Morphological differentiation of eggs and comparative efficacy of oviposition and gravid traps for *Aedes* vectors at different habitats

Jacob Edward Bova

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Masters of Science in

Entomology

Sally L. Paulson, Chair

Carlyle C. Brewster

Dana M. Hawley

April 25th, 2014

Blacksburg, Virginia

Key Words: LACV, *Aedes*, Eggs, Diapause, Surveillance

Morphological differentiation of eggs and comparative efficacy of oviposition and gravid traps for *Aedes* vectors at different habitats

Jacob Edward Bova

ABSTRACT

Mosquito surveillance is an integral part of understanding mosquito-borne disease, including and not limited to the La Crosse virus. The primary container-inhabiting Aedeni vectors include *Aedes triseritatus*, *Aedes albopictus*, and *Aedes japonicus*. To better understand the efficacy of gravid traps and oviposition traps as surveillance methods for these mosquitoes, field studies were conducted in three different habitat types.

Ae. triseriatus, *Ae. albopictus*, *Ae. japonicus*, and *Aedes aegypti* eggs were described with the aid of color images and SEM. All eggs were broadly cigar shaped with *Ae. triseriatus* and *Ae. japonicus* eggs being dull or matte black while *Ae. albopictus* and *Ae. aegypti* eggs were shiny jet black. *Ae. triseriatus* eggs were larger, lighter in color, and have a rougher appearance when compared to *Ae. japonicus*. *Ae. albopictus* and *Ae. aegypti* can be differentiated by the distinct presence of a micropylar collars in *Ae. aegypti*.

Ovitrap and gravid trap efficacy for the surveillance of *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* were measured in three different habitats on two different sites. Both sites contained the same 2ha habitats with varying degrees of forest canopy disturbance. *Ae. triseriatus* was the most abundant mosquito on all sites. Ovitraps and gravid traps were efficient in collecting *Ae. triseriatus*, while ovitraps were not efficient in collecting *Ae. japonicus* and *Ae. albopictus*.

Ae. albopictus diapause eggs were imaged by light microscopy and SEM. Diapause eggs have deeper choronic cells and an over-all rougher exterior when compared to *Aedes albopictus* non-diapause eggs.

Acknowledgements

I would like to thank my advisor and mentor, Dr. Sally L. Paulson for her patience and guidance. Her influence and knowledge was instrumental in my scientific development, and my experience working under her will always remain with me. My graduate committee members, Dr. Carlyle C. Brewster and Dr. Dana M. Hawley have also been incredibly important in the completion of my master's degree. Dr. Brewster's statistical advice cannot be overstated.

I would like to thank the Anderson lab for providing *Aedes aegypti* mosquitoes and the Kuhar lab for lab space to conduct experiments. Dr. Greg Paulson provided expertise with SEM imaging and was a pleasure to work with.

Special thanks goes out to Dr. Camille Harris for her friendship and support in the field and virus testing. Fan Yang was incredibly helpful throughout the duration of my studies, and I will treasure all the friendships made in the entomology department. I also owe a debt of gratitude to the office staff, Kathy Shelor and Sarah Kenley for their guidance.

Finally I would like to thank my family for their unwavering support and Christy Smith for her love and encouragement.

Table of Contents

	Page
Abstract	*ii*
Acknowledgments	*iv*
List of Figures	*vii*
List of Tables	*ix*
Chapter 1	
Introduction and Literature Review	1
1.1 Introduction to mosquito-borne viruses in southwestern Virginia	1
1.1.1 Introduction to La Crosse virus	1
1.1.1.1 La Crosse virus genome and replication:	2
1.1.1.2 Enzootic maintenance cycle of La Crosse virus:	2
1.1.2 Introduction to West Nile virus:	3
1.1.2.1 Introduction of West Nile virus to the United States	4
1.1.2.2 *Flavivirus* genome and replication:	4
1.1.2.3 Enzootic maintenance cycle of West Nile virus	4
1.1.2.4 Human infection with West Nile virus	5
1.1.3 Introduction to Eastern Equine Encephalitis virus	5
1.1.4 Saint Louis encephalitis virus	6
1.2 Introduction to three *Aedes* mosquitoes of southwestern Virginia	6
1.2.1. *Aedes triseriatus*	6
1.2.2. *Aedes albopictus*	7
1.2.3. *Aedes japonicus*	9
1.4. Mosquito surveillance methods	9
1.5. Eggshell morphology	11
1.5.1. Structure and appearance of three sympatric *Aedes* species in southwestern Virginia	11
1.6 Invasive mosquito species and competition	12
1.7. Diapause in *Aedes albopictus*	13
1.8 Literatures cited	17
Chapter 2	
Morphological Differentiation of Eggs of Container-inhabiting *Aedes*	
2.1 Introduction	29
2.2 Materials and methods	30
2.3 Results	32
2.4 Discussion	32
2.5 Literature cited	34
2.6 Figures	36
2.7 Tables	41

Chapter 3
A Comparison Between Two Sampling Methods for Container-Inhabiting Mosquitoes in a La Crosse Virus Endemic Area
3.1 Introduction 42
3.2 Materials and methods 43
3.3 Results 45
3.4 Discussion 47
3.5 Literature cited 50
3.6 Figures 55
3.7 Tables 60

Chapter 4
Description of the Diapause Egg of *Aedes albopicutus* Using Auto Montage Technology
4.1 Introduction 61
4.2 Materials and methods 62
4.3 Results & Discussion 63
4.4 Literature cited 65
4.6 Figures 67

List of Figures

Figure		Page
2.1.	*Aedes triseriatus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.	36
2.2.	*Aedes japonicus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.	37
2.3.	*Aedes aegypti* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.	38
2.4.	*Aedes albopictus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.	39
2.5.	Differentiation of the eggs of container-inhabiting *Aedes*. (A) *Ae. aegypti*: shiny, jet black eggs with prominent micropylar collars (arrow). (B) *Ae. albopictus*: very shiny, jet black eggs, strongly tapered from the widest point. (C) *Ae. triseriatus*: large, matt black eggs. (D) *Ae. japonicus*: matt black eggs, smaller than *Ae. triseriatus.*	40
3.1.	Location of the Southern Appalachian Silviculture and Biodiversity (SASAB) study sites. BB1 and BB2 are circled.	55
3.2.	Site BB1 with labeled trap types and locations. Adult mosquitoes and mosquito eggs were collected from Julian week 27 to 39 2011. The control habitat (CON) is undisturbed forest >80 years. The clearcut habitat (CCT) was selectively harvested for trees with a diameter >5cm. The shelterwood habitat (SW) had a complete removal of canopy vegetation in 2007.	56
3.3.	Log mean number of eggs per trap per week of *Ae. triseriatus, Ae. japonicus,* and *Ae. albopictus*. Columns with with different letters are significantly different ($p<0.001$).	57
3.4.	Log mean number of adults per trap per week of *Ae. triseriatus, Ae. japonicus,* and *Ae. albopictus*. Columns with with different letters are significantly different ($p<0.05$).	57
3.5.	Seasonal abundance of *Ae.* triseriatus, *Ae. japonicus,* and *Ae. albopictus* as measured by eggs.	58
3.6.	Seasonal abundance of *Ae.* triseriatus, *Ae. japonicus,* and *Ae. albopictus* as measured by adults.	58

3.7. Habitat preference as measured by eggs. Means with different letters are significantly different (p<0.001). 59

3.8. Habitat preference as measured by adults. Means with different letters are significantly different (p<0.001). 59

4.1. Auto-montage image of diapause (A) and non-diapause (B) eggs of *Ae. albopictus.* 67

List of Tables

Table		Page
2.1.	*Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* egg measurements	41
3.1.	Total numbers of mosquitoes collected in ovitraps and gravid traps	60

Chapter 1

Introduction and Literature Review

1.1 Introduction to mosquito-borne viruses in southwestern Virginia

Mosquito-borne viruses are spread to vertebrate hosts by the bite of an infected mosquito. The most prevalent viruses in southwestern Virginia are Lacrosse virus (LACV) and West Nile virus (WNV). St. Louis encephalitis virus (SLEV) and Eastern equine encephalitis virus (EEEV) occur in Virginia; however, incidence is low and the primary vector of EEEV does not occur in southwestern Virginia. These viruses are all encephalitides capable of infecting human hosts and in some cases may be deadly.

1.1.1 Introduction to La Crosse virus:

The La Crosse virus (LACV) is a mosquito-borne Bunyavirus in the California serogroup. It is endemic to much of the mid-west and east to southwestern Virginia. It was first identified in 1964 after the death of a girl (Grimstad 1988) and its natural cycle was rapidly identified in the years to follow. The primary vector is *Aedes triseriatus* and primary reservoir hosts are chipmunks (*Tomias striatus*) and grey squirrels (*Sciuris carolinensis*) are important for the natural maintenance of this virus (Thompson et al. 1965, Watts et al. 1973, Pantuwatana et al. 1974, Beaty and Thompson 1975, Balfour et al. 1976, Rosen 1981). Humans are tangential or dead-end hosts of LACV and most infections asymptomatic; however, neurological symptoms can be severe. Most cases are reported in children, ages 15 years and younger. Most people with mild infections report fevers, lethargy, headaches, nausea and vomiting. If the virus passes the blood-brain barrier and becomes neuroinvasive, it can progress to encephalitis. Symptoms may include severe headaches, seizures, coma, permanent brain damage, neurological sequelae and in less than 1%, death (CDC 2014). There is no vaccine for LACV and around 80-100 probable

cases of California serogroup neuroinvasive cases are reported every year to the CDC. Most of these are presumed to be LACV (CDC 2014). The last confirmed human case of LACV in Virginia was in 2009; however, there was one probable case in 2011 (CDC 2014).

1.1.1.1 La Crosse virus genome and replication:

The LACV is in the California serogroup of the virus family: Bunyaviridae. It has a tripartite negative sense ssRNA genome, consisting of a small, medium, and large segments with helical symmetry. The small segment has 984 nucleotides and codes for a nucleocapsid protein. The medium segment contains 4526 nucleotides and codes for the G1 and G2 glycoproteins and nonstructural proteins. The large segment contains 6980 nucleotides and codes for the polymerase, used in viral replication, in addition to nonstructural proteins. The genome is surrounded by a lipid envelope and enters the host's cells through receptor-mediated endocytosis and membrane fusion. Replication occurs in the cytoplasm. The virus-encoded RNA-dependent RNA polymerase initiates transcription of the negative sense RNA into mRNA. Virus maturation and glycosylation of G1 and G2 glycoproteins and virus maturation begins in the endoplasmic reticulum and ends in the Golgi apparatus. Nucleocapsids then accumulate in the cisternae and exit the cell via exosytosis (Elliot 1990, Bishop 1996).

1.1.1.2 Enzootic maintenance cycle of La Crosse virus:

The native eastern treehole mosquito *Aedes triseriatus* is the primary vector of LACV. This mosquito is commonly collected in areas where human cases occur and virus has been isolated from field-collected mosquitoes (Pantuwatana et al. 1974, Beaty and Thompson 1975). Eastern chipmunks (*Tomias striatus*) and eastern grey squirrels (*Sciuris carolinensis*) are thought to be the primary amplifying reservoirs of the virus (Nasci 1985, Richards et al. 2006). However, LACV has been found in foxes (*Vulpes vulpes* and *Urocyon cineroargenteus*), fox squirrels

(*Sciuris niger*), and white-tailed deer (*Odocoileus virginianus*) (Moulton 1971, Issel 1972, Gauld 1975, Amundson 1981, Amundson 1985). The virus is amplified during summer months and prevalence of infection of both vertebrate reservoirs and mosquito vectors is higher during July and August (Clark et al. 1983).

Transmission occurs either horizontally or vertically between mosquito vectors and vertebrate reservoirs. Horizontal transmission occurs when an infected mosquito vector takes a bloodmeal from a non-infected susceptible vertebrate host. The vertebrate host can then act as an amplifying reservoir by developing a sufficiently high viremia to infect a non-infected, susceptible mosquito vector. Vertical, or transovarial transmission, of LACV occurs primarily in *Aedes triseriatus*. If the infection of LACV disseminates to the ovaries of a female mosquito, the virus will be passed on to her progeny (Grimstad 1988). This usually does not occur until the second ovarian cycle. The transmission rate can be as high as 98%. Through vertical transmission, the virus remain viable over the winter in the infected eggs of *Ae. triseriatus* (Miller 1977).

In addition to the primary vector, several mosquito species in southwestern Virginia are competent vectors of LACV. These include the introduced mosquitoes, *Aedes albopictus* and *Aedes japonicus (Grimstad 1988, Hawley 1988a, Sardelis et al. 2002a)*. Harris et al. (unpublished data) have demonstrated that *Culex* species may also be accessory vectors.

1.1.2 Introduction to West Nile virus:

West Nile virus (WNV) is a positive sense ssRNA mosquito-borne *Flavivirus* within the *Flaviviridae* family of the Japanese encephalitis serogroup. It was first detected in Uganda in 1937 and has since spread to much of the Old and New World, including the United States. This virus is the pathogenic agent that causes West Nile fever (WNF) and West Nile encephalitis

(WNE). The maintenance cycle in the United States consists of avian amplifying hosts and *Culex* and *Aedes* mosquito vectors. Human cases fluctuate by year in the United States and confirmed human cases are thought to be a minute percentage when the many asymptomatic cases are taken into account.

1.1.2.1 Introduction of West Nile Virus to the United States

The first epidemic of WNV in the United States occurred in and around New York City in the late summer of 1999. It was at first thought to be a variation St. Louis virus but was later identified as WNV (Lanciotti R.S 1999). By the end of the initial period of transmission, 62 cases of WNF were confirmed with seven fatalities (Fine 1999b, a, Nash et al. 2001). An estimated 2.6% of the residents of Queens may have been infected; however, due to most cases being asymptomatic most went unreported (Garmendia et al. 2001). The likely vectors of the epidemic were *Culex pipiens* and *Culex restuans* (Nash et al 2001). Since its introduction in 1999, it has spread to all 48 of the contiguous United States (Davis et al. 2005).

1.1.2.2 *Flavivirus* genome and replication:

The West Nile virus genome consists of a single positive sense ssRNA. It has a 40-60nm icosahedral nucleocapsid and is enveloped. The virus enters the cell through endocytosis and begins translation in the cytoplasm. The viral replicase is synthesized and used to create genome length negative-sense templates. This template is used to create a 10:1 ratio of positive and negative sense genome length fragments. The virus exit the cell through exocytosis (Fauquet 2005, Gould 2006).

1.1.2.3 Enzootic maintenance cycle of West Nile virus

The enzootic maintenance cycle of WNV is primarily maintained by horizontal transmission between avian vertebrate hosts and *Culex* mosquitoes. Several avian orders are

competent amplifying reservoirs but the American crow, blue jay, western scrub-jay, yellow-billed magpie, and the house sparrow are considered the most significant. However, American robins are important in the spread of WNV due to *Culex pipiens*' preference to feed on it (Kilpatrick et al. 2006b, Kilpatrick et al. 2006a). Most mammals, such as humans and horses are dead end hosts because their viremia levels are not sufficient to infect a susceptible mosquito vector that feeds on them (Dauphin et al. 2004). In Virginia, the primary vector mosquito species are *Culex restuans* and *Cx. pipiens* (Blitvich 2008).

1.1.2.4 Human infection with West Nile virus:

Human symptoms range from asymptomatic cases to severe encephalitis and meningitis. Around 20% of people infected will develop West Nile fever, and exhibit mild symptoms such as fever, headache, and myalgia. Severe cases of WNV may be neuroinvasive and can manifest as encephalitis, meningitis, and meningoencephalitis. These neuroinvasive symptoms occur in less than 1% of cases. Neurological sequelae may occur in patients with severe neuroinvasive infections (CDC 2013).

The virus was first identified in Virginia from a dead crow on September 28, 2000 in Prince Edward County. Since then the number of human cases has fluctuated yearly. In Virginia, there were 9 probable and confirmed cases of WNV in 2011 (VDH 2014).

1.1.3 Introduction to Eastern Equine Encephalitis virus

The EEEV is an *Alphavirus* in the *Togaviridae* family. It was first identified from the brain of a fatally infected horse in 1933 and is one of the most deadly mosquito-borne viruses with fatality rates reaching 80% in humans (Adams et al. 2008).The enzootic cycle of EEEV is still unclear but likely involves the mosquito vector *Culiseta melanura* and avian amplifying vertebrate reservoirs. *Culiseta melanura* is primarily found in eastern Virginia along the coast

(Scott and Burrage 1984).The most recent confirmed human case of EEEV in Virginia was in 2003 and there was a probable case in 2012 (CDC 2013). *Ae. albopictus* is a competent vector for EEEV and is found in southwestern Virginia (Mitchell et al. 1992).

