continue to spread to adjacent regions where it has yet to be detected.

Larvae were detected at a wide range of altitudes, including mountain summits (1500 meters) where winter low temperatures can dip below -18 degrees C. This temperature tolerance occurs within its home range as well, which can reach into northern Japan. Such a distribution may be indicative of a different colonization pattern than we have previously seen with invasive mosquito species in the continental US. *Aedes albopictus* and *Aedes aegypti* are two of the better-known invasive mosquitoes in the US, and both have established successfully; however, their distribution is limited to regions of warmer temperatures and relatively high humidity (Nawrocki and Hawley 1987). A similar winter temperature barrier may not exist for *Oc. japonicus*, and indeed, it has been found up the eastern seaboard of the US and throughout Ontario, Canada (Andreadis et al. 2001, Sardelis and Turell 2001, Thielman and Hunter 2006).

Data also reveal that oviposition sites for *Oc. japonicus* encompass a wide range of container types. Larvae were collected from both artificial containers located in suburban settings, and in natural containers in extremely rural areas. Every watershed sampled was positive for *Oc. japonicus*, and rock pools in particular supported large numbers of larvae. This observation agrees with previous findings on *Oc. japonicus* habitat in its native range. The extensive use of rock pools as oviposition sites in the US as well may point to river corridors as main distribution

routes of this species to new areas. This would contrast with colonization patterns exhibited by mosquito species that have previously invaded the US, such as *Ae. albopictus*. *Aedes albopictus* primarily uses artificial containers (tires, buckets, etc.) for oviposition sites and in consequence, *Ae. albopictus* distribution routes in the US mainly followed major roadways along which used tires and other goods are transported (Moore and Mitchell 1997). Its use of urban habitat also allowed for ease of monitoring as this species spread to new areas. If *Oc. japonicus* is expanding its range via river corridors, its colonization pattern may differ drastically from that of *Ae. albopictus*. Such a colonization pattern may result in *Oc. japonicus* establishment in more isolated areas, where detection of a new species may be delayed. It is likely that this species has already established where its presence has not yet been noted; however, its ability to oviposit in a wide range of container types suggests that this species will establish in urban areas and exploit urban corridors as well.

The wide range of container habitats used by *Oc. japonicus* leads to sharing containers with an equally wide range of cohabiting species. In rock pools, *Oc. japonicus* was frequently found with *Oc. atropalpus*, while it was most commonly found with *Oc. triseriatus* in tree holes. In artificial containers, *Oc. japonicus* commonly occurred with *Cx. restuans* but samples also revealed it to be found alongside a wide range of other mosquito species. Many of the species that co-occur with *Oc. japonicus* have disparate habitat requirements, ranging from pristine rock pools to artificial containers with extremely high organic content. All of these habitat types supported *Oc. japonicus*, as did depauperate containers.

Given that *Oc. japonicus* larvae are found alongside other larval mosquito species and that they can persist in low-nutrient environments, there is potential for interspecific resource competition in regions where *Oc. japonicus* has successfully established. Juliano et al. (2004) have shown that resource competition between the invasive mosquitoes *Ae. albopictus* and *Ae. aegypti* played a role in the displacement of *Ae. aegypti* in several southeastern regions of the US. Analyses did not indicate that interspecific competition was occurring in my study (presence of other mosquito species did not influence size of adult female *Oc. japonicus*), but a majority of containers that contained more than one mosquito species were those that were larger and more nutrient-rich, potentially revealing more about habitat quality than interspecific interactions between mosquito species; however, variability in female size in the field, and while detritus amounts are only an estimate of the nutrients available to mosquito larvae, female size was affected by the amount of detritus in a container with depauperate conditions producing smaller females. Since adult size is known to reflect nutrients available to immature mosquitoes (Christophers 1960), resource-poor containers would produce smaller females (Fish and Carpenter 1982, Nasi and Mitchell 1994). Interspecific competition may occur under such conditions, as this survey was not exhaustive and multiple species do likely develop in nutrient-limited habitats. Unfortunately, little is currently known about the competitive ability of this species, and both field monitoring and laboratory-based experimental research are necessary for further understanding.