1.1.4 Saint Louis Encephalitis virus

The SLEV is a *Flavivirus* in the *Flaviviridae* family. It was first identified from an epidemic in the fall of 1933 around St. Louis, MO. Since then, there have been more than 50 outbreaks in the United States and Canada (Lumsden 1958, Chamberlain 1980, Monath T.P. 1987, Lanciotti R.S 1999). The SLEV enzootic maintenance cycle is horizontal involving avian amplifying reservoir hosts and *Culex* mosquito vectors (McLean 1980). *Culex pipiens* and *Ae. albopictus* are common mosquitoes in southwestern Virginia and are both competent vectors of SLEV (Mitchell 1980, Hawley 1988b, Mitchell et al. 1992).

1.2 Introduction to three *Aedes* mosquitoes of southwestern Virginia

There are three sympatric container-inhabiting *Aedes* mosquitoes in southwestern Virginia. Those species are *Ae. triseriatus*, *Ae. albopictus*, and *Aedes japonicus* (Barker et al. 2003, Grim et al. 2007). All are competent vectors for LACV (Pantuwatana et al. 1974, Gerhardt et al. 2001, Sardelis et al. 2002a). *Aedes albopictus* is a competent vector for many viruses including WNV, SELV, EEEV, and possibly CHIKV in Virginia (Bonizzoni et al. 2013a).

1.2.1. *Aedes triseriatus*

Aedes triseriatus (Say), the native eastern treehole mosquito is the primary vector of the LACV (Pantuwatana et al. 1974, Watts et al. 1974, Beaty and Thompson 1975, Miller 1977). It is found in heavily wooded areas throughout the eastern half of the United States and breeds in treeholes and artificial containers (Grimstad 1988, Barker et al. 2003). The mosquito has an affinity for taking blood meals from eastern chipmunks (*Tamias striatus*) and grey squirrels

(*Sciuris carolinensis*), the primary amplifying hosts for LACV (Wright 1970, Nasci 1985, Richards et al. 2006).

In addition to transmitting LACV horizontally, female *Ae. triseriatus* mosquitoes can also vertically transmit the virus to their progeny through the egg (Miller 1977). This provides an overwintering mechanism for the virus in the hatch-resistant diapause eggs (Miller 1979, Shroyer 1980). The eggs are oviposited directly above the water line in treeholes and artificial containers, generally less than one meter above the ground (Scholl 1977, Aziz and Hayes 1987, Nasci 1988). The infection rates of *Ae. triseriatus* mosquitoes are higher in the late summer. Virus prevalence in natural cycles starts low in early summer and increases throughout the summer months through horizontal amplification. As the incidence of virus increases throughout the summer months, there is an increased risk of tangential human infection (Szumlas et al. 1996).

1.2.2. *Aedes albopictus*

Aedes albopictus (Skuse) is the most invasive mosquito in the world and a competent vector for over 30 viruses (Gratz 2004, Benedict et al. 2007). It originated in Asia and has been transported as desiccation-resistant diapause eggs that can survive long distance transport in various containers such as used tires (Sprenger and Wuithiranyagool 1986, Hawley et al. 1987). The spread is thought to be human-mediated due to the low levels of diversity in invasive populations (Usmani-Brown et al. 2009, Zhong et al. 2013). Once it has been established in a region it is incredibly difficult to eliminate and appropriate surveillance and control strategies should be implemented as soon as possible (Holder et al. 2010).

Ae. albopictus was first introduced to Hawaii in the end of the 18th century and in Texas, the continental United States, in 1985 (Sprenger and Wuithiranyagool 1986) and can now be found as far north as Illinois and New Jersey and south to Texas and Florida (Hawley et al. 1987,

Juliano and Lounibos 2005). Since its introduction to the continental United States, it has spread as far north as the 15° of latitude and south to Florida (Focks et al. 1994, Mogi et al. 2012, Bonizzoni et al. 2013b). Western populations have been found in California, New Mexico, and occasionally as far north as Washington (Bonizzoni et al. 2013b). In the 1990's, *Ae. albopictus* spread into Mexico, southward throughout Central America, and as far south as northern Argentina in South America. The species is also found in many Caribbean Islands (Caminade et al. 2012, Medlock et al. 2012).

Ae. albopictus was first identified on the African continent in 1989 and has established itself in Nigeria, Cameroon, Equatorial Guinea, and Gabon (Simard et al. 2005, Paupy et al. 2009). Populations are also present in all countries surrounding the Mediterranean Sea, much of the Middle East, and is progressing northward (Caminade et al. 2012).

Populations of *Ae. albopictus* have shown the ability to rapidly evolve photoperiodism to northern climatic critical photoperiods (CPP). The CPP increases with latitude and marks the day length that triggers the initiation of a diapause response in a population. When populations of *Ae. albopictus* were initially tested for CPP response in the 1980's they showed a weak response, as opposed to native Japanese populations; however, in as little time as 20 years US populations showed a strong response (Focks et al. 1994). This indicates the ability for this species to rapidly adapt to northern ranges upon being established in a country and has implications for the spread of the species as temperatures continue to rise due to climate change (Urbanski et al. 2012).

The first field isolates of LACV from *Ae. albopictus* were found in Tennessee (Gerhardt et al. 2001) and the species has long been known to be a competent vector of WNV (Philip 1943). It is also a competent vector of EEEV, SLEV, and CHIKV ((Mitchell 1980, Hawley 1988a, Benedict et al. 2007). *Ae. albopictus* is considered a bridge or accessory vector for most of these

viruses due to its catholic feeding behavior (Grimstad 1988, Streit 1993, Weaver and Reisen 2010). *Ae. albopictus* is an avid man biter. In fact, biting rates can be high enough in some areas to deter parents form allowing their children time outside. It has been suggested that this mosquito-induced lack of physical activity may be an contributing cause of childhood obesity (Worobey et al. 2013).

1.2.3. *Aedes japonicus*

Aedes japonicus (Theobald) has spread rapidly throughout the United States and even to Hawaii since introduction in 1998 (Peyton et al. 1999, Larish and Savage 2005, Widdel et al. 2005, Bevins 2007a, Morris et al. 2007, Hughes et al. 2008, Neitzel et al. 2009, Kaufman et al. 2012b). A recent study indicates multiple introductions of *Ae. japonicus* into the United States probably through the used tire trade with Asia (Fonseca et al. 2010). They were found to be most abundant during midsummer in southwestern Virginia (Grim et al. 2007). They are container-inhabiting mosquitoes and may oviposit their eggs in artificial and natural containers and lay desiccation-resistant eggs above the water line. The eggs are capable of undergoing diapause and surviving temperate winters (Andreadis et al. 2001, Juliano and Lounibos 2005). *Ae. japonicus* is a competent vector for WNV, EEEV, and LACV (Turell et al. 2001, Sardelis et al. 2002b, Sardelis et al. 2002a)

1.4. Mosquito surveillance methods

Each life-stage of a mosquito can be utilized for mosquito surveillance. Adult traps attract blood-fed gravid females, host-seeking females, and adults looking for a suitable place to rest (Reiter 1983). Larval sampling may be done using dippers or by allowing oviposition traps to fill

with water and collecting any larvae that develop (Pantuwatana et al. 1974). *Aedes* eggs may be collected using CDC oviposition traps, which imitate the mosquito's natural oviposition site.

Host-seeking adult female mosquitoes can be collected using attractants such as pheromones, CO_2, and light. Examples of this type of trap include the CDC light trap and the BG Sentinel Trap. These traps require the use a battery-operated fan and usually an incandescent light source in combination with CO_2 and/or octenol (Burkett et al. 2001, Burkett et al. 2002). The BG sentinel trap relies primarily on a battery operated fan and the BG-Lure, a human skin-mimicking pheromone attractant. It is used to attract anthropophilic mosquito species using kairomones like ammonia, lactic acid, and caproic acid (Rose 2004). The CDC Light trap uses battery powered fan and an incandescent light bulb and an insulated compartment to place dry ice to use as a CO_2 source (Burkett et al. 2001, Burkett et al. 2002). Blood-fed adult female mosquitoes can be collected using Reiter gravid traps, which use oviposition attractants such as water or infusions. This is significant because blood-fed females are assumed to have higher minimum field infection rates when compared to host seeking adults and other mosquito life stages (Reiter 1983). Resting boxes imitate natural shelters and battery powered aspirators can be used to collect them (Loomis 1959, Perdew and Meek 1990). Because these traps selectively attract different stages in the gonotrophic cycle of the mosquitoes, they can be used concurrently on the same site (Meyer 1991). However, any sample that travels through the battery-powered fan can be damaged and may make identification difficult (Saul et al. 1977, Apperson et al. 2002).

Surveillance for *Aedes* eggs is implemented by using oviposition traps, or ovitraps. These traps are plastic cups nailed to a tree or post. An oviposition substrate, like seed germination paper, is placed inside the cup and collected. The eggs can be collected and taken back to the

laboratory and identified and/or reared to the larval and adult stages (Loor and Defoliart 1969). Due to LACV vertical transmission, virus isolation can be performed on these samples.

1.5. Eggshell morphology

The eggshell of *Aedes* mosquitoes protects the oocyte, egg, embryo, and pharate larvae from desiccation and adverse environmental factors, while still allowing for sufficient gas exchange. The micropyle is a small pore on every egg that allows for the entry of sperm. The dorsal side of *Aedes* eggs is glued to the oviposition substrate (Ivanova-Kazaas, 1949; Christophers, 1960; Horsfall et al., 1972; Harbach and Knight, 1980).

In *Aedes* eggs, the endochorion and exochorion can be differentiated and are physically separate (Hardwood 1958). The exterior reticulum forms variable exochorionic cells, tubercles, and ridge patterns. These may be the imprints from the ovarian follicles (Harwood, 1958; Powell et al. 1986; Linley, 1989).

1.5.1 Structure and appearance of three sympatric *Aedes* species in southwestern Virginia

The exterial reticular patterns of *Ae. albopictus* and *Aedes aegypti* are described by Linley 1989a with the aid of scanning electron microscopy (SEM). Both are shiny black in color and broadly cigar shaped. *Ae. albopictus* eggs are slightly larger than *Ae. aegypti* eggs and have similar exochorionic cell structure and placement when viewed under a standard dissecting microscope. However, *Ae. aegypiti* can be differentiated from *Ae. albopictus* by the presence of a distinct mycropylar collar in *Ae. aegypti.*

Ae. triseriatus eggs and *Ae. japonicus* eggs are both larger than *Ae. albopictus* and *Ae. aegypti* eggs and have deeper chorionic cells. *Ae. triseriatus* eggs were described by Linley (1989b) using SEM. *Ae. triseriatus* eggs are dull black almost grey in color and have deeply

excavated outer chorionic cells. Haddow et al. (2009) described the egg of *Ae. japonicus* as dull black in color with irregular deep external chorionic cells. The egg of *Ae. japonicus* and egg of *Ae. triseriatus* can be differentiated by color and size. Auto-Montage microscopy can also be used to differentiate between sympatric *Aedes eggs* (Obenauer et al. 2009).

1.6 Invasive mosquito species and competition

Ae. triseriatus, Ae. albopictus, and *Ae. triseriatus* co-occur in much of their eastern United States range and may compete with each other or even displace entire populations (Rochlin et al. 2013). Most studies are conducted on larval competition, as these three species are commonly found in the same natural and artificial containers. The presence of *Ae. albopictus* may limit the spread of *Ae. japonicus,* while these two invasive species combined may be displacing the native *Ae. triseriatus* in certain habitats.

Ae. albopictus and *Ae. triseriatus* competition favors *Ae. albopictus* in larval habitats, but their preference for different habitats, urban for *Ae. albopictus* vs. forested for *Ae triseriaus*, will sometimes limit their interaction (Novak et al. 1993, Teng and Apperson 2000). More urban populations of *Ae. triseriatus* experience a sharper decline in the presence of *Ae. albopictus* (Livdahl and Willey 1991, Lounibos et al. 2001).

Ae. japonicus and *Ae. triseriatus* larvae breed in both natural treeholes and artificial containers such as tires and are commonly found to co-occur. After its introduction into an area, *Ae. japonicus* populations have increased, while *Ae. triseriatus* populations have decreased (Joy and Sullivan 2005, Bevins 2007b, Burger and Davis 2008, Andreadis and Wolfe 2010, Kaufman et al. 2012a). However, these studies have primarily focused primarily on artificial containers. Kaufman (2012) found only a minor presence of *Ae. japonicus* in treeholes. There is little

evidence that interactions of *Ae. japonicus* and *Ae. triseriatus* in the heavily forested preferred habitats of *Ae. triseriatus* will favor *Ae. japonicus* (Kaufman and Fonseca 2014).There seems to be only slight larval competition between *Ae. triseriatus* and *Ae. japonicus.* Larval development by these two species is limited primarily by larval density (Alto 2011, Hardstone and Andreadis 2012, Lorenz et al. 2013).

In the United States, *Ae. japonicus* was introduced in the northeastern and *Ae. albopictus* was introduced in the south. Their range now overlaps with the exceptions of the northern and southern extremes (Beebe et al. 2013, Bonizzoni et al. 2013b, Rochlin et al. 2013, Kampen and Werner 2014, Kaufman and Fonseca 2014). *Ae. albopictus* has been shown to outcompete multiple other mosquito species in the larval stage; however, *Ae. japonicus* develop faster in colder temperatures (Joy and Sullivan 2005, Bevins 2007b, Alto 2011). In New Jersey they were found to prefer different developmental containers, thus limiting the interspecies larval competition (Bartlett-Healy et al. 2012).

The introduction of these two species may also impact transmission of LACV. When developing with *Ae. japonicus* and *Ae. albopictus*, *Ae. triseriatus* may be a more more efficient vector (Bevins 2008a, b). Competition between species can increase stress and limit body size, thus impacting vectorial capacity (Alto et al. 2005, Muturi et al. 2011). *Ae. albopictus* and *Ae. japonicus* may also act as bridge vectors of LACV and are more commonly collected at LACV homes than *Ae. triseriatus* (Haddow and Odoi 2009).

1.7. Diapause in *Aedes albopictus*

Insects utilize diapause as an escape mechanism to avoid prolonged periods of unfavorable environmental conditions. This allows the insect to remain in place, as opposed to

certain insects that migrate to avoid these conditions. Diapause is a hormonally preprogrammed active process or syndrome. It is similar to quiescence but differs when diapause is not broken due to immediate exposure to favorable conditions. Quiescence is utilized by *Ae. albopictus* when they oviposit eggs above the water line of natural and artificial containers. These eggs will immediately hatch when submerged in water, thus breaking quiescence; as opposed to diapause eggs, oviposited by *Ae. albopictus*, which will fail to hatch even when initially submerged. These eggs will only break diapause after a certain amount of time has passed, temperatures have fluctuated, and are then finally submerged (Perez and Noriega 2013, Poelchau et al. 2013a).

There are three distinct phases or stages of diapause: prediapause, diapause, and postdiapause. *Ae. albopictus* undergoes facultative photoperiodic diapause; facultative because it is not predetermined by each generation's life-stage and photoperiodic because diapause is initiated by shortened fall day-lengths. Larvae and pupae sense shorter day-lengths and upon eclosion, mating, and a bloodmeal, the adult prediapause female mosquito will oviposit diapause eggs (Wang 1966). The prediapause adult females alter their normal behavior by taking up to 33% more sugar meals as opposed to non-prediapause females (Villiard; unpublished data).