*Ochlerotatus japonicus* could potentially affect vector-borne disease transmission in the US. This study took place in a region that has seen a recent

increase in human cases of Lacrosse virus (LACV), a pediatric arboviral encephalitis primarily spread to humans by the mosquito vector, *Oc. triseriatus* (Jones et al. 1999, Erwin et al. 2002). The virus cycles between small mammals and mosquitoes, with incidental cases occurring in humans. These cases occasionally manifest as severe clinical symptoms in children and young adults. Laboratory transmission rates of LACV by *Oc. japonicus* were comparable to those of the historic vector, *Oc. triseriatus* (Sardelis et al. 2002b). The extent to which *Oc. japonicus* will play a role in disease transmission, if any, is unclear; however, its rapid and successful establishment in a region known to have endemic transmission of LACV (Szumilas et al. 1996, Jones et al. 1999, Erwin et al. 2002, Utz et al. 2003) suggest this question merits a closer look.

Overall, not only is *Oc. japonicus* a new species in the US, but it is a species with different physiological constraints and different habitat preferences than the mosquito species that have previously invaded the US. These differences may herald different invasion patterns, and invasion into different regions, than we have previously seen with invasive mosquito species in the US. Previous work has shown that *Oc. japonicus* appears to be naturalizing in many areas, and the data from this study support the contention that it will become a dominant invader.

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## Tables

Table 1: Larval *Ochlerotatus japonicus* collections with corresponding habitat types and associated species.

Habitat	No. of sites	No. of sites containing <i>Oc. japonicus</i>	% of sites occupied	Associated species
Tree holes	5	5	100	<i>Oc. triseriatus</i>
Rock pools	24	20	83.3	<i>Cx. territans</i> , <i>Oc. atropalpus</i> , <i>Cx. restuans</i>
Small artificial containers (< 0.2 L)	24	12	50	<i>Cx. restuans</i> , <i>Oc. triseriatus</i> , <i>Ae. albopictus</i> , <i>Cx. pipiens</i>
Large artificial containers (> 0.2 L)	20	19	95	<i>An. punctipennis</i> , <i>Cx. pipiens</i> , <i>Ae. albopictus</i> , <i>Cx. territans</i>
Surface water pools	2	0	0	<i>Cx. pipiens</i>

## Legends

**Fig. 1.** Highlighted counties were surveyed for *Ochlerotatus japonicus* in this study (compliments of the United States Geological Survey).

**Fig. 2.** Comparative abundance of container-breeding mosquito species sampled during 2005 and 2006. *Ochlerotatus japonicus* was the most commonly encountered species, comprising 48% of total mosquito larvae sampled (n = 4,961).