The genetics and biochemistry that initiate prediapause in *Ae. albopictus* have only recently been studied and there remains much to learn. The clock genes involved in circadian clocks may play a roll in photoperiodism and both of the circadian genes *period* (*per*) and *timeless* (*tim*) have been characterized in *Ae. albopictus*. The circadian clocks are regulated by light-sensitive and insensitive flaviprotiens called *cryptochromes* which bind to proteins coded for by the genes *timeless* (*tim*) and *period* (*per*). Light-sensitive positive regulator *cyrptochrome 1* (CRY1) and light-insensitive negative regulator *cytochrome 2* (CRY2) have both been identified in *Ae. albopictus.* CRY1 binds to TIM proteins, which are coded for by the *timeless*

(*tim*) gene, and degrade themselves in the presence of light. This binding and degradation increases the amount of *tim* and *per* mRNA throughout the day while at night CRY2 is uninhibited and works to suppress transcriptional regulation at the core of the circadian clock (Konopka and Benzer 1971, Zhu et al. 2005). The CPP and could be sensed by this process and initiate the cascade of effects resulting in the production of a prediapause adult female *Ae. albopictus (Meuti and Denlinger 2013).*

Upon initiation of the prediapause stage, there are major transcriptional shifts in the adult female and resulting prediapause embryos. Mature oocytes dissected from prediapause gravid females had 10 upregulated transcipts which are associated with DNA replication and transcription. Additional transcips were related to ecdyson signaling, gluconeogenesis pathways, and the *methuselah* family of genes. The prediapause females then oviposit prediapause eggs which are larger and contain higher amounts of surfaces hydrocarbons as opposed to nondiapause eggs (Urbanski et al. 2010). Prediapause embryos showed a transcriptional shift associated with endocrine signaling, metabolism, the cell cycle, DNA binding and repair.

The later embryonic stage of prediapause had a higher gene expression associated with energy production, metabolism and cuticle formation. These transcriptional shifts allow the prediapause embryos to initiate lipid accumulation and inhibition of the citric acid cycle (Poelchau et al. 2011, 2013b, Poelchau et al. 2013c). This is in preparation of the lowered metabolism and developmental arrest that occurs in the subsequent pharate larval diapause stage. The diapause phase occurs when the *Ae. albopictus* pharate larvae cease development and drastically lower their metabolism. The transcriptional changes present are associated with lipid metabolism and beta-oxidation (Poelchau et al. 2011). The pharate larvae are not in a state of complete biological stasis but are progressing towards post diapause.

Postdiapause is a state almost identical to quiescence when the diapause pharate larvae will react to favorable environmental stimuli. Postdiapause enables *Ae. albopictus* populations to synchronize their spring emergence. Postdiapause is usually induced when temperatures rise and remain above a certain threshold (Denlinger and Armbruster 2014). There are fitness costs of undergoing diapause due to the depletion of energy reserves (Hahn and Denlinger 2011).

1.8 Literature cited

Adams, A. P., J. F. Aronson, S. D. Tardif, J. L. Patterson, K. M. Brasky, R. Geiger, M. de la Garza, R. Carrion, Jr., and S. C. Weaver. 2008. Common marmosets (Callithrix jacchus) as a nonhuman primate model to assess the virulence of eastern equine encephalitis virus strains. J Virol 82: 9035-9042.

Alto, B. W. 2011. Interspecific larval competition between invasive *Aedes japonicus* and native *Aedes triseriatus* (Diptera: Culicidae) and adult longevity. Journal of Medical Entomology 48: 232-242.

Alto, B. W., L. P. Lounibos, S. Higgs, and S. A. Juliano. 2005. Larval competition differentially affects arbovirus infection in *Aedes* mosquitoes Ecology 86: 3279-3288.

Amundson, T. E., Yuill, T.M. and DeFoliart, G.R. . 1985. Experimental La Crosse virus infection of red fox (*Vulpes fulva*), raccoon (*Procyon lotor*), oppossum (*Didelphis virginiana*). American Journal of Tropical Medicine and Hygiene 30: 586-595.

Amundson, T. E. a. Y., T.M. . 1981. Natural La Crosse infection in the red fox (*Vulpes fulva*), gray fox (*Urocyon cinereoargenteus*), raccoon (*Procyn lotor*), and oppossum (*Didelphis virginiana*). The American journal of tropical medicine and hygiene 30: 706-714.

Andreadis, T. G., and R. J. Wolfe. 2010. Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in the Northeastern United States. Journal of Medical Entomology 47: 43-52.

Andreadis, T. G., J. F. Anderson, L. E. Munstermann, R. J. Wolfe, and D. A. Florin. 2001. Discovery, distribution, and abundance of the newly introduced mosquito *Ochlerotatus japonicus* (Diptera: Culicidae) in Connecticut, USA. J Med Entomol 38: 774-779.

Apperson, C. S., B. A. Harrison, T. R. Unnasch, H. K. Hassan, W. S. Irby, H. M. Savage, S. E. Aspen, D. W. Watson, L. M. Rueda, B. R. Engber, and R. S. Nasci. 2002. Host-feeding habits of Culex and other mosquitoes (Diptera: Culicidae) in the Borough of Queens in New York City, with characters and techniques for identification of *Culex* mosquitoes. J Med Entomol 39: 777-785.

Aziz, N., and J. Hayes. 1987. Oviposition and biting patterns of *Aedes triseriatus* in the flood plains of Fort Bend County, Texas. J Am Mosq Control Assoc 3: 397-399.

Balfour, H. H., Jr., C. K. Edelman, H. Bauer, and R. A. Siem. 1976. California arbovirus (La Crosse) infections. III. Epidemiology of California encephalitis in Minnesota. Journal of Infectious Diseases 133: 293-301.

Barker, C. M., S. L. Paulson, S. Cantrell, and B. S. Davis. 2003. Habitat preferences and phenology of *Ochlerotatus triseriatus* and *Aedes albopictus* (Diptera: Culicidae) in southwestern Virginia. J Med Entomol 40: 403-410.

Bartlett-Healy, K., I. Unlu, P. Obenauer, T. Hughes, S. Healy, T. Crepeau, A. Farajollahi, B. Kesavaraju, D. Fonseca, G. Schoeler, R. Gaugler, and D. Strickman. 2012. Larval mosquito habitat utilization and community dynamics of *Aedes albopictus* and *Aedes japonicus* (Diptera: Culicidae). Journal of Medical Entomology 49: 813-824.

Beaty, B. J., and W. H. Thompson. 1975. Emergence of La Crosse virus from endemic foci. Fluorescent antibody studies of overwintered *Aedes triseriatus*. American Journal of Tropical Medicine and Hygiene 24: 685-691.

Beebe, N. W., L. Ambrose, L. A. Hill, J. B. Davis, G. Hapgood, R. D. Cooper, R. C. Russell, S. A. Ritchie, L. J. Reimer, N. F. Lobo, D. Syafruddin, and A. F. v. d. Hurk. 2013. Tracing the tiger: population genetics provides valuable insights into the *Aedes* (Stegomyia) *albopictus* invasion of the Australasian Region. PLoS Neglected Tropical Diseases 7: e2361.

Benedict, M. Q., R. S. Levine, W. A. Hawley, and L. P. Lounibos. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne and Zoonotic Diseases 7: 76-85.

Bevins, S. N. 2007a. Establishment and abundance of a recently introduced mosquito species *Ochlerotatus japonicus* (Diptera: Culicidae) in the Southern Appalachians, USA. J Med Entomol 44: 945-952.

Bevins, S. N. 2007b. Timing of resource input and larval competition between invasive and native container-inhabiting mosquitoes (Diptera: Culicidae). Journal of Vector Ecology 32: 252-262.

Bevins, S. N. 2008a. Effects of expanded mosquito range. Science 321: 1634.

Bevins, S. N. 2008b. Invasive mosquitoes, larval competition, and indirect effects on the vector competence of native mosquito species (Diptera: Culicidae). Biological Invasions 10: 1109-1117.

Bishop, D. H. L. 1996. Biology and molecular biology of Bunyaviruses, pp. 19-53. . *In* R.M. Elliot [ed], The Bunyaviridae. Plenum Press, New York.

Blitvich, B. J. 2008. Transmission dynamics and changing epidemiology of West Nile virus. Anim Health Res Rev 9: 71-86.

Bonizzoni, M., G. Gasperi, X. Chen, and A. A. James. 2013. The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives. Trends Parasitol 29: 460-468.

Burger, J. F., and H. Davis. 2008. Discovery of *Ochlerotatus japonicus japonicus* (Theobald) (Diptera: Culicidae) in southern New. Hampshire, U.S.A. and its subsequent increase in abundance in used tire casings. Entomological News 119: 439-444.

Burkett, D. A., W. J. Lee, K. W. Lee, H. C. Kim, H. I. Lee, J. S. Lee, E. H. Shin, R. A. Wirtz, H. W. Cho, D. M. Claborn, R. E. Coleman, and T. A. Klein. 2001. Light, carbon dioxide, and octenol-baited mosquito trap and host-seeking activity evaluations for mosquitoes in a malarious area of the Republic of Korea. J Am Mosq Control Assoc 17: 196-205.

Burkett, D. A., W. J. Lee, K. W. Lee, H. C. Kim, H. I. Lee, J. S. Lee, E. H. Shin, R. A. Wirtz, H. W. Cho, D. M. Claborn, R. E. Coleman, W. Y. Kim, and T. A. Klein. 2002. Late season commercial mosquito trap and host seeking activity evaluation against mosquitoes in a malarious area of the Republic of Korea. Korean J Parasitol 40: 45-54.

Caminade, C., J. M. Medlock, E. Ducheyne, K. M. McIntyre, S. Leach, M. Baylis, and A. P. Morse. 2012. Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. J R Soc Interface 9: 2708-2717.

Chamberlain, R. W. 1980. History of St. Louis encephalitis, p 3–61. In Monath TP , St. Louis encephalitis. American Public Health Association, Washington, DC. .

Clark, G. G., H. L. Pretula, W. H. Rohrer, R. N. Harroff, and T. Jakubowski. 1983. Persistance of La Crosse virus (California encephalitis serogroup) in north-central Illinois. American Journal of Tropical Medicine and Hygiene 32: 175-184.

Dauphin, G., S. Zientara, H. Zeller, and B. Murgue. 2004. West Nile: worldwide current situation in animals and humans. Comp Immunol Microbiol Infect Dis 27: 343-355.

Davis, C. T., G. D. Ebel, R. S. Lanciotti, A. C. Brault, H. Guzman, M. Siirin, A. Lambert, R. E. Parsons, D. W. Beasley, R. J. Novak, D. Elizondo-Quiroga, E. N. Green, D. S.

Young, L. M. Stark, M. A. Drebot, H. Artsob, R. B. Tesh, L. D. Kramer, and A. D. Barrett. 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype. Virology 342: 252-265.

Denlinger, D. L., and P. A. Armbruster. 2014. Mosquito diapause. Annu Rev Entomol 59: 73-93.

Elliot, R. M. 1990. Molecular biology of the Bunyaviridae. Journal of General Virology 71: 501-522.

Fauquet, C. M., Mayo, M.A., Maniloff, J. *et al*. 2005. Virus Taxonomy: Classification and Taxonomy of Viruses, Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, Amsterdam, Boston and Heidelberg.

Fine, A., Layton, M., Miller, J. et al. 1999a. Update: West Nile-like viral encephalitis--New York, 1999. MMWR Morb Mortal Wkly Rep 48: 890-892.

Fine, A., Layton, M., Miller, J. *et al*. 1999b. From the Centers for Disease Control and Prevention. Update: West Nile-like viral encephalitis--New York, 1999. JAMA 282: 1714.

Focks, D. A., S. B. Linda, G. B. Craig, Jr., W. A. Hawley, and C. B. Pumpuni. 1994. *Aedes albopictus* (Diptera: Culicidae): a statistical model of the role of temperature, photoperiod, and geography in the induction of egg diapause. Journal of Medical Entomology 31: 278-286.

Fonseca, D. M., A. K. Widdel, M. Hutchinson, S. E. Spichiger, and L. D. Kramer. 2010. Fine-scale spatial and temporal population genetics of *Aedes japonicus*, a new US mosquito, reveal multiple introductions. Mol Ecol 19: 1559-1572.

Garmendia, A. E., H. J. Van Kruiningen, and R. A. French. 2001. The West Nile virus: its recent emergence in North America. Microbes Infect 3: 223-229.

Gauld, L. W., Yuill, T.M., Hanson, R.P. and Sinha, S.K. . 1975. Isolation of La Crosse virus (California encephalitis group) from the chipmunk (Tamias striatus), an amplifier host American Journal of Tropical Medicine and Hygiene 24: 999-1005.

Gerhardt, R. R., K. L. Gottfried, C. S. Apperson, B. S. Davis, P. C. Erwin, A. B. Smith, N. A. Panella, E. E. Powell, and R. S. Nasci. 2001. First isolation of La Crosse virus from naturally infected *Aedes albopictus*. Emerging Infectious Diseases 7: 807-811.

Gould, E. A. a. G. T. S. 2006. Taxonomy and evolution of the Flaviviridae. In Molecular Biology of the Flavivirus (eds M. Kalitzky and P. Borowski), Horizon Bioscience, Wymondham, UK, pp. 17-45.

Gratz, N. G. 2004. Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology 18: 215-227.

Grim, D. C., B. T. Jackson, and S. L. Paulson. 2007. Abundance and bionomics of *Ochlerotatus j. japonicus* in two counties in southwestern Virginia. J Am Mosq Control Assoc 23: 259-263.

Grimstad, P. R. 1988. California group virus disease., pp. 99-136 *In* T.P. Monath [ed], The arboviruses: epidemiology and ecology. Volume II. CRC Press, Inc., Boca Raton, Florida USA.

Haddow, A. D., and A. Odoi. 2009. The incidence risk, clustering, and clinical presentation of la crosse virus infections in the eastern United States, 2003-2007. PLoS ONE: e6145.

Hahn, D. A., and D. L. Denlinger. 2011. Energetics of insect diapause. Annu Rev Entomol 56: 103-121.

Hardstone, M. C., and T. G. Andreadis. 2012. Weak larval competition between the invasive mosquito *Aedes japonicus japonicus* (Diptera: Culicidae) and three resident container-inhabiting mosquitoes in the laboratory. Journal of Medical Entomology 49: 277-285.

Hawley, W. A. 1988a. The biology of *Aedes albopictus*. Journal of American Mosquito Control Association 4: 1-39.

Hawley, W. A. 1988b. The biology of *Aedes albopictus*. 4: 1-40.

Hawley, W. A., P. Reiter, R. S. Copeland, C. B. Pumpuni, and G. B. Craig, Jr. 1987. *Aedes albopictus* in North America: probable introduction in used tires from northern Asia. Science, USA 236: 1114-1116.

Holder, P., S. George, M. Disbury, M. Singe, J. M. Kean, and A. McFadden. 2010. A Biosecurity Response to *Aedes albopictus* (Diptera: Culicidae) in Auckland, New Zealand. Journal of Medical Entomology 47: 600-609.

Hughes, T. H., P. M. Irwin, A. Kaufman, H. Sage, B. B. Pagac, Jr., and S. M. Paskewitz. 2008. First records of *Aedes japonicus japonicus* in Wisconsin. J Am Mosq Control Assoc 24: 583-584.

Issel, C. J., Trainer, D.O. and Thompson, W.H. . 1972. Serologic evidence of infections of white-tailed deer in Wisconsin with three California group arboviruses (La Crosse, Trivittatus, and Jamestown Canyon). American Journal of Tropical Medicine and Hygiene 21: 985-988.

Joy, J. E., and S. N. Sullivan. 2005. Occurrence of tire inhabiting mosquito larvae in different geographic regions of West Virginia. J Am Mosq Control Assoc 21: 380-386.

Juliano, S. A., and L. P. Lounibos. 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecology Letters 8: 558-574.

Kampen, H., and D. Werner. 2014. Out of the bush: the Asian bush mosquito *Aedes japonicus japonicus* (Theobald, 1901) (Diptera, Culicidae) becomes invasive. Parasites and Vectors 7: (4 February 2014).

Kaufman, M. G., and D. M. Fonseca. 2014. Invasion biology of *Aedes japonicus japonicus* (Diptera: Culicidae). Annual Review of Entomology 59: 31-49.

Kaufman, M. G., W. W. Stanuszek, E. A. Brouhard, R. G. Knepper, and E. D. Walker. 2012a. Establishment of *Aedes japonicus japonicus* and its colonization of container habitats in Michigan. Journal of Medical Entomology 49: 1307-1317.

Kaufman, M. G., W. W. Stanuszek, E. A. Brouhard, R. G. Knepper, and E. D. Walker. 2012b. Establishment of *Aedes japonicus japonicus* and its colonization of container habitats in Michigan. J Med Entomol 49: 1307-1317.

Kilpatrick, A. M., L. D. Kramer, M. J. Jones, P. P. Marra, and P. Daszak. 2006a. West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior. PLoS Biol 4: e82.