**Fig. 3.** Larval mosquito species found in artificial containers and the corresponding county (n = 3,397).

**Fig. 4.** Larval mosquito species found in natural containers and the corresponding county (n = 1,564).

**Figures**

Figure 1

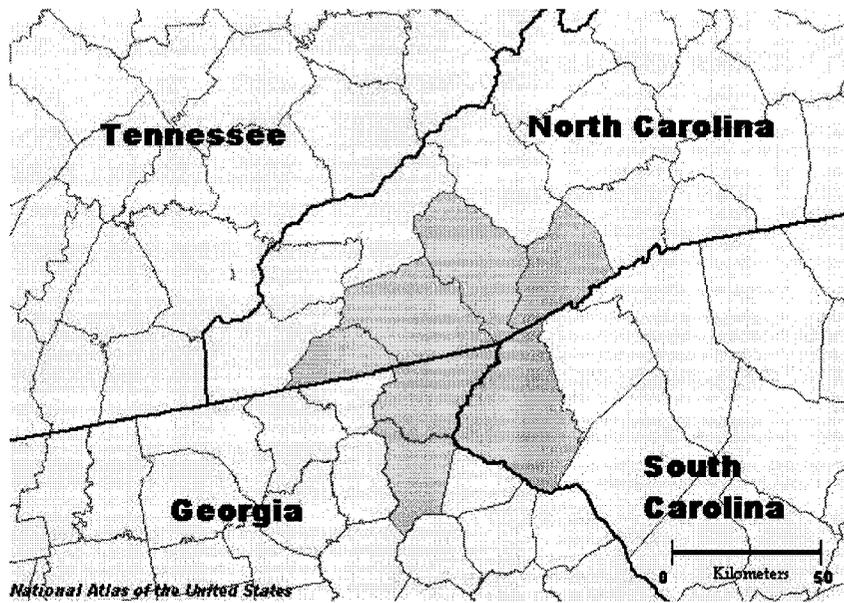


Figure 2

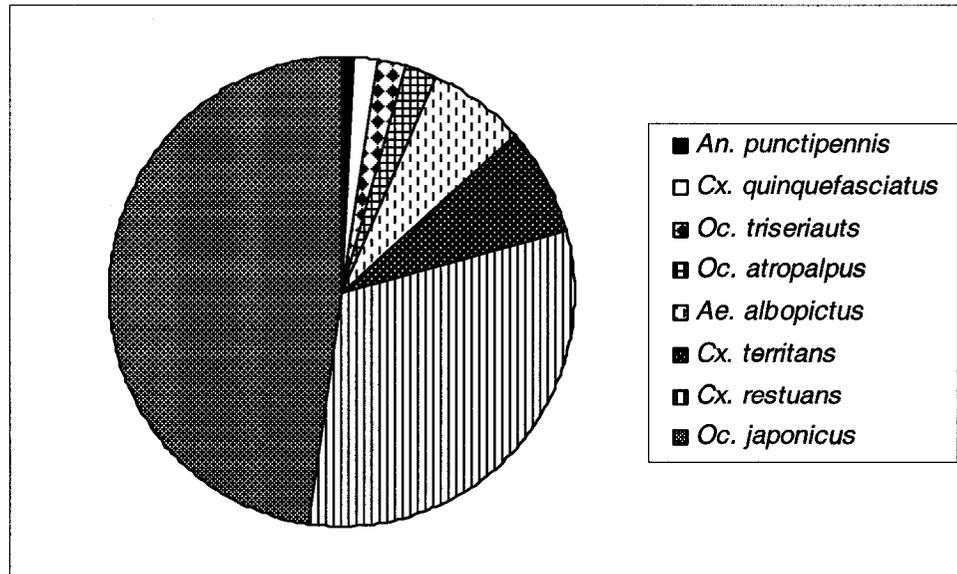


Figure 3

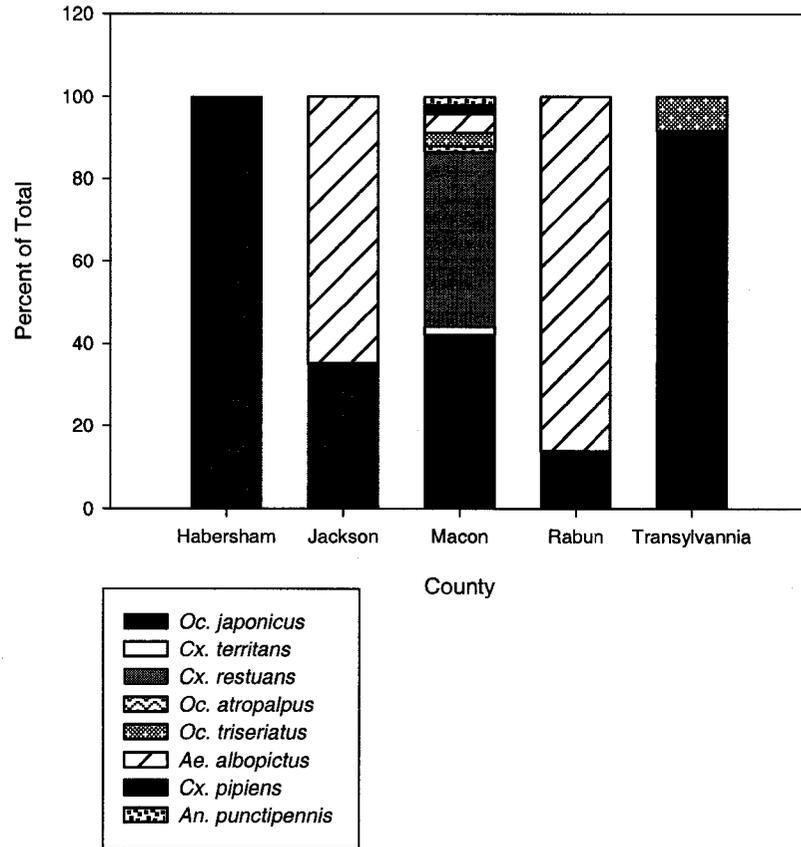
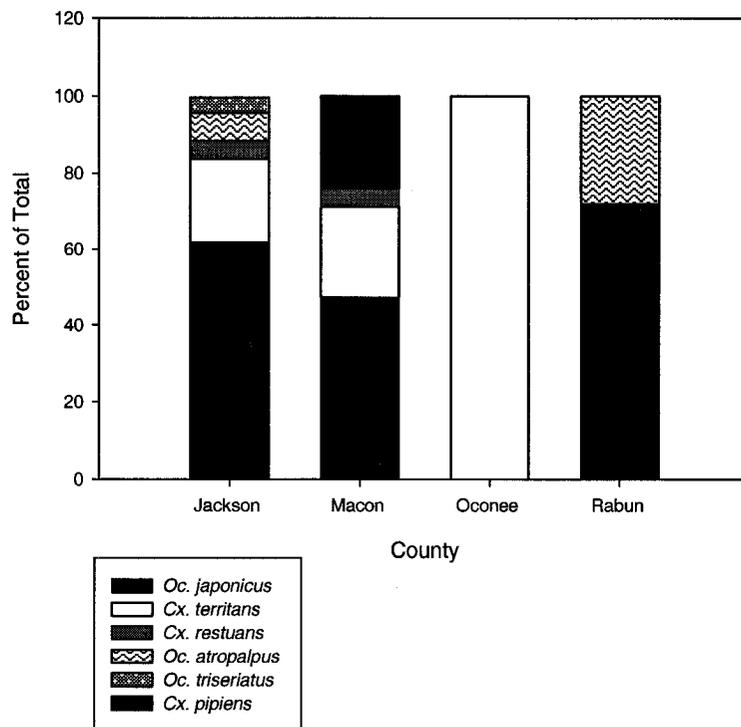


Figure 4



## **APPENDICES**

## **Appendix 1: Transovarial Transmission**

Adult mosquito size reflects the amount of nutrition available during larval development (Christophers 1960). Additionally, size is known to correlate with horizontal transmission efficiency of a pathogen (Takahashi 1976, Grimstad and Haramis 1984); however, the effect adult mosquito size has on vertical pathogen transmission is unknown. La Crosse virus (LACV) is an arbovirus that is horizontally transmitted between its main mosquito vector, *Ochlerotatus triseriatus*, and small mammals. This virus is also vertically transmitted with high efficiency from female mosquitoes to their offspring. Here, I report on the relationship between *Oc. triseriatus* size, fecundity, and LACV transovarial transmission rates.

### **Methods**

Mosquito eggs from laboratory-adapted colonies were supplied by the Arthropod-borne Infectious Disease Lab (AIDL) in Fort Collins, Colorado. Eggs were hatched simultaneously in separate 1 L plastic containers containing 300 mL of tap water and hatching solution (1:100; Difco [Detroit, MI] brain-heart infusion). Larvae were counted and added to respective treatments three days after hatching commenced. The replacement series design employed five treatments, with differing ratios of *Ae. albopictus* and *Oc. triseriatus* for each treatment, while overall density of mosquito larvae per treatment remained constant (Table 1). *Aedes albopictus* is an invasive mosquito species known to be a dominant competitor for larval resources; its

presence in the larval environment is known to influence *Oc. triseriatus* size (Novak et al. 1993). Varying ratios of the two species produced adult *Oc. triseriatus* representing a wide range of sizes. Each treatment contained three replicates.

Larval rearing methods and viral preparation are reported in previous studies (Gabitzsch et al. 2006). Emergent adult female *Oc. triseriatus* fed on virus-infected Vero cells diluted in a fresh sheep-blood preparation and placed both in an artificial membrane feeder and as droplets on mosquito netting. Female *Oc. triseriatus* were allowed to feed for one to two hours. Blood-meals were analyzed immediately before and after feedings with TCID<sub>50</sub> viral titers  $\approx 8.51 \times 10^5$  / ml. Mosquitoes were sorted on a chill-table, and females that successfully fed (determined visually by distention) were held for 14 days for viral incubation. During this time females were placed in 30 cm x 30 cm flight cages containing males from the same respective treatments and allowed to mate. Mosquitoes were sustained on sugar cubes, water, and raisins, and females were given a second, non-infectious bloodmeal after 14 days using the same protocols. Females were then separated into individual mesh-covered 550 ml paper-board containers and allowed to oviposit on unbleached 2 cm x 15 cm paper strips held in oak-leaf infused water. After one gonotrophic cycle, females had one wing removed and measured to estimate size. Wing length is an accepted criterion to approximate overall mosquito size (Christophers 1960). La Crosse virus infection was determined with an indirect immunofluorescence assay using a fluorescein labeled anti-LACV antibody on mosquito head-squashes (Beaty and Thompson 1978).

Oviposition strips were collected and individual eggs counted to estimate clutch size. Eggs were then hatched following the same protocol as above, reared

until fourth instars, and then analyzed for infection following protocol (Gabitzsch et al. 2006). General linearized mixed models (SAS, Proc Glimmix) were used to analyze the effect treatment had on clutch size and vertical transmission. Pearson correlation coefficients were used to examine relationships between wing length and clutch size, as well as wing length and vertical transmission rates.

### **Results**

Treatment significantly affected wing lengths of emergent females ( $F=4.14$ ,  $p=0.011$ ), with females increasing in size as the proportion of invasives per treatment increased. Treatment did not significantly affect vertical transmission. Wing length was found to correlate with fecundity ( $r=0.600$ ,  $p<0.0001$ ). Clutch size increased as female wing length increased (Figure 1). Proportion of vertically infected progeny was not related to female wing length (Table 2). On average, 67.5% of progeny were transovarially infected with LACV (Table 2).

### **Summary**

Previous studies have found relationships between vector size and horizontal pathogen transmission efficiency in the LACV/*Oc. triseriatus* system; however, LACV also exhibits high vertical transmission rates from female mosquitoes to offspring, and little is known about how vector size influences vertical transmission. Results reported here demonstrate that larger females have larger clutch sizes, which has been previously documented (Lyimo and Takken 1993); however, there was no correlation between vector size and proportion of transovarially infected offspring. La Crosse virus infections in *Oc. triseriatus* mosquitoes have few detectable fitness consequences, and vertical transmission is extremely efficient (Patrican and Defoliart

1985). This efficiency is not impacted by adult size, although smaller females lay fewer eggs overall, which could potentially affect vector population sizes. In this study, *Ae. albopictus* presence affected adult *Oc. triseriatus* size and fecundity in the laboratory. Field studies may clarify how these effects influence native mosquitoes at the population and community scales, and what ramifications those have, if any, on pathogen transmission parameters.