Kilpatrick, A. M., P. Daszak, M. J. Jones, P. P. Marra, and L. D. Kramer. 2006b. Host heterogeneity dominates West Nile virus transmission. Proc Biol Sci 273: 2327-2333.

Konopka, R. J., and S. Benzer. 1971. Clock mutants of *Drosophila melanogaster*. Proc Natl Acad Sci U S A 68: 2112-2116.

Lanciotti R.S, R. J. T., Deubel V., Smith J., Parker M., Steele K., Crise B., Volpe K.E., Crabtree M.B., Scherret J.H., Hall R.A., MacKenzie J.S., Cropp C.B., Panigrahy B., Ostlund E., Schmitt B., Malkinson M., Banet C., Weissman J., Komar N., Savage H.M., Stone W., McNamara T., Gubler D.J 1999. Origin of the West Nile virus

responsible for an outbreak of encephalitis in the northeastern United States. Science, USA 286: 2333-2337.

Larish, L. B., and H. M. Savage. 2005. Introduction and establishment of *Aedes* (Finlaya) *Japonicus japonicus* (Theobald) on the island of Hawaii: implications for arbovirus transmission. J Am Mosq Control Assoc 21: 318-321.

Livdahl, T. P., and M. S. Willey. 1991. Prospects for an invasion: competition between *Aedes albopictus* and native *Aedes triseriatus*. Science 253: 189-191.

Loomis, E. C., and D.H. Green. 1959. Ecological observations on *Culex tarsalis* Coquillett and other mosquitoes in the Delta Region of the Central Valley of California. Annals of the Entomological Society of America 52: 524-533.

Loor, K. A., and G. R. Defoliart. 1969. An oviposition trap for detecting the presence of *Aedes triseriatus* (Say). Mosquito News 29: 487-488 pp.

Lorenz, A. R., E. D. Walker, and M. G. Kaufman. 2013. Does autocthonous primary production influence oviposition by *Aedes japonicus japonicus* (Diptera: Culicidae) in container habitats? Journal of Medical Entomology 50: 69-78.

Lounibos, L. P., G. F. O'Meara, R. L. Escher, N. Nishimura, M. Cutwa, T. Nelson, R. E. Campos, and S. A. Juliano. 2001. Testing Predictions of Displacement of Native Aedes by the Invasive Asian Tiger Mosquito *Aedes Albopictus* in Florida, USA. Biological Invasions 3: 151-166.

Lumsden, L. L. 1958. St. Louis encephalitis in 1933; observations on epidemiological features. . Public Health Rep 73: 340-353.

McLean, R. G., Bowen, G.S. 1980. Vertebrate hosts, p 381–450. In Monath TP , St. Louis encephalitis. American Public Health Association, Washington, DC.

Medlock, J. M., K. M. Hansford, F. Schaffner, V. Versteirt, G. Hendrickx, H. Zeller, and W. Van Bortel. 2012. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector Borne Zoonotic Dis 12: 435-447.

Meuti, M. E., and D. L. Denlinger. 2013. Evolutionary links between circadian clocks and photoperiodic diapause in insects. Integr Comp Biol 53: 131-143.

Meyer, R. P. 1991. Urbanization and the efficiency of carbon dioxide and gravid traps for sampling Culex quinquefasciatus. J Am Mosq Control Assoc 7: 467-470.

Miller, B. R., G.R. DeFoliart, and T.M. Yuill. 1979. *Aedes triseriatus* and La Crosse virus: lack of infection in eggs of first ovarian cycle following oral infection of females The American journal of tropical medicine and hygiene 28: 897-901.

Miller, B. R., G.R. DeFoliart, and T.M. Yuill. . 1977. Vertical transmision of La Crosse virus (California encephalitis group): transovarial and filial infection rates in *Aedes triseriatus* (Diptera: Culicidae). Medical Entomology 14: 437-440.

Mitchell, C. J., M. L. Niebylski, G. C. Smith, N. Karabatsos, D. Martin, J. P. Mutebi, G. B. Craig, Jr., and M. J. Mahler. 1992. Isolation of eastern equine encephalitis virus from *Aedes albopictus* in Florida. Science 257: 526-527.

Mitchell, C. J., Francy, D.B., Monath, T.P. . 1980. Arthropod vectors, pp. 313–380. In Monath TP, St Louis encephalitis. American Public Health Association, Washington, DC

Mogi, M., P. Armbruster, and D. M. Fonseca. 2012. Analyses of the northern distributional limit of *Aedes albopictus* (Diptera: Culicidae) with a simple thermal index. Journal of Medical Entomology 49: 1233-1243.

Monath T.P., T. T. F. 1987. St. Louis encephalitis: lessons from the last decade. The American journal of tropical medicine and hygiene 37.

Morris, J. A., R. L. Lampman, G. Ballmes, J. Funes, J. Halvorsen, and R. J. Novak. 2007. First record of *Aedes japonicus japonicus* in Illinois: defining its spatial distribution and associated mosquito species. J Am Mosq Control Assoc 23: 243-251.

Moulton, D. W. a. T., W.H. . 1971. California group virus infections in small, forest-dwelling mammals of Wisconsin. American Journal of Tropical Medicine and Hygiene 20: 474-482.

Muturi, E. J., C. H. Kim, B. W. Alto, M. R. Berenbaum, and M. A. Schuler. 2011. Larval environmental stress alters *Aedes aegypti* competence for Sindbis virus. Trop Med Int Health 16: 955-964.

Nasci, R. S. 1985. Local variation in blood feeding by *Aedes triseriatus* and *Aedes hendersoni* (Diptera: Culicidae). . Journal of Medical Entomology 22: 619-623.

Nasci, R. S. 1988. Biology of *Aedes triseriatus* (Diptera: Culicidae) developing in tires in Louisiana. J Med Entomol 25: 402-405.

Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, and M. Layton. 2001. The outbreak

of West Nile virus infection in the New York City area in 1999. N Engl J Med 344: 1807-1814.

Neitzel, D. F., K. A. Johnson, S. Brogren, and M. M. Kemperman. 2009. First collection records of *Aedes japonicus* in Minnesota. J Am Mosq Control Assoc 25: 367-369.

Novak, M. G., L. G. Higley, C. A. Christianssen, and W. A. Rowley. 1993. Evaluating larval competition between *Aedes albopictus* and *A. triseriatus* (Diptera: Culicidae) through replacement series experiments. Environmental Entomology 22: 311-318.

Obenauer, P. J., L. J. Buss, and P. E. Kaufman. 2009. Utilizing Auto-Montage, Technology for Identifying Field-Collected Container-Inhabiting Mosquito Eggs1. Journal of the American Mosquito Control Association 25: 517-520.

Pantuwatana, S., W. H. Thompson, D. M. Watts, T. M. Yuill, and R. P. Hanson. 1974. Isolation of La Crosse virus from field collected *Aedes triseriatus* larvae. American Journal of Tropical Medicine and Hygiene 23: 246-250.

Paupy, C., H. Delatte, L. Bagny, V. Corbel, and D. Fontenille. 2009. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes Infect 11: 1177-1185.

Perdew, P. E., and C. L. Meek. 1990. An improved model of a battery-powered aspirator. J Am Mosq Control Assoc 6: 716-719.

Perez, M. H., and F. G. Noriega. 2013. *Aedes aegypti* pharate 1st instar quiescence: a case for anticipatory reproductive plasticity. J Insect Physiol 59: 318-324.

Peyton, E. L., S. R. Campbell, T. M. Candeletti, M. Romanowski, and W. J. Crans. 1999. *Aedes* (Finlaya) *japonicus japonicus* (Theobald), a new introduction into the United States. J Am Mosq Control Assoc 15: 238-241.

Philip, C. B., Smadel, J. E. 1943. Transmission of West Nile virus by infected *Aedes albopictus*. Proc Soc Exp Biol Med 53: 49-50.

Poelchau, M. F., J. A. Reynolds, D. L. Denlinger, C. G. Elsik, and P. A. Armbruster. 2011. A de novo transcriptome of the Asian tiger mosquito, Aedes albopictus, to identify candidate transcripts for diapause preparation. BMC Genomics 12: (20 December 2011).

Poelchau, M. F., J. A. Reynolds, C. G. Elsik, D. L. Denlinger, and P. A. Armbruster. 2013a. RNA-Seq reveals early distinctions and late convergence of gene expression between diapause and quiescence in the Asian tiger mosquito, *Aedes albopictus*. J Exp Biol 216: 4082-4090.

Poelchau, M. F., J. A. Reynolds, D. L. Denlinger, C. G. Elsik, and P. A. Armbruster. 2013b. Transcriptome sequencing as a platform to elucidate molecular components of the diapause response in the Asian tiger mosquito *Aedes albopictus*. Physiological Entomology 38: 173-181.

Poelchau, M. F., J. A. Reynolds, C. G. Elsik, D. L. Denlinger, and P. A. Armbruster. 2013c. Deep sequencing reveals complex mechanisms of diapause preparation in the invasive mosquito, *Aedes albopictus*. Proceedings of the Royal Society of London. Series B, Biological Sciences 280: 20130143.

Reiter, P. 1983. A portable battery-powered trap for collecting gravid *Culex* mosquitoes. Mosquito News 43: 496-498.

Richards, S. L., L. Ponnusamy, T. R. Unnasch, H. K. Hassan, and C. S. Apperson. 2006. Host-feeding patterns of *Aedes albopictus* (Diptera: Culicidae) in relation to availability of human and domestic animals in suburban landscapes of central North Carolina. J Med Entomol 43: 543-551.

Rochlin, I., R. Gaugler, E. Williges, and A. Farajollahi. 2013. The rise of the invasives and decline of the natives: insights revealed from adult populations of container-inhabiting *Aedes* mosquitoes (Diptera: Culicidae) in temperate North America. Biological Invasions 15: 991-1003.

Rose, A., Geier, M., Eiras, Á.E., Kröckel, U., Ball, T.S., Grunewald, J., and Bergbauer, R. 2004. Field-testing a novel type of trap for host-seeking mosquitoes. The Program and Abstract Book. Osijek, Croatia, p 15.

Rosen, L. 1981. Transovarial transmission of arboviruses by mosquitoes. Mede-cine Tropicale 41: 23-29.

Sardelis, M. R., M. J. Turell, and R. G. Andre. 2002a. Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

Sardelis, M. R., D. J. Dohm, B. Pagac, R. G. Andre, and M. J. Turell. 2002b. Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J Med Entomol 39: 480-484.

Saul, S. H., P. R. Grimstad, and G. B. Craig, Jr. 1977. Identification of *Culex* species by electrophoresis. Am J Trop Med Hyg 26: 1009-1012.

Scholl, P. J., and G.R. DeFoliart. 1977. *Aedes triseriatus* and *Aedes hendersoni*: vertical and temporal distribution as measured by oviposition. Environmental Entomology 6: 355-358.

Scott, T. W., and T. G. Burrage. 1984. Rapid infection of salivary glands in *Culiseta melanura* with eastern equine encephalitis virus: an electron microscopic study. Am J Trop Med Hyg 33: 961-964.

Shroyer, D. A., and G. B. Craig, Jr. 1980. Egg hatchability and diapause in *Aedes triseriatus* (Diptera: Culicidae). I. Population Size. Journal of Medical Entomology 15: 89-98.

Simard, F., E. Nchoutpouen, J. C. Toto, and D. Fontenille. 2005. Geographic distribution and breeding site preference of *Aedes albopictus* and *Aedes aegypti* (Diptera: culicidae) in Cameroon, Central Africa. J Med Entomol 42: 726-731.

Sprenger, D., and T. Wuithiranyagool. 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. Journal of the American Mosquito Control Association 2: 217-219.

Streit, T. G., G.B. Craig Jr., and P.R. Grimstad. 1993. La Crosse virus transmission by *Aedes albopictus*. Arthropod-Borne Virus Exchange: 18-19.

Szumlas, D. E., C. S. Apperson, E. E. Powell, P. Hartig, D. B. Francy, and N. Karabotsos. 1996. Relative abundance and species composition of mosquito populations (Diptera:Culicidae) in a La Crosse virus-endemic area in western North Carolina. J Med Entomol 33: 598-607.

Teng, H. J., and C. S. Apperson. 2000. Development and survival of immature *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) in the laboratory: effects of density, food, and competition on response to temperature. Journal of Medical Entomology 37: 40-52.

Thompson, W. H., B. Kalfayan, and R. C. Anslow. 1965. Isolation of California Encephalitis Group Virus from a Fatal Human Illness. American Journal of Epidemiology 81: 245-253.

Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 38: 130-134.

Urbanski, J., M. Mogi, D. O,ÄôDonnell, M. DeCotiis, T. Toma, and P. Armbruster. 2012. Rapid Adaptive Evolution of Photoperiodic Response during Invasion and Range Expansion across a Climatic Gradient. The American Naturalist 179: 490-500.

Urbanski, J. M., J. B. Benoit, M. R. Michaud, D. L. Denlinger, and P. Armbruster. 2010. The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, *Aedes albopictus*. Proc Biol Sci 277: 2683-2692.

Usmani-Brown, S., L. Cohnstaedt, and L. E. Munstermann. 2009. Population genetics of *Aedes albopictus* (Diptera: Culicidae) invading populations, using mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 5 sequences. Annals of the Entomological Society of America 102: 144-150.

Wang, R. L. 1966. Observations on the influence of photoperiod on egg diapause in *Aedes albopictus* Skuse. Acta Entomologica Sinica 15: 75-77.

Watts, D. M., S. Pantuwatana, G. R. DeFoliart, T. M. Yuill, and W. H. Thompson. 1973. Transovarial transmission of LaCrosse virus (California encephalitis group) in the mosquito, *Aedes triseriatus*. Science, USA 182: 1140-1141.

Watts, D. M., W. H. Thompson, T. M. Yuill, G. R. Defoliart, and R. P. Hanson. 1974. Overwintering of La Crosse virus in *Aedes triseriatus*. American Journal of Tropical Medicine and Hygiene 23: 694-700.

Weaver, S. C., and W. K. Reisen. 2010. Present and future arboviral threats. Antiviral Res 85: 328-345.

Widdel, A. K., L. J. McCuiston, W. J. Crans, L. D. Kramer, and D. M. Fonseca. 2005. Finding needles in the haystack: single copy microsatellite loci for *Aedes japonicus* (Diptera: Culicidae). Am J Trop Med Hyg 73: 744-748.

Worobey, J., D. M. Fonseca, C. Espinosa, S. Healy, and R. Gaugler. 2013. Child outdoor physical activity is reduced by prevalence of the Asian tiger mosquito, *Aedes albopictus*. Journal of the American Mosquito Control Association 29: 78-80.

Wright, R. E., and G. R. DeFoliart 1970. Associations of Wisconsin mosquitoes and woodland vertebrate hosts. Annals of the Entomological Society of America 63: 720-728.

Zhong, D., E. Lo, R. Hu, M. E. Metzger, R. Cummings, M. Bonizzoni, K. K. Fujioka, T. E. Sorvillo, S. Kluh, S. P. Healy, C. Fredregill, V. L. Kramer, X. Chen, and G. Yan. 2013. Genetic analysis of invasive *Aedes albopictus* populations in Los Angeles County, California and its potential public health impact. PLoS One 8: e68586.

Zhu, H., Q. Yuan, A. D. Briscoe, O. Froy, A. Casselman, and S. M. Reppert. 2005. The two CRYs of the butterfly. Curr Biol 15: R953-954.

Chapter 2

Morphological Differentiation of Eggs of Container-inhabiting *Aedes*

2.1 Introduction

Aedes triseriatus (Say), *Aedes japonicus* (Theobald), *Aedes albopictus* (Skuse), and *Aedes aegypti* (Linnaeus) are among the mosquitoes of greatest public health concern in the United States. All are competent vectors of arboviral diseases as well as being significant biting pests (Szumlas et al. 1996, Turell et al. 2001, Sardelis et al. 2002b, Sardelis et al. 2002a, Leisnham and Juliano 2012, Bonizzoni et al. 2013). *Ae. triseriatus* is a native mosquito while the other three are exotic invasive species. It is believed that *Ae. aegypti* was introduced to the New World from Africa by European trade activities during the 14th and 15th centuries and is now found throughout the southeastern United States.(Powell and Tabachnick 2013). The invasions by *Ae. albopictus* and *Ae. japonicus* are more recent. Both species are believed to have been introduced to the U.S. from Asia by the used tire trade in the late 20th century (Lounibos 2002). *Ae. albopictus* is established throughout the southeast U.S. (Moore 1999) while the range of *Ae. japonicus* is still expanding (Thorn et al. 2012). Thus, these four container-inhabiting mosquitoes are sympatric in many locations.

Container-inhabiting *Aedes* females oviposit one egg at a time, gluing the ‘eggs to the substrate directly above the water line in artificial and natural containers of water (Clements 1999). The habit of utilizing artificial containers has contributed to the successful colonization and range expansion of *Ae. albopictus* and *Ae. japonicus* in the United States (Lounibos 2002). However, this characteristic of container-inhabiting mosquitoes has been exploited to design an effective sampling method, the ovitrap (Loor and DeFoliart 1969). Ovitraps are easily and cheaply constructed and are sensitive at detecting the presence of gravid females in an area (Silver 2008). This is particularly useful because these species are poorly attracted to light traps

and CO_2 baited traps (Craig 1983). Ovitraps are widely used for population monitoring, disease surveillance, and evaluation of control activities. However, because different combinations of the 4 species are sympatric in various locations and, indeed, may occur in the same ovitrap, it can be difficult to determine the relative species composition. Oviposition substrates are often brought into the lab and reared to adult for enumeration and speciation. However, this is time consuming and will underestimate the counts due to a phenomenon called installment hatching. For many *Aedes* eggs, only a portion will hatch with the first inundation, with more hatching with each subsequent flooding (Gillett 1955), an adaptation to the ephemeral habitat of a container. A method to differentiate the eggs directly would be more efficient.

The eggshell surrounds and protects the oocyte, egg, embryo and pharate larva, while reducing water loss and allowing for gas exchange. It is comprised of the exochorion, innerchorion and a serosal cuticle. The exochorion is highly variable in color and has numerous tubercles and surface markings (Harbach 1980, Clements 1999). Surface characteristics such as shape of the exochorionic cells, the mycropylar collar, and color can used to differentiate among *Ae. triseriatus*, *Ae. albopictus*, *Aedes ae.* and *Ae. japonicus* in the eastern United States. (Linley 1989a, b, Haddow et al. 2009).

Our objective was find morphological characteristics that will permit the reliable identification of eggs to species using a standard stereomicroscope.

2.2 Materials and methods

Eggs of *Ae. japonicus*, *Ae. triseriatus*, and *Ae. albopictus* were obtained from colonies established from mosquitoes collected in Montgomery County, VA. The eggs of *Ae. aegypti* were from the Rockefeller strain, a strain that has been maintained in laboratories for decades.

Female mosquitoes were allowed to feed on sheep blood via a water-jacketed artificial membrane feeder (Rutledge et al. 1964) with stretched parafilm for a membrane (Failloux et al. 1991). An oviposition cup was filled partially with water and lined with seed germination paper (Steinly et al. 1991), which was used as an oviposition substrate. When eggs were laid, the papers were removed from the oviposition cup and stored in a plastic Ziploc bag with a partially wetted paper towel to prevent desiccation.

Eggs were prepared for light microscopy by immersing the papers in water and then allowing them to dry for 8-10 minutes to partially dry the eggs to reduce reflection from excess water. The optimal times for imaging or differentiating species was found to be when the eggs were moist but dry enough to clearly see external chorionic features such as outer chorionic cells, conspicuous mycropylar collars, and color.

Photographs were taken using a Wild Photomakroskop M 400 microscope on JVC KY-F75U 1/2" 3-CCD digital capture video camera and Syncroscopy Auto-Montage Pro imaging software. This software produces a perfectly focused composite single image from a series of images focused at different heights on the specimen.

Measurements were made using the Auto-Montage Pro measurements option. A total of 20 eggs of each species were measured. The eggs were taken from at least 4 different oviposition papers to ensure that they were the progeny of different females. The length was measured from the anterior to posterior pole and the width of the egg was measured at its widest point, usually where dehiscence occurs. This is about a quarter of the way down from the anterior pole. Data were analyzed by ANOVA using JMP Pro.

2.3 Results

All eggs are broadly cigar shaped, tapering anteriorly and posteriorly. *Ae. triseriatus* eggs are significantly longer and wider than the eggs of the other species ($P<0.0001$)(Table 2.1). The eggs of the 4 species can easily be divided into 2 groups based on color. *Ae. triseriatus* (Fig. 2.1) and *Ae. japonicus* (Fig. 2.2) eggs are dull or matte black in color with conspicuous chorionic cells whereas *Ae. albopictus* (Fig. 2.3) and *Ae. aegypti* (Fig. 2.4) eggs are shiny jet black with less conspicuous chorionic cells. In addition to larger size, the eggs of *Ae. triseriatus* are lighter in color with deeper, more irregular chorionic cells giving them a rougher appearance when compared to the egg of *Ae. japonicus*. Three characteristics can be used to differentiate between the eggs of *Ae. aegypti and Ae. albopictus*: 1) *Ae. aegypti* eggs have very prominent micropylar collars while *Ae. albopictus* eggs do not; the eggs of Ae. albopictus are more strongly tapered from the widest point; and 2) *Ae. albopictus* eggs have large central tubercles inside the chorionic cells, giving them a slightly smoother, shinier appearance than eggs of *Ae. aegypti*.

Using these criteria, the eggs of the 4 species of container-inhabiting mosquitoes can be differentiated reliably for field studies without the need to rear them to adults (Fig. 2.5).

2.4 Discussion

Detailed descriptions of all 4 species based on scanning electron microscopy (SEM) have been published previously (Linley 1989a & 1989b; Haddow et al. 2009). However, many of the fine structure differences that have been described cannot be seen using standard stereomicroscopy. The Auto-Montage (AM) software uses a conventional stereomicroscope to take a series of images at different planes of focus and combines them to produce a montage image (Boyde 2004). The photomicrographs produced have better resolution and increased

depth of field than ordinary photographs (Rueda 2004). Obenauer et al. (2009) published AM images of *Ae. triseriatus, Ae. albopictus,* and *Ae. aegypti* and noted that this method could be useful for mosquito surveillance. By studying AM and SEM images, I was able to identify characteristics to reliably discriminate among the 4 sympatric container-inhabiting mosquito species using only a standard stereomicroscope (Fig 2.5).

To carry out mosquito surveillance activities, mosquitoes can be collected at different stages of their life cycle, i.e., as larvae, adults or eggs. Each approach has advantages and disadvantages. Ovitraps are a routine surveillance method commonly used in North America for container-inhabiting mosqutioes (Silver 2008). Compared to larval surveys, oviposition surveys can significantly reduce costs and number of working days required (Fay and Eliason 1966; Jakob and Bevier 1969). Ovitraps can be particularly useful when vector populations are low (Focks 2003). Adults of the 4 container-inhabiting species in this study are difficult to sample. Light traps, such as the CDC trap, yield poor catches of these diurnal mosquitoes (Hanson et al. 1988; Service 1993). Baiting with CO_2 improves the efficacy but adds to the expense and labor.

A major drawback of using ovitraps is the difficulty in identifying mosquito eggs. Generally, the eggs must be hatched and reared to adult for identification. However, because the eggs of these species hatch by installment, that is, a portion of the eggs will remain dormant even in the presence of hatching stimulus, counting the eggs will provide a more accurate estimate of population size (Clements 1999). Our results eliminate the need to rear the mosquitoes, reducing labor and time while providing an accurate count of the vector mosquitoes.

2.5 Literature Cited

Bonizzoni, M., G. Gasperi, X. Chen, and A. A. James. 2013. The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives. Trends in Parasitology 29: 460-468.

Clements, A. N. 1999. The biology of mosquitoes, Volume 2: Sensory, reception, and behaviour. Chapman and Hall, Cambridge. .

Denlinger, D. L., and P. A. Armbruster. 2014. Mosquito diapause. Annu Rev Entomol 59: 73-93.

Haddow, A. D., J. K. Moulton, R. R. Gerhardt, L. J. McCuiston, and C. J. Jones. 2009. Description of the egg of *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) using variable pressure scanning electron microscopy. J Med Entomol 46: 9-14.

Hahn, D. A., and D. L. Denlinger. 2011. Energetics of insect diapause. Annu Rev Entomol 56: 103-121.

Harbach, R. E., and K. L. Knight. . 1980. Taxonomist's glossary of mosquito anatomy. Plexus, Marlton, NJ. .

Leisnham, P. T., and S. A. Juliano. 2012. Impacts of climate, land use, and biological invasion on the ecology of immature *Aedes* mosquitoes: implications for La Crosse emergence. EcoHealth 9: 217-228.

Linley, J. R. 1989a. Comparative fine structure of the eggs of *Aedes albopictus*, *Ae. aegypti*, and *Ae. bahamensis* (Diptera: Culicidae). Journal of Medical Entomology 26: 510-521.

Linley, J. R. 1989b. Scanning electron microscopy of the egg of *Aedes* (Protomacleaya) *triseriatus* (Diptera: Culicidae). Journal of Medical Entomology 26: 474-478.

Sardelis, M. R., M. J. Turell, and R. G. Andre. 2002a. Laboratory transmission of La Crosse virus by Ochlerotatus j. japonicus (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

Sardelis, M. R., D. J. Dohm, B. Pagac, R. G. Andre, and M. J. Turell. 2002b. Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J Med Entomol 39: 480-484.

Szumlas, D. E., C. S. Apperson, E. E. Powell, P. Hartig, D. B. Francy, and N. Karabotsos. 1996. Relative abundance and species composition of mosquito populations

(Diptera:Culicidae) in a La Crosse virus-endemic area in western North Carolina. J Med Entomol 33: 598-607.

Thorn, M., W. C. Varnado, and J. Goddard. 2012. First record of *Aedes japonicus* japonicus in Mississippi. Journal of the American Mosquito Control Association 28: 43-44.

Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 38: 130-134.

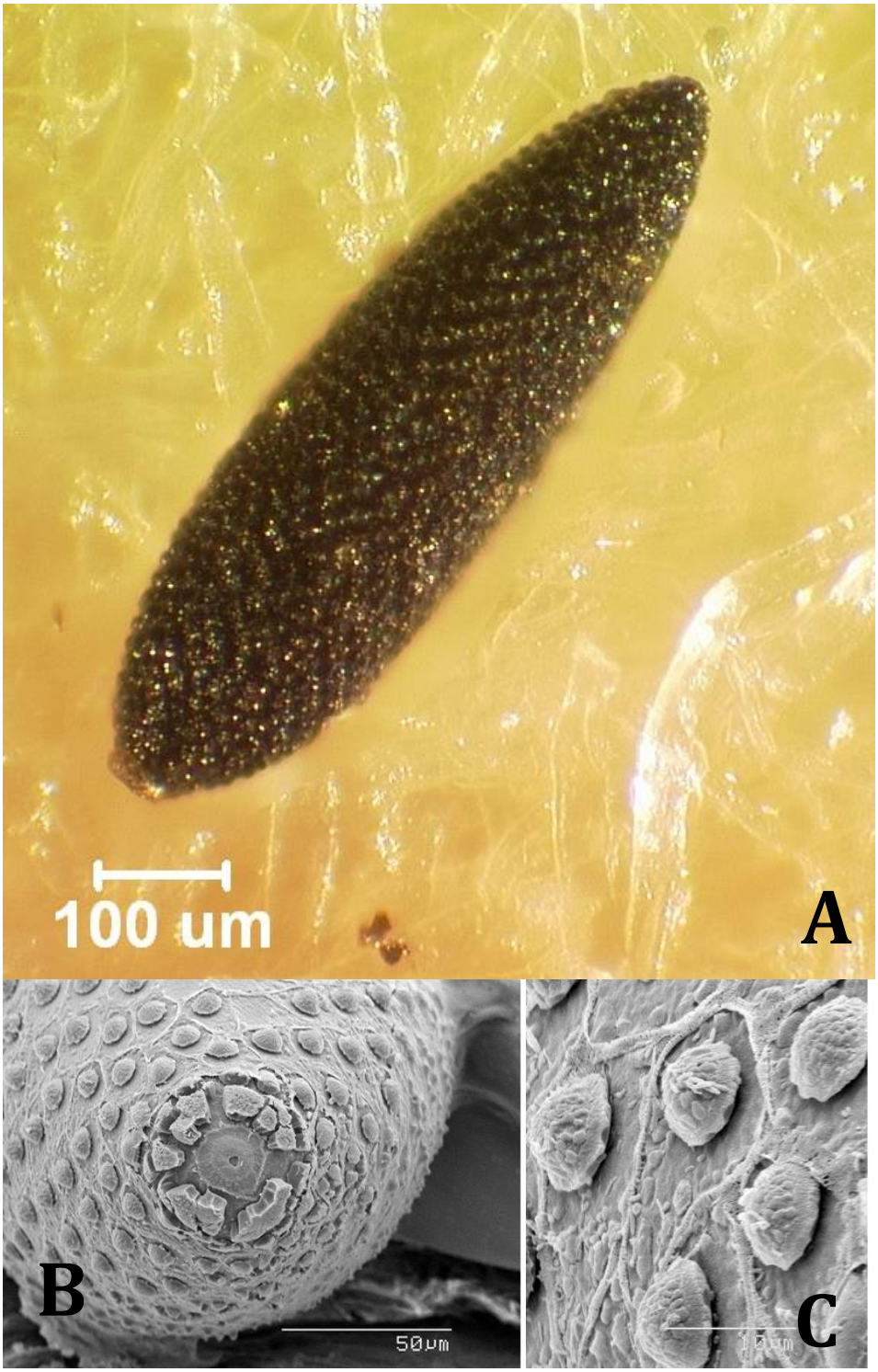

Fig. 2.1. *Aedes triseriatus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.

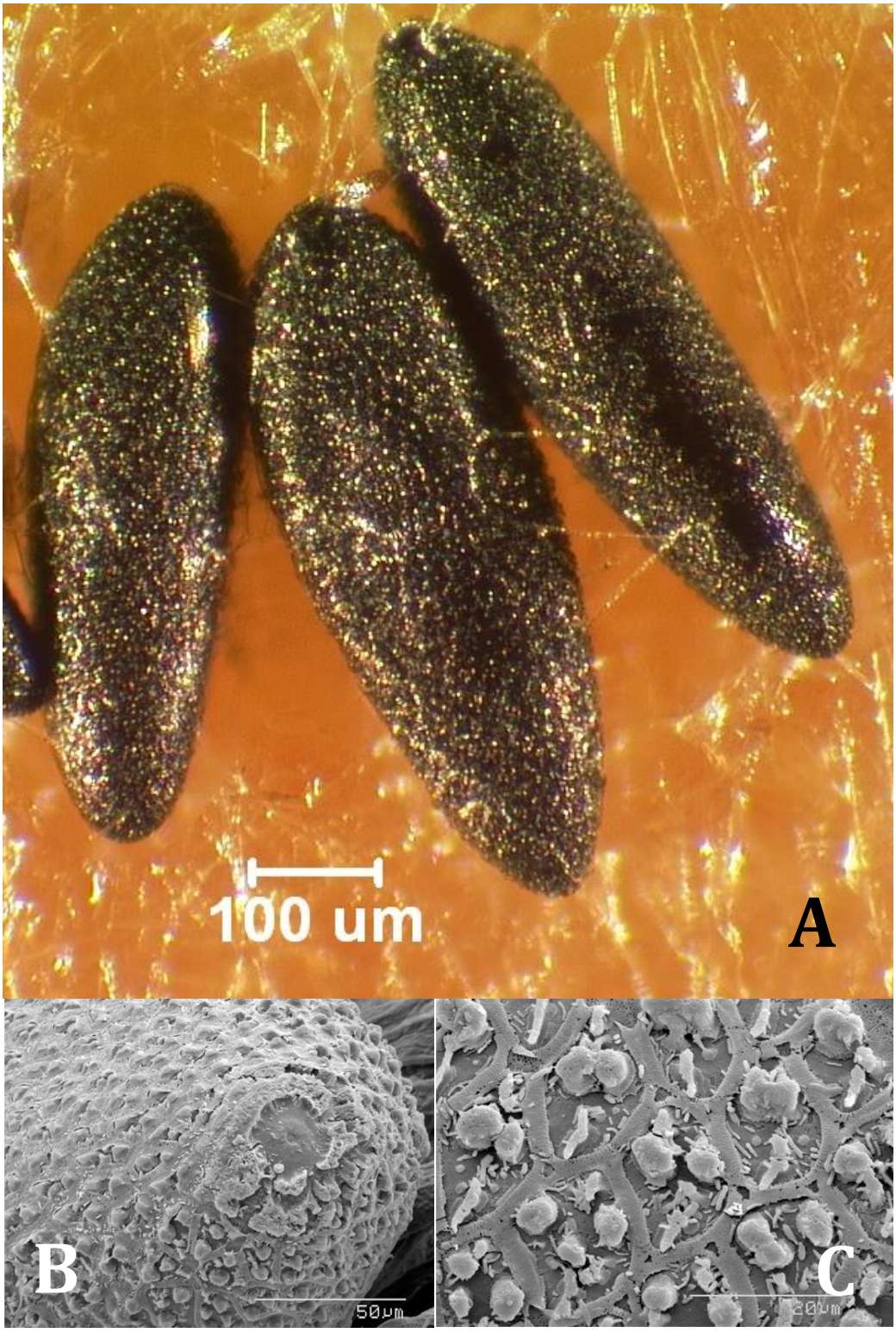

Fig. 2.2. *Aedes japonicus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.