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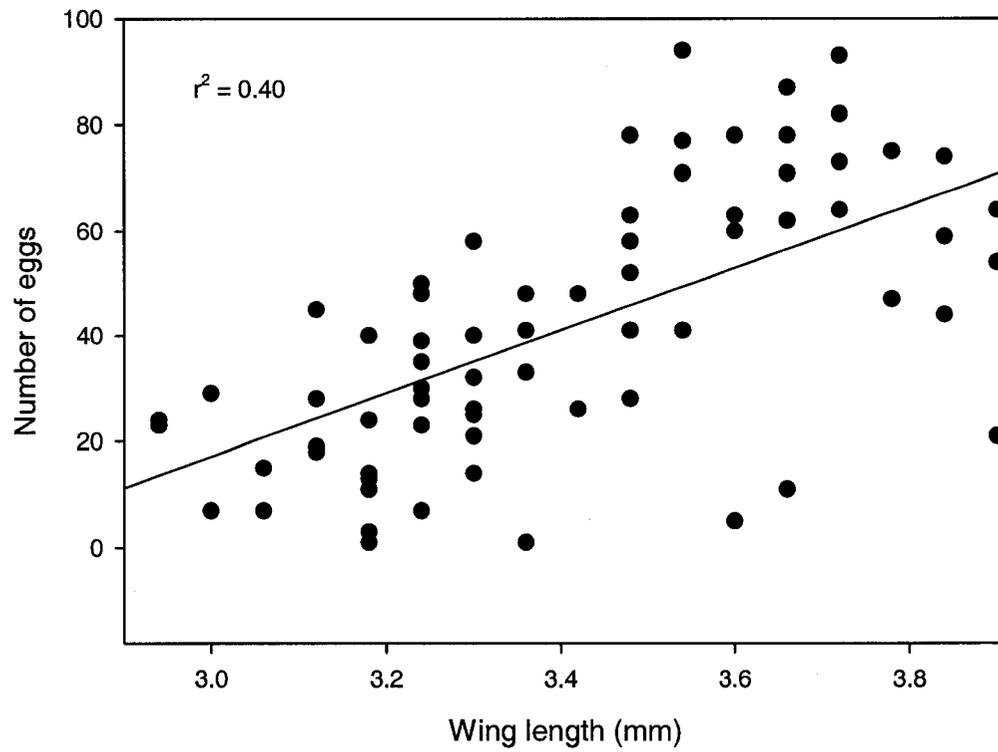
Table 1: Replacement series design varying the number of each mosquito species per treatment while density remains constant.

<b>Treatment</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>
# of <i>Oc. triseriatus</i>	48	36	24	12	0
# of <i>Ae. albopictus</i>	0	12	24	36	48
Total # of mosquito larvae	48	48	48	48	48

Table 2: Descriptive statistics for all variables examined.

	<b>n</b>	<b>Mean</b>	<b>Standard Deviation</b>	<b>Range</b>
Wing Length (mm)	93	3.37	0.27	2.82 - 3.90
Clutch Size	71	40.71	25.28	0 - 94
% of Offspring Infected	2891	67.40%	20.00%	16.0 - 100%

Figure 1: Linear regression of wing length and clutch size per female ( $r^2 = 0.40$ ).



## **Appendix 2: *Ochlerotatus triseriatus* and *Aedes albopictus* field sampling**

Human transport and commerce have introduced new species to areas around the globe. Some invaders substantially alter native biodiversity and can affect many ecological scales. *Aedes albopictus*, is one of those species. A container-inhabiting mosquito species native to Asia, its rapid establishment in the US led to declines in resident mosquito species, such as *Aedes aegypti* (Juliano and Lounibos 2005). Larval resource limitations caused by interspecific competition are believed to be the mechanism behind these declines (Juliano et al. 2004). Resident mosquitoes that manage to persist within the same larval habitat as *Ae. albopictus* often emerge as smaller, nutritionally deprived adults (Lounibos 2001).

*Ochlerotatus triseriatus* is a mosquito species native to the US that also oviposits in water-filled containers and is yet to be replaced by *Ae. albopictus*, but it is not immune to effects of interspecific larval competition. Here, I report on field surveys of larval mosquito habitats in an area where *Ae. albopictus* and *Oc. triseriatus* ranges overlap. The goal was to examine morphological effects of interspecific larval competition on the two species. Both of these species are known vectors of medically important pathogens and competitive effects could impact pathogen transmission dynamics.