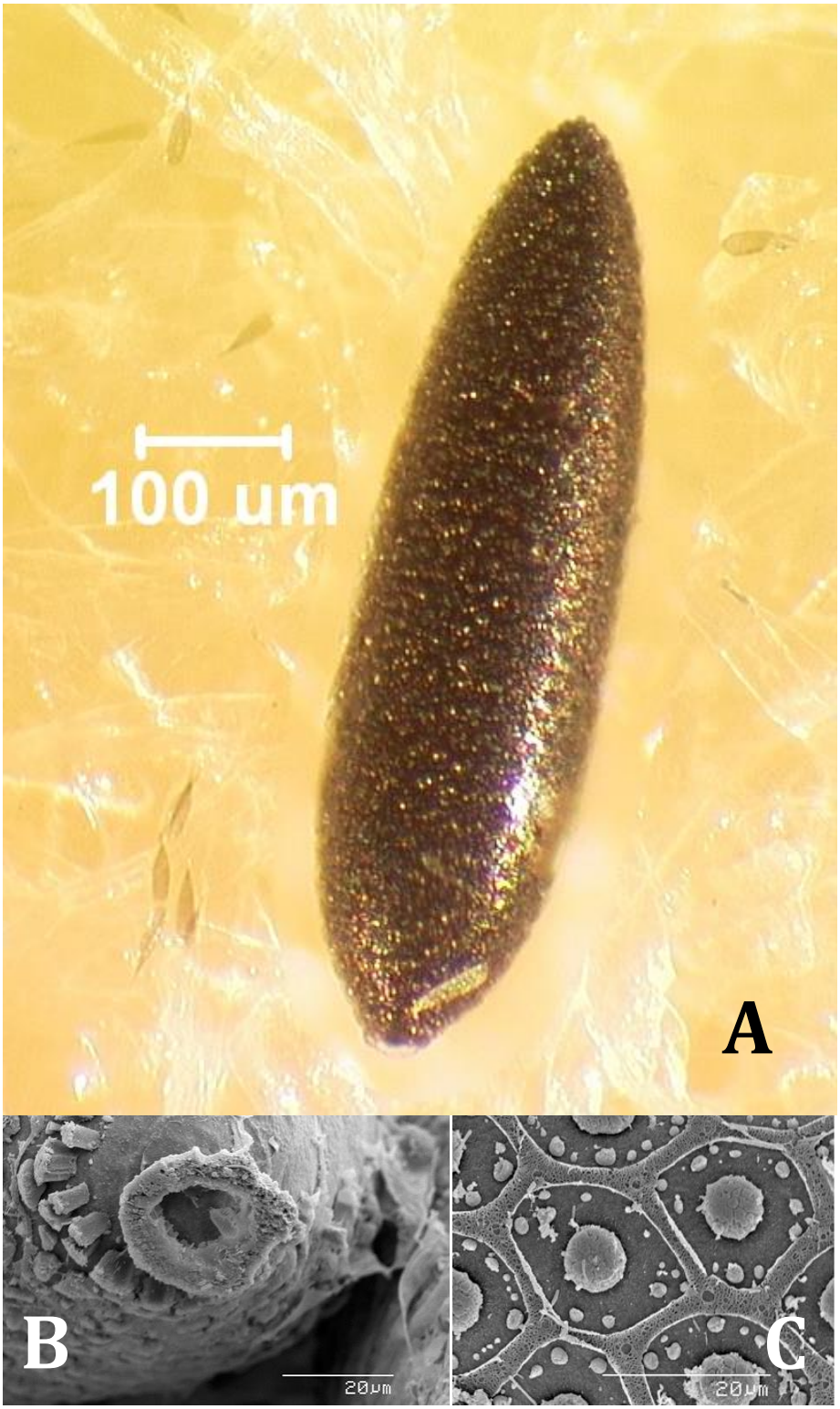

Fig. 2.3. *Aedes aegypti* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.

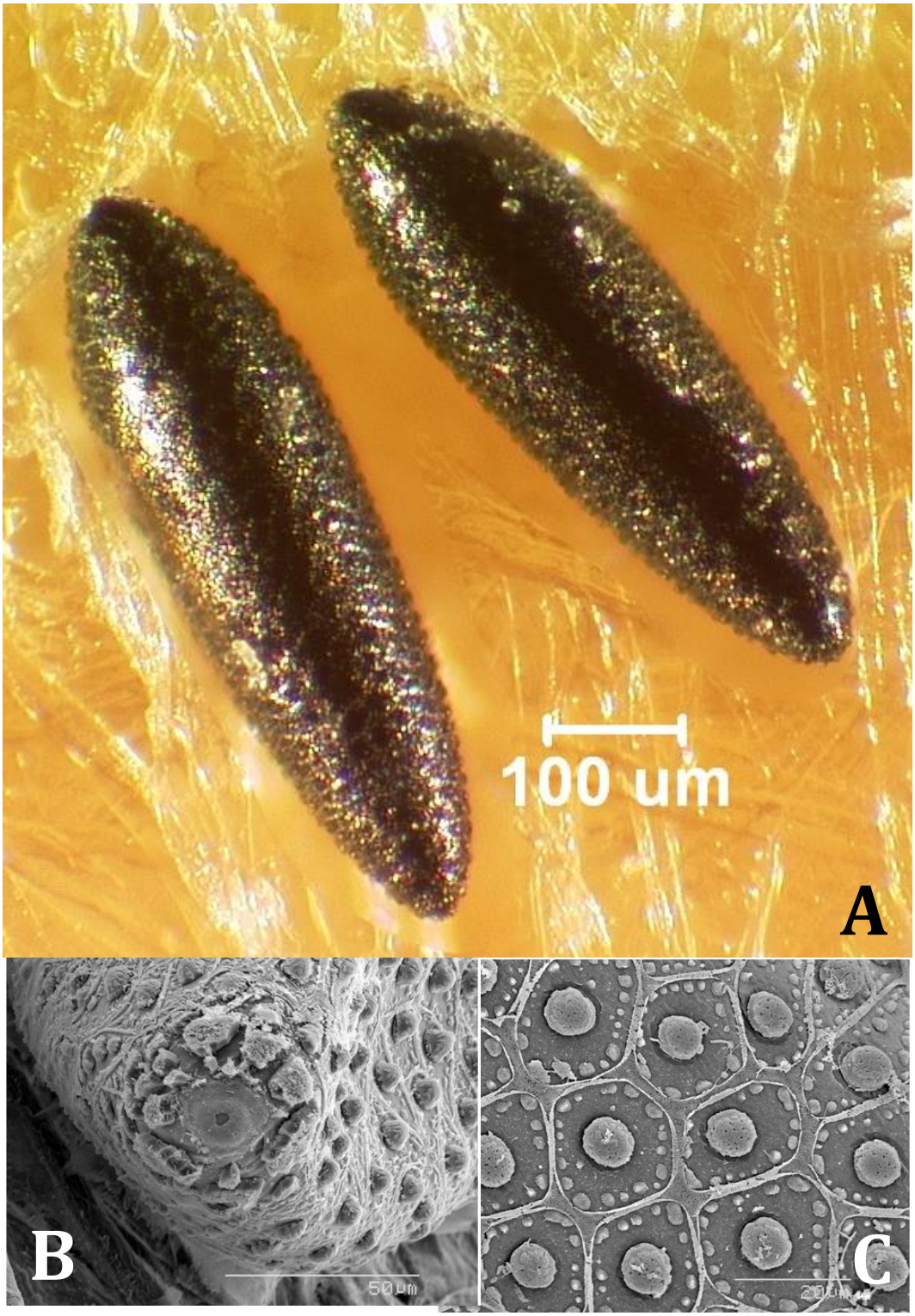

Fig. 2.4. *Aedes albopictus* egg. (A) AM image. (B) Mycropylar apparatus. (C) Chorionic cell detail.

Fig. 2.5. Differentiation of the eggs of container-inhabiting *Aedes*. (A) *Ae. aegypti*: shiny, jet black eggs with prominent micropylar collars (arrow). (B) *Ae. albopictus*: very shiny, jet black eggs, strongly tapered from the widest point. (C) *Ae. triseriatus*: large, matt black eggs. (D) *Ae. japonicus*: matt black eggs, smaller than *Ae. triseriatus.*

Table 2.1 *Ae. triseriatus*, *Ae. albopictus*, and *Ae. japonicus* egg measurements

Species	Length (μm)		Width (μm)		L/W Ratio	
	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE	Range
Ae. triseriatus	690.5 ± 4.6a*	652.2-721.6	217.6 ± 2.0a	200.3-230.6	3.18 ± 0.02a	3.08-3.39
Ae. japonicus	604.2 ± 6.0b	565.5-6.48.4	182 ± 3.1b	1.83.2-203.7	3.33 ± 0.05b	3.02-3.68
Ae. albopicus	597.6 ± 5.0b	560.2-631.5	183.5 ± 3.9b	152.8-213.4	3.29 ± 0.06a	2.91-3.67
Ae. aegypti	605.0 ±4.1b	573.1-631.9	178.1 ± 3.2b	153.0-201.9	3.42 ± 0.06b	3.03-3.96

Chapter 3

A Comparison Between Two Sampling Methods for Container- Inhabiting Mosquitoes in a La Crosse Virus Endemic Area

3.1 Introduction

Three species of container-inhabiting *Aedes* mosquitoes are sympatric in southwestern Virginia. These species are the native *Aedes triseriatus* (Say), the eastern treehole mosquito and two invasive mosquitos, *Aedes albopictus* (Say) and *Aedes japonicus* (Theobald) (Barker et al. 2003, Grim et al. 2007). *Aedes triseriatus* is the primary vector of La Crosse virus (LACV) and the two exotic species are considered accessory vectors. La Crosse encephalitis (Family Bunyaviridae, Genus Bunyavirus, California serogroup, LACV) is the most common and important endemic mosquito-borne disease of children in the U.S.A. (Rust et al. 1999). The majority of human cases have occurred in the upper Midwestern states with more recent cases being reported in West Virginia and Tennessee (Haddow et al 2011). *Ae. japonicus* and *Ae. albopictus* are also competent vectors for Eastern equine encephalitis virus (EEEV) which is endemic to Virginia; however it is predominantly found on the east coast. They are also competent vectors for West Nile Virus. *Ae. albopictus* is a competent vector of St. Louis encephalitis virus (SLEV) which is endemic to Virginia (Turell et al. 2001, Sardelis et al. 2002b, Sardelis et al. 2002a). It is important to understand the population dynamics of all three species as it may impact the epidemiology of the viruses in three different habitat types.

To study the population dynamics of these species, I used 2 types of traps that are commonly used in LACV surveillance. Because *Ae. triseriatus* females are not attracted to light, use of oviposition traps has become a routine method to sample this species (Hanson et al. 1988). The virus is maintained through transovarial transmission so the eggs can be collected, hatched

and reared and the resulting adults tested for virus infection (Watts et al. 1973, Pantuwatana et al. 1974, Balfour and et al. 1975). Gravid traps are used to collect adult mosquitoes that have taken at least one blood meal. These traps are often baited with an infusion to be more attractive (Reiter 1983). Recent field isolates tested positive for LACV in southwestern Virginia (Harris et al. *in review*).

Land variation and deforestation have been shown to affect vector-borne disease with malaria, WNV and Lyme borreliosis (Patz et al. 2004). However, the effects of forest fragmentation and forest canopy disturbance (FCD) on mosquito-borne disease is not well understood, The objective of this study was to compare the two sampling methods, ovitraps, and gravid traps, to assess mosquito population density and phenology in 3 different forest habitat types.

3.2 Materials and Methods

This study was conducted from June 2011 to August 2011 at two sites that were part of the Southern Appalachian Silviculture and Biodiversity (SASAB) project (Fig. 3.1). These sites were chosen because recent mosquito isolates from theses sites tested positive for LACV (Harris et al. *in review*). The SASAB project is a long-term study, which consists of seven treatment habitat types, two-hectares in size, applied in a complete randomized block design to multiple sites in the southern Appalachian mountain range (Belote et al. 2009, Homyack 2009, Belote et al. 2012). For this study, three different treatment habitats were used in two different sites (BB1 and BB2) in the Jefferson National Forest. Each treatment habitat had a different level of forest canopy disturbance (FCD): undisturbed control, a clearcut intermediate FCD, and a shelterwood high level FCD. The undisturbed control habitats had not been harvested in 80-120 years. In the

clearcut habitats, trees with a diameter greater than 5-cm were removed 16 years ago. Complete canopy removal of the shelterwood habitats occurred in 2007. Four oviposition traps (Loor and Defoliart 1969) and two Reiter gravid traps (Hausherr's Machine Works, Toms River, NJ) were placed in each of the habitats at least 30 m apart. Thus, a total of 12 gravid traps and 24 oviposition traps were used at each site, BB1 and BB2 (Fig. 3.2).

The ovitraps were constructed from black plastic cups (450-ml) (WNA, Inc. (SKU 16SPOLY), Covington, KY) with several drain holes punched halfway down the sides. The oviposition substrate was a strip of seed germination paper (12.5 x 5.0 cm) (Anchor Paper Company (No. SD 3815L), Saint Paul, MN) (Steinly et al. 1991). The oviposition traps were nailed to trees at knee height and approximately 200ml of hay infusion (Jackson and Paulson, 2006) was used as an attractant in each trap. Four oviposition traps were placed in each treatment habitat type at least 30m from the edge of the two-hectare plots. The seed germination papers were collected and replaced weekly for the three-month collection period and stored in sealable plastic bags to prevent desiccation. Eggs were counted and identified to species then were reared to adults using the methods of Mustermann and Wasmuth (1985). Adults were then sorted by species, trap type, and trap date and stored at -80°C for later virus assay.

Gravid traps were set up and sampled after 24 hours twice every week. Hay infusion was used as an attractant. Two gravid traps were placed in the center of each treatment habitat type at least 30m apart. Adult mosquitoes were collected from the trap with a hand held aspirator (Hausherr's Machine Works, Toms River, NJ) and placed in vials labeled with date and site. The vials were then put in a small cooler with ice to be taken back to the lab to be identified. The adult mosquitoes were identified to species using the keys and descriptions of Slaff and

Apperson (1989) and Darsie (2002); however, only *Ae. triseriatus, Ae. albopictus, Ae. japanicus* were included in this study.

Adult mosquitoes were pooled into groups of no more than 50 and screened for virus by plaque assay on VERO cells using M199 (Beaty 1995, Nasci et al. 2000) as a simple diagnostic. If cytopathic effect was present the isolates were sent to The Centers of Disease Control and Prevention to be tested for virus using molecular techniques.

3.3 Results

Mosquito abundance and phenology. In 2011, 24 ovitraps and 12 gravid traps were sampled repeatedly throughout June, July, and August for three sympatric container-inhabiting mosquito species, *Ae. triseriatus, Ae. albopictus*, and *Ae. japonicus.* The relative abundance of total *Ae. triseriatus* was significantly higher than that of *Ae. japonicus* and *Ae. albopictus,* whether measure by eggs in ovitraps or adults in gravid traps (Fig. 3.3; $p<0.001$ and Fig. 3.4; $p<0.05$). *Ae. triseriatus* comprised 99.3% of total eggs collected while a total of 460 *Aedes japonicus* and 295 *Aedes albopictus* eggs comprise <1% of total eggs collected. *Ae. triseriatus* comprised 84.4% of total adult mosquitoes collected while *Ae. japonicus* and *Ae.* albopictus comprised 5.2% respectively (Table 3.1). Total oviposition activity of *Ae. japonicus* and *Ae. albopictus* did not differ significantly (Fig. 3.3, $p>0.05$) while adult *Ae. japonicus* and *Ae. albopictus* relative abundance did differ significantly (Fig 3.4, $p<0.05$).

Ae. triseriatus adults and eggs peaked in July (Fig. 3.5). The oviposition activity of *Ae. triseriatus* peaked in week 32 (early July) with 556.0 eggs/ trap-week, while peak relative abundance of adult *Ae. triseriatus* occurred two weeks later, week 34 (late July) with 8.04 adults/ trap-day. *Ae. japonicus* adults and eggs peaked in late June, week 30 with 4.7 eggs/ trap-week,

and 1.4 adults/ trap-day (Fig. 3.5 and 3.6). No *Ae. japonicus* eggs were collected in mid-late August, weeks 37-39. There was no distinct peak in *Ae. albopictus* ovipostion behavior but had three weeks of similar intensity; weeks 35-37 with 2.6, 2.5, and 2.6 eggs/trap-week . The relative abundance of *Ae. albopictus* adults peaked two weeks later in late August, week 39 with 2.0 adults/trap-night (Fig. 3.6). Both adults and eggs of *Ae. triseriatus* and *Ae. japonicus* show similar phenologies, with numbers peaking in June and July and declining in August, while *Ae. albopictus* adults and eggs were initially low in June and July and sharply increased in late August (Fig. 3.5 and 3.6).

Mosquito habitat preferences. BB1 and BB2 sites did not differ significantly for adults/trap-day or eggs/trap-week ($p>0.05$) and so the data were combined.

Total mosquito relative oviposition activity varied among habitats with more eggs/trap-week obtained from the control and clearcut habitats when compared to the shelterwood habitats (Fig 3.7; $p<0.05$). The control and clearcut habitats also had a greater relative intensity of total adult mosquitoes with more adults/trap-night (Fig 3.8; $p<0.001$).

The relative oviposition preference of the three mosquito species varied among the three habitats (Fig. 3.7). The total number of *Ae. triseriatus* eggs collected were greatest in the control habitats and differed significantly from the shelterwood habitats ($p<0.05$) but did not differ significantly from the clearcut habitats ($p>0.05$). The highest prevalence of *Ae. japonicus* and *Ae. albopictus* eggs were collected from the clearcut habitats. The number of *Ae. japonicas* collected from the clearcut differed significantly from both the control and shelterwood habitats ($p<0.05$). Total *Ae. albopictus* eggs collected from the clearcut habitats differed significantly from the shelterwood habitats ($p<0.05$); however, there was no statistical significance when *Ae. albopictus* eggs from the clearcut habitats were compared to the control habitats ($p>0.05$).

Adult relative abundance varied by mosquito species and habitat type. *Ae. triseriatus* and *Ae. japonicus* adult relative abundance was greatest on the control and clearcut habitats ($p<0.05$), while there was no statistical significance among *Ae. albopictus* adults collected (Fig. 3.8; $p>0.05$). The highest prevalence of *Ae. triseriatus* adults were collected from the clearcut habitats but did not differ significantly from the control habitats. *Ae. japonicus* adults collected from the clearcut habitats were statistically greater than those collected from the shelterwood habitats, and there were more adults collected from the clearcut habitats than the sheltwood habitats but not significantly so ($p>0.05$).

Virus infection rates. Of the 1,064 pools of mosquitoes assayed for virus, one pool of three *Ae. japonicus* adults showed cytopathic effects. These mosquitoes were collected July 18th, week 35, on the BB1 clearcut habitat. An identification of the virus is not yet available.

3.4 Discussion

The invasive mosquito species, *Ae. japonicus* and *Ae. albopictus,* are rapidly expanding their range along the east coast of the United States (Farajollahi and Crans 2012, Gaspar et al. 2012, Kaufman et al. 2012, Bonizzoni et al. 2013, Rochlin et al. 2013); however, *Ae. triseriatus* was the dominant species when relative abundance of both ovitrap and gravid trap collections were compared on our sites. A previous study by Barker et al. (2003) reported *Ae. triseriatus* ovitrap abundance at 90.1% in multiple different habitats across Virginia. Our ovitrap collections in the Jefferson National Forest in 2011 yielded a higher relative abundance at 99.8%, which is similar to relative abundance as reported by Szumlas (1996) in western North Carolina at >98% *Ae. triseriatus* abundance. This result is not surprising as *Ae. triseriatus* prefers forested habitats as oviposition sites (Barker et al. 2003, Ellis 2008).

Multiple studies indicate the invasive *Ae. japonicus* and *Ae. albopictus* are effective competitors with *Ae. triseriatus* where they are sympatric and in some habitats may lead to displacement of the native species (Andreadis and Wolfe 2010, Alto 2011, Armistead et al. 2012). However, it was the relative abundance of both invasive species was relatively low at our study sites. This may be due to the close association of *Ae. albopictus* with urban areas (Hawley 1988a, Bonizzoni et al. 2013, Rochlin et al. 2013) and rock pools being the preferred oviposition site of *Ae. japonicus* (Tanaka et al. 1979, Andreadis et al. 2001). Both invasive species are thought to spread via human movements (Hawley et al. 1987, Peyton et al. 1999, Lounibos 2002) and may not have spread in sufficient numbers or fully adapted to the rural heavily forested sites. Because *Ae. triseriatus* is the primary vector of the LACV, high abundance of the species could indicate an area perfect for the natural transmission cycle of the virus.

Gravid traps have been successfully utilized in the previous studies to sample for blood-fed *Ae. japonicus* and *Ae. albopictus* (Andreadis et al. 2001, Scott et al. 2001, Falco et al. 2002, Obenauer et al. 2010); however, ovitraps may not be sufficient to estimate relative abundance of *Ae. japonicus* and *Ae. albopictus*. An *Ae. triseriatus* female will oviposit between 20-60 eggs per gonotrophic cycle as measured in the field via ovitrap (Kitron et al. 1989). In our study, the ratio of eggs collected by ovitrap to the number of adult mosquitoes sampled by gravid trap was 61.9. This ratio agrees closely with the fecundity of *Ae. triseriatus*, suggesting that either trap is effective for this species. For *Ae. japonicus* and *Ae. albopictus*, relative egg abundance collected via ovitrap was not comparable to adults collected via gravid trap. In the laboratory, the average fecundity of an *Ae. japonicus* female mosquito was approximately 114 (Oliver and Howard 2005). However, the ratio of eggs to adults in this study was only 2.88. A similar result was found for *Ae. albopictus*. One *Ae. albopictus* female mosquito will oviposit 60-80 eggs per

gonotrophic cycle depending on the blood-source (Xue et al. 2009) but the ratio of eggs to adult in the field was 2.85. The low ratios suggest that ovitraps are not a sensitive method for collecting these 2 species. This also may be due to the use of seed germination paper as an oviposition substrate. Habitat adult to egg ratios were not significantly different from the total ratios ($p>0.05$).

Previous studies have indicated *Ae. japonicus* is active earlier in the spring and later in the fall (Andreadis et al. 2001, Falco et al. 2002, Barker et al. 2003, Joy and Sullivan 2005, Grim et al. 2007). *Ae. japonicus* adult and egg collection from our sites in 2011 closely resembled *Ae. triseriatus* seasonal patterns, with populations peaking in June and July. Many of the studies on *Ae. japonicus* are conducted in northern latitudes and as in the early years of the *Ae. japonicus* invasion.

Adult *Ae. albopictus* and *Ae. albopictus* egg numbers peaked in late August, and these data share similar trends as previous studies (Barker et al. 2003, Joy 2004, Joy and Sullivan 2005). Temperate *Ae. albopictus* populations undergo photoperiodic diapause to survive cooler temperatures of the winter (Wang 1966, Hawley 1988b, Yang 1988). This is a rapidly evolving adaptation to northern climates (Urbanski et al. 2012) and populations peak late in the summer/early fall to help compensate for numbers lost during the winter.

All three species preferred control and clearcut habitats to the shelterwood. There is much less canopy in the shelterwood with much of the habitat in direct sunlight. Mosquitoes most likely avoid this area because of the risk of desiccation. Due to a lack of natural oviposition sites in the clearcut habitats, ovitraps may have had less competition.

3.5 Literature Cited

Alto, B. W. 2011. Interspecific larval competition between invasive *Aedes japonicus* and native *Aedes triseriatus* (Diptera: Culicidae) and adult longevity. J Med Entomol 48: 232-242.

Andreadis, T. G., and R. J. Wolfe. 2010. Evidence for reduction of native mosquitoes with increased expansion of invasive *Ochlerotatus japonicus japonicus* (Diptera: Culicidae) in the Northeastern United States. Journal of Medical Entomology 47: 43-52.

Andreadis, T. G., J. F. Anderson, L. E. Munstermann, R. J. Wolfe, and D. A. Florin. 2001. Discovery, distribution, and abundance of the newly introduced mosquito *Ochlerotatus japonicus* (Diptera: Culicidae) in Connecticut, USA. J Med Entomol 38: 774-779.

Armistead, J. S., N. Nishimura, J. R. Arias, and L. P. Lounibos. 2012. Community ecology of container mosquitoes (Diptera: Culicidae) in Virginia following invasion by *Aedes japonicus*. Journal of Medical Entomology 49: 1318-1327.

Balfour, H. H., Jr., and et al. 1975. Isolates of California encephalitis (La Crosse) virus from field-collected eggs and larvae of *Aedes triseriatus*: identification of the overwintering site of California encephalitis. Journal of Infectious Diseases 131: 712-716.

Barker, C. M., S. L. Paulson, S. Cantrell, and B. S. Davis. 2003. Habitat preferences and phenology of *Ochlerotatus triseriatus* and *Aedes albopictus* (Diptera: Culicidae) in southwestern Virginia. J Med Entomol 40: 403-410.

Beaty, B. J., Calisher, C. H., Shope, R. E. 1995. Arboviruses, p. 189–212 In Lennete E. T., Lennete D. A., editors. (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 7th ed. American Public Health Association, Washington, DC.

Belote, R. T., N. J. Sanders, and R. H. Jones. 2009. Disturbance alters local-regional richness relationships in Appalachian forests. Ecology 90: 2940-2947.

Belote, R. T., R. H. Jones, and T. F. Wieboldt. 2012. Compositional stability and diversity of vascular plant communities following logging disturbance in Appalachian forests. Ecol Appl 22: 502-516.

Bonizzoni, M., G. Gasperi, X. Chen, and A. A. James. 2013. The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives. Trends in Parasitology 29: 460-468.

Ellis, A. M. 2008. Linking movement and oviposition behaviour to spatial population distribution in the tree hole mosquito *Ochlerotatus triseriatus*. J Anim Ecol 77: 156-166.

Falco, R. C., T. J. Daniels, and M. C. Slamecka. 2002. Prevalence and distribution of *Ochlerotatus japonicus* (Diptera: Culicidae) in two counties in Southern New York State. Journal of Medical Entomology 39: 920-925.

Farajollahi, A., and S. C. Crans. 2012. A checklist of the mosquitoes of New Jersey with notes on established invasive species. Journal of the American Mosquito Control Association 28: 237-239.

Gaspar, J. P., T. McKay, and M. J. Huss. 2012. First report of *Aedes japonicus* in natural and artificial habitats in Northeastern Arkansas. Journal of the American Mosquito Control Association 28: 38-42.

Grim, D. C., B. T. Jackson, and S. L. Paulson. 2007. Abundance and bionomics of *Ochlerotatus j. japonicus* in two counties in southwestern Virginia. J Am Mosq Control Assoc 23: 259-263.

Haddow, A.D., C.J. Jones, and A. Odoi. 2011. Assessing risk of focal arboviral infections: Are we the big or little picture? PLoS ONE 4(9): e6954. doi:10.1371/journal.pone.0006954

Hawley, W. A. 1988. The biology of *Aedes albopictus*. . Journal of American Mosquito Control Association 4: 1-39.

Hawley, W. A., P. Reiter, R. S. Copeland, C. B. Pumpuni, and G. B. Craig, Jr. 1987. *Aedes albopictus* in North America: probable introduction in used tires from northern Asia. Science, USA 236: 1114-1116.

Homyack, J. A., and C. A. Haas. . 2009. Long-term effects of experimental forest harvesting on abundance and reproductive demography of terrestrial salamanders. Biological Conservation 142.1 110-121.

Joy, J. E. 2004. Larval mosquitoes in abandoned tire pile sites from West Virginia. J Am Mosq Control Assoc 20: 12-17.

Joy, J. E., and S. N. Sullivan. 2005. Occurrence of tire inhabiting mosquito larvae in different geographic regions of West Virginia. J Am Mosq Control Assoc 21: 380-386.

Kaufman, M. G., W. W. Stanuszek, E. A. Brouhard, R. G. Knepper, and E. D. Walker. 2012. Establishment of *Aedes japonicus japonicus* and its colonization of container habitats in Michigan. J Med Entomol 49: 1307-1317.

Kitron, U. D., D. W. Webb, and R. J. Novak. 1989. Oviposition behavior of *Aedes triseriatus* (Diptera: Culicidae): prevalence, intensity and aggregation of eggs in oviposition traps. Journal of Medical Entomology 26: 462-467.

Loor, K. A., and G. R. Defoliart. 1969. An oviposition trap for detecting the presence of *Aedes triseriatus* (Say). Mosquito News 29: 487-488 pp.

Lounibos, L. P. 2002. Invasions by insect vectors of human disease. Annual Review of Entomology 47: 233-266.

Mustermann, L. E. a. L. M. W. 1985. *Aedes triseriatus*. P. Singh R. F. Moore Handbook of insect rearing vol. 2 Elsevier Amsterdam, The Netherlands.: 15-24.

Nasci, R. S., C. G. Moore, B. J. Biggerstaff, N. A. Panella, H. Q. Liu, N. Karabatsos, B. S. Davis, and E. S. Brannon. 2000. La Crosse encephalitis virus habitat associations in Nicholas County, West Virginia. J Med Entomol 37: 559-570.

Obenauer, P. J., P. E. Kaufman, D. L. Kline, and S. A. Allan. 2010. Detection of and monitoring for *Aedes albopictus* (Diptera: Culicidae) in suburban and sylvatic habitats in North Central Florida using four sampling techniques. Environmental Entomology 39: 1608-1616.

Oliver, J., and J. J. Howard. 2005. Fecundity of naturally blood-fed *Ochlerotatus japonicus*. J Med Entomol 42: 254-259.

Pantuwatana, S., W. H. Thompson, D. M. Watts, T. M. Yuill, and R. P. Hanson. 1974. Isolation of La Crosse virus from field collected *Aedes triseriatus* larvae. American Journal of Tropical Medicine and Hygiene 23: 246-250.

Patz, J. A., P. Daszak, G. M. Tabor, A. A. Aguirre, M. Pearl, J. Epstein, N. D. Wolfe, A. M. Kilpatrick, J. Foufopoulos, D. Molyneux, and D. J. Bradley. 2004. Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence. Environ Health Perspect 112: 1092-1098.

Peyton, E. L., S. R. Campbell, T. M. Candeletti, M. Romanowski, and W. J. Crans. 1999. *Aedes* (Finlaya) *japonicus japonicus* (Theobald), a new introduction into the United States. J Am Mosq Control Assoc 15: 238-241.

Reiter, P. 1983. A portable battery-powered trap for collecting gravid Culex mosquitoes. Mosquito News 43: 496-498.

Rochlin, I., R. Gaugler, E. Williges, and A. Farajollahi. 2013. The rise of the invasives and decline of the natives: insights revealed from adult populations of container-inhabiting *Aedes* mosquitoes (Diptera: Culicidae) in temperate North America. Biological Invasions 15: 991-1003.

Sardelis, M. R., M. J. Turell, and R. G. Andre. 2002a. Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). Journal of Medical Entomology 39: 635-639.

Sardelis, M. R., D. J. Dohm, B. Pagac, R. G. Andre, and M. J. Turell. 2002b. Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). J Med Entomol 39: 480-484.

Scott, J. J., F. L. Carle, and W. J. Crans. 2001. *Ochlerotatus japonicus* collected from natural rockpools in New Jersey. Journal of the American Mosquito Control Association 17: 91-92.