**Methods:**

This study was conducted in June and July of 2005, in a region encompassing Macon, Clay, and Jackson counties in North Carolina, as well as Rabun County in Georgia. Mosquito-borne disease cases in humans have increased in this region in recent years (Jones et al. 1999). Sample sites were encountered by walking 100 step wandering grids. When a container was encountered the location was marked using a Garmin™ GPS unit. Water temperature was recorded and container size was measured to estimate volume. The entire contents of the container were removed and taken back to the laboratory for processing. All fourth instar larvae and pupae were removed from samples and allowed to emerge as adults for species identification (Darsie and Ward 2005) and wing length measurements. By limiting adult size data to only third and fourth instars, data included only those individuals exposed to natural competitive interactions while developing. Mosquito wing length is an accepted proxy for overall body size (Nasci 1990). Wings were mounted on a glass slide and measured from the alular notch to the distal margin (excluding fringe scales) using a calibrated eye-piece micrometer. Representative specimens were submitted to the C.P. Gillete Museum at Colorado State University. Remaining mosquitoes were allowed to develop until fourth instars and then identified to species (Darsie and Ward 2005). Statistical analyses consisted of t-tests comparing mosquito wing lengths from monospecific samples with wing lengths of mosquitoes co-occurring with other larval species.

**Results:**

Previous work predicts that *Ae. albopictus* will outcompete *Oc. triseriatus* when the two species co-occur in the same container habitat, leading to smaller emergent *Oc. triseriatus* females (Lounibos 2001). Wing lengths of female *Oc. triseriatus* emerging from habitats containing only conspecifics did not differ from those that emerged from habitats containing *Ae. albopictus* ( $t = -0.36$ ,  $p = 0.71$ ; Table 1; Figure 1a). There also was not a significant difference between wing lengths of female *Ae. albopictus* found only with conspecifics when compared with females found in containers with *Oc. triseriatus* ( $t = -0.56$ ,  $p = 0.579$ ; Figure 1b).

**Summary:**

Previous research predicts that *Oc. triseriatus* mosquitoes will be small and nutritionally deprived when emerging from larval habitats shared with *Ae. albopictus*, an invasive species and a dominant larval resource competitor (Lounibos 2001). Results presented here found no evidence of this pattern in the field, with *Oc. triseriatus* females approximately the same size, regardless of the presence of the invasive species; however, small sample sizes resulted from patchy distributions of both species in the sample region, and results may not be representative of all competitive dynamics.

*A posteriori* power tests reveal that extremely large sample sizes would need to be collected to detect size differences between mosquitoes emerging from monospecific larval habitats, and mosquitoes emerging from habitats containing more than one species. While larval habitat quality was highly variable, many of the

containers sampled contained large amounts of detritus. Detritus is a primary nutritional resource for container-breeding mosquito larvae (Leonard and Juliano 1995). Western North Carolina is heavily forested and receives high amounts of precipitation, resulting in detritus-rich container habitats; therefore, nutrient-limited conditions and competitive interactions may not be primary determinates of morphology and container-breeding mosquito populations in this region. Exploring other regions or other habitat types (i.e. only artificial containers) may offer different results or additional insights.

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Table 1: Mean wing lengths (in millimeters) of both mosquito species.

Species	Mean wing length	Standard deviation	Range
<i>Oc. triseriatus</i>	3.57	0.207	3.00 - 4.20
<i>Ae. albopictus</i>	2.49	0.207	2.04 - 2.82

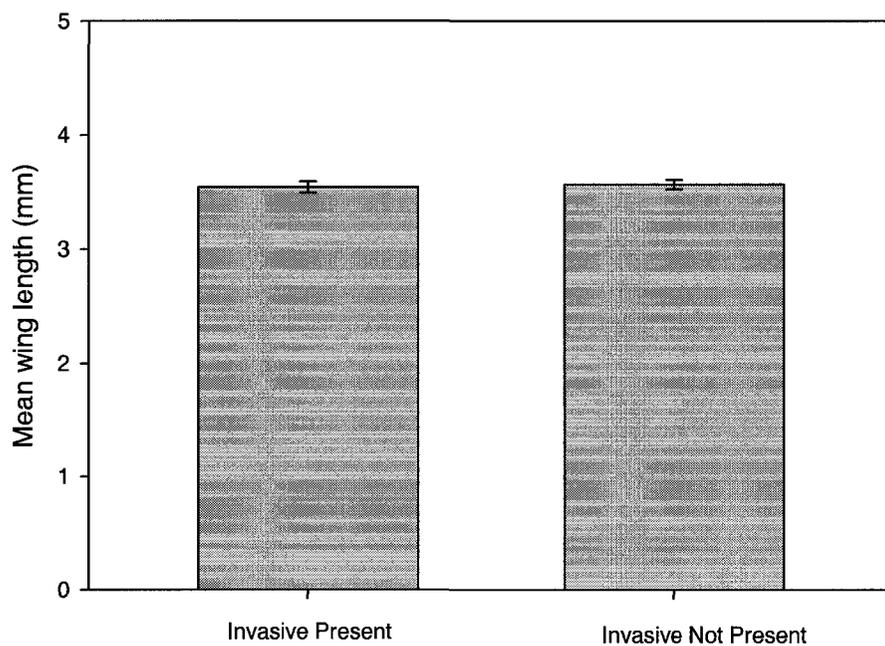
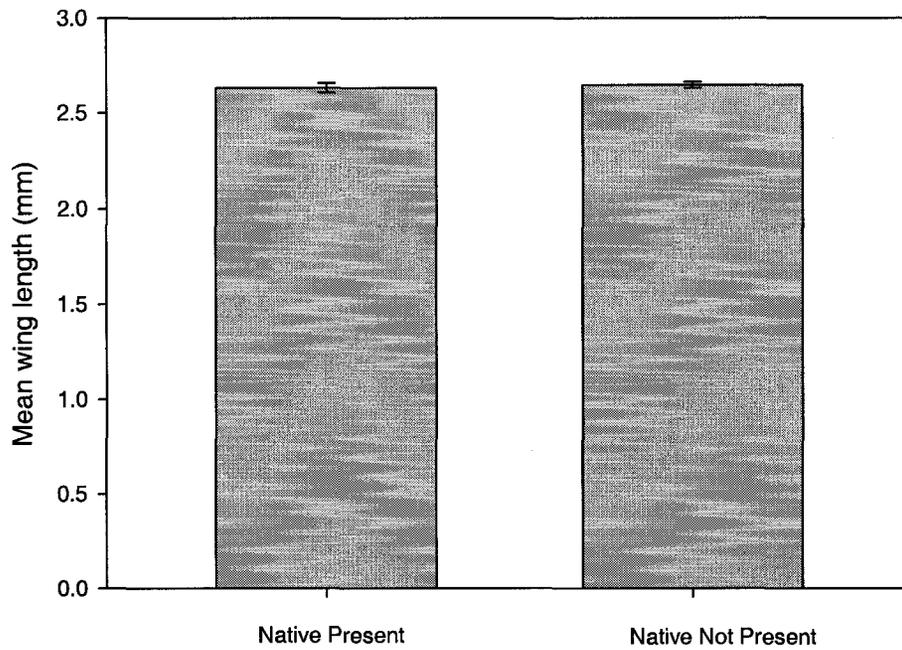


Figure 1a: *Ochlerotatus triseriatus* wing lengths in the presence of, and in the absence of, the invasive species, *Aedes albopictus* (n=34).



Graph 1b: *Aedes albopictus* wing lengths in the presence of, and in the absence of, the native species, *Ochlerotatus triseriatus* (n=42).