Tanaka, K., K. Mizusawa, and E. S. Saugstad. 1979. A revision of the adult and larval mosquitoes of Japan (including the Ryukyu Archipelago and the Ogasawara Islands) and Korea (Diptera: Culicidae). 16: vii: 987.

Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. J Med Entomol 38: 130-134.

Urbanski, J., M. Mogi, D. O‚ÄôDonnell, M. DeCotiis, T. Toma, and P. Armbruster. 2012. Rapid Adaptive Evolution of Photoperiodic Response during Invasion and Range Expansion across a Climatic Gradient. The American Naturalist 179: 490-500.

Wang, R. L. 1966. Observations on the influence of photoperiod on egg diapause in *Aedes albopictus* Skuse. . Acta Entomologica Sinica 15: 75-77.

Watts, D. M., S. Pantuwatana, G. R. DeFoliart, T. M. Yuill, and W. H. Thompson. 1973. Transovarial transmission of LaCrosse virus (California encephalitis group) in the mosquito, *Aedes triseriatus*. Science, USA 182: 1140-1141.

Xue, R. D., D. R. Barnard, and A. Ali. 2009. Influence of multiple blood meals on gonotrophic dissociation and fecundity in *Aedes albopictus*. Journal of the American Mosquito Control Association 25: 504-507.

Yang, Z. Z. 1988. Egg diapause in the mosquito *Aedes albopictus* (Skuse) (Diptera: Culicidae). Acta Entomologica Sinica 31: 287-294.

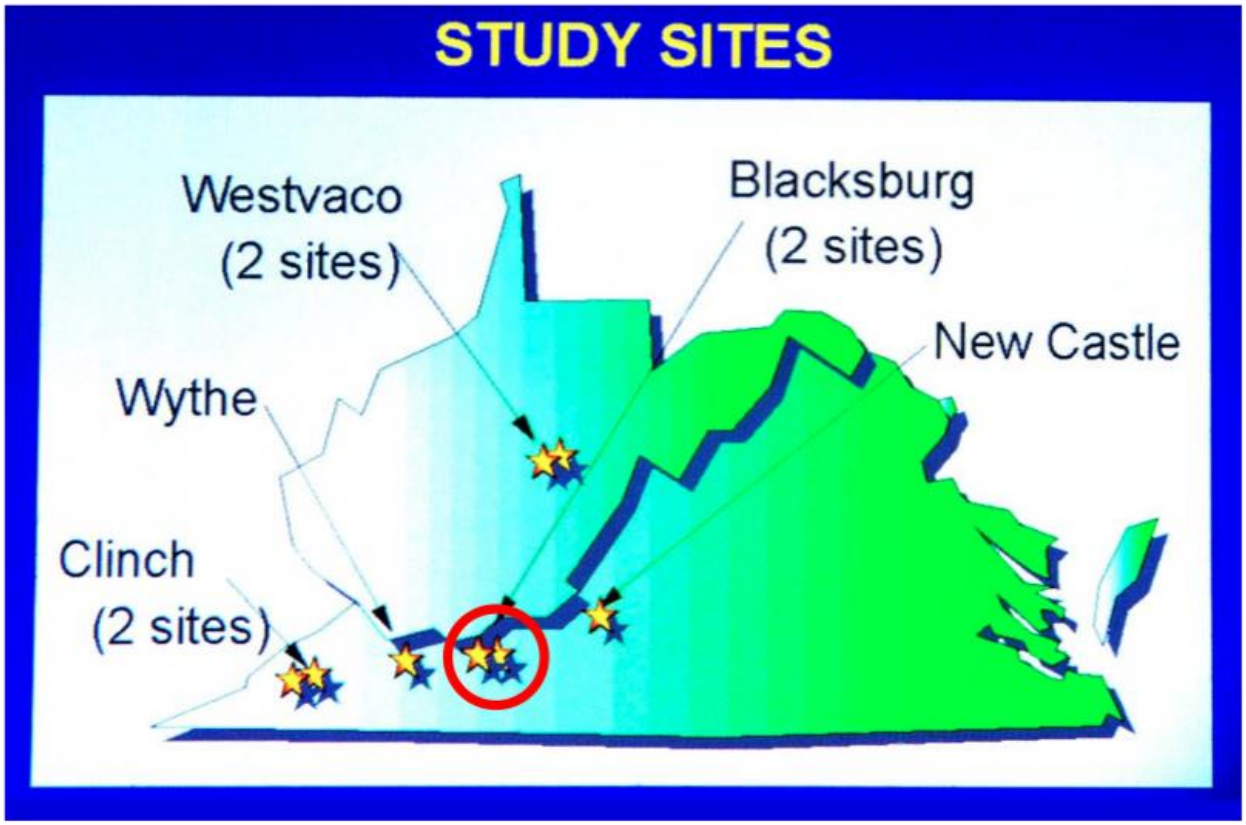

Fig. 3.1. Location of the Southern Appalachian Silviculture and Biodiversity (SASAB) study sites. BB1 and BB2 are circled.

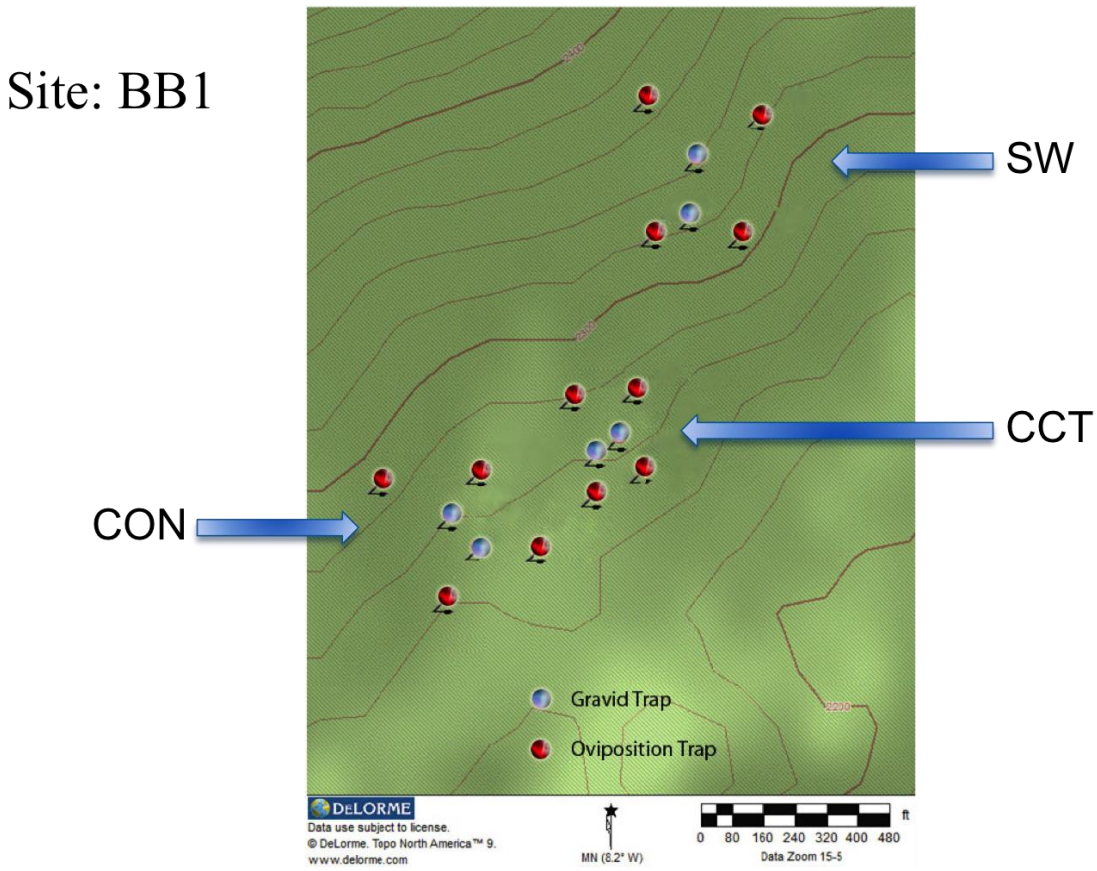

Fig. 3.2. Site BB1 with labeled trap types and locations. Adult mosquitoes and mosquito eggs were collected from Julian week 27 to 39 2011. The control habitat (CON) is undisturbed forest >80 years. The clearcut habitat (CCT) was selectively harvested for trees with a diameter >5cm. The shelterwood habitat (SW) had a complete removal of canopy vegetation in 2007.

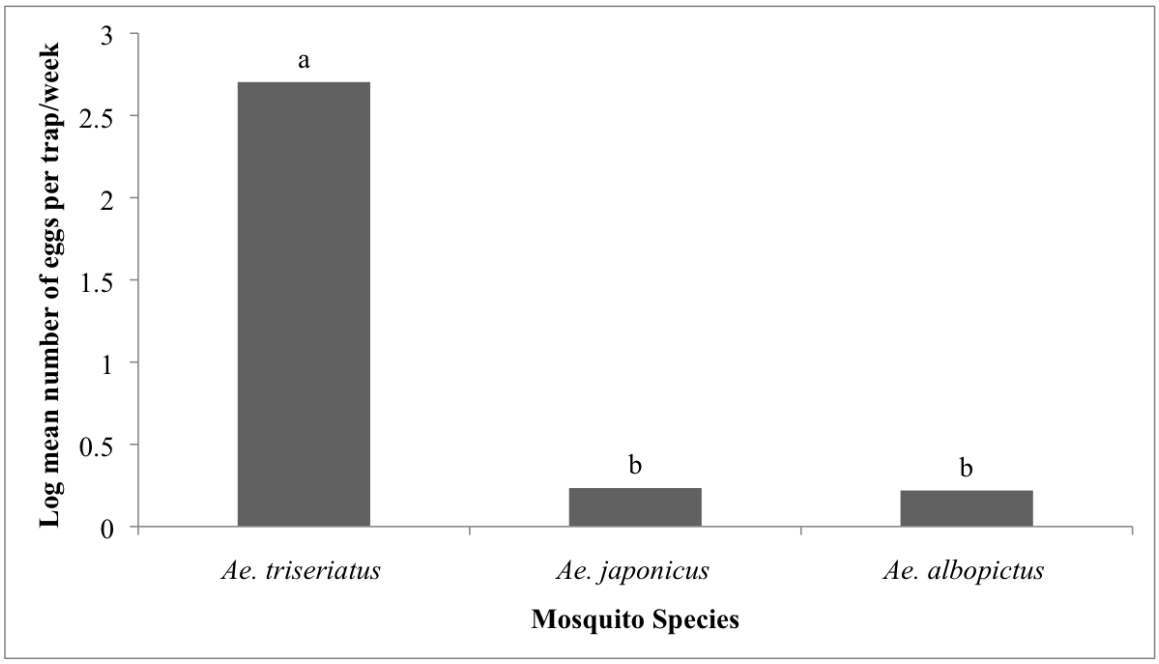

Fig. 3.3. Log mean number of eggs per trap per week of *Ae. triseriatus, Ae. japonicus*, and *Ae. albopictus*. Columns with with different letters are significantly different (p<0.001).

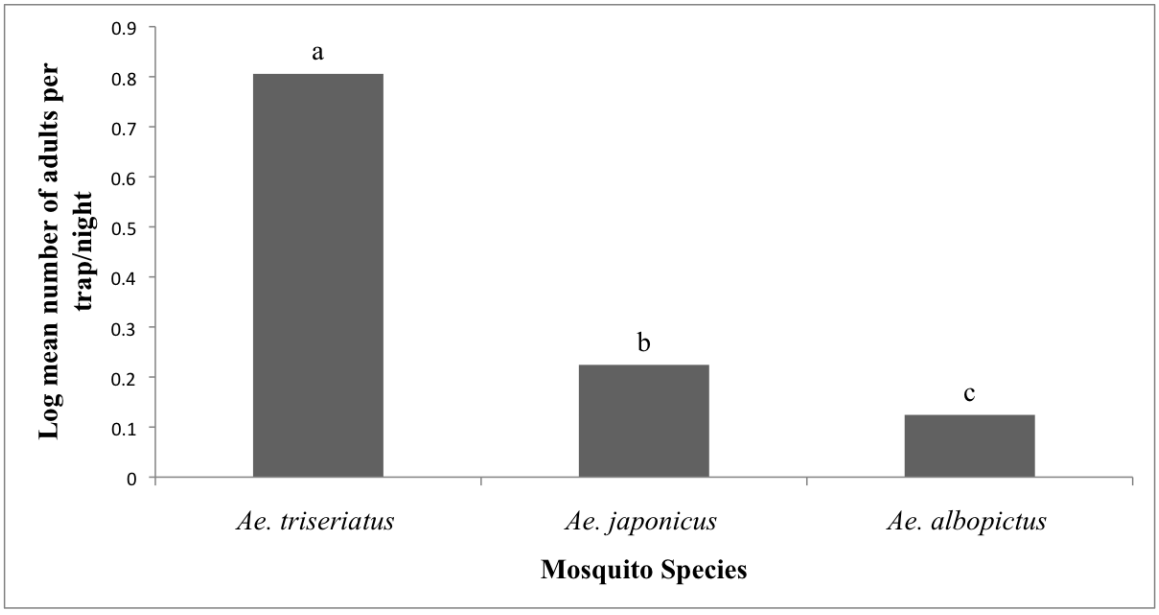

Fig. 3.4. Log mean number of adults per trap per week of *Ae. triseriatus, Ae. japonicus*, and *Ae. albopictus*. Columns with with different letters are significantly different (p<0.05).

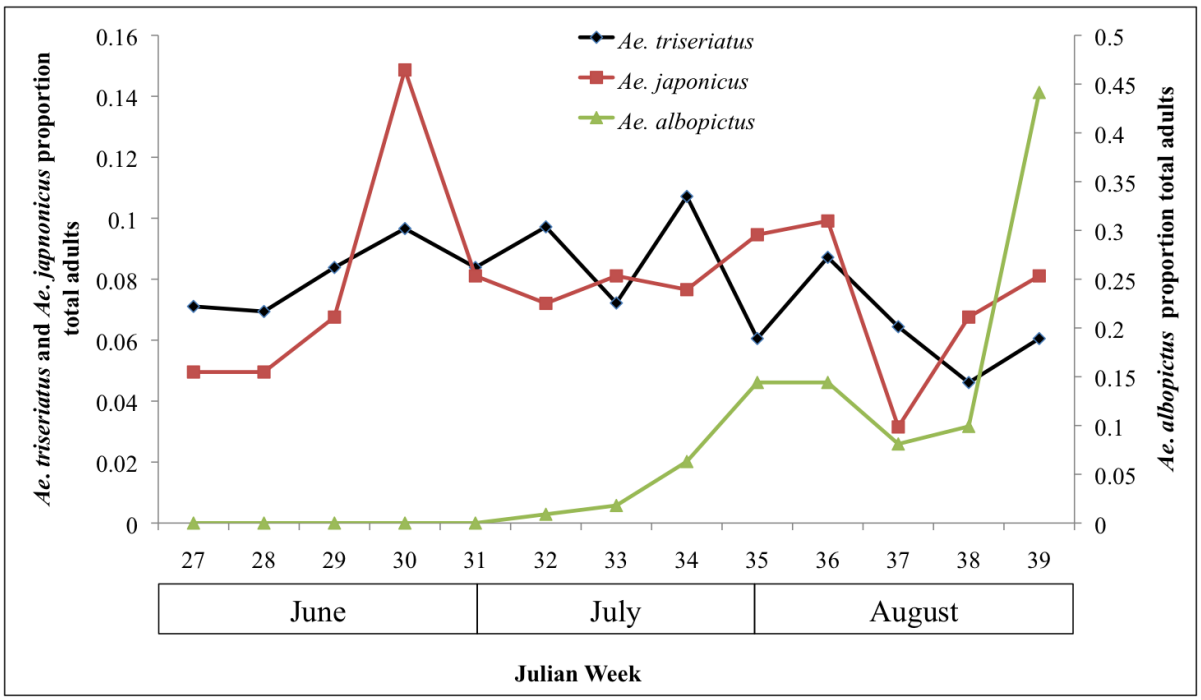

Fig. 3.5. Seasonal abundance of *Ae.* triseriatus, *Ae. japonicus*, and *Ae. albopictus* as measured by eggs.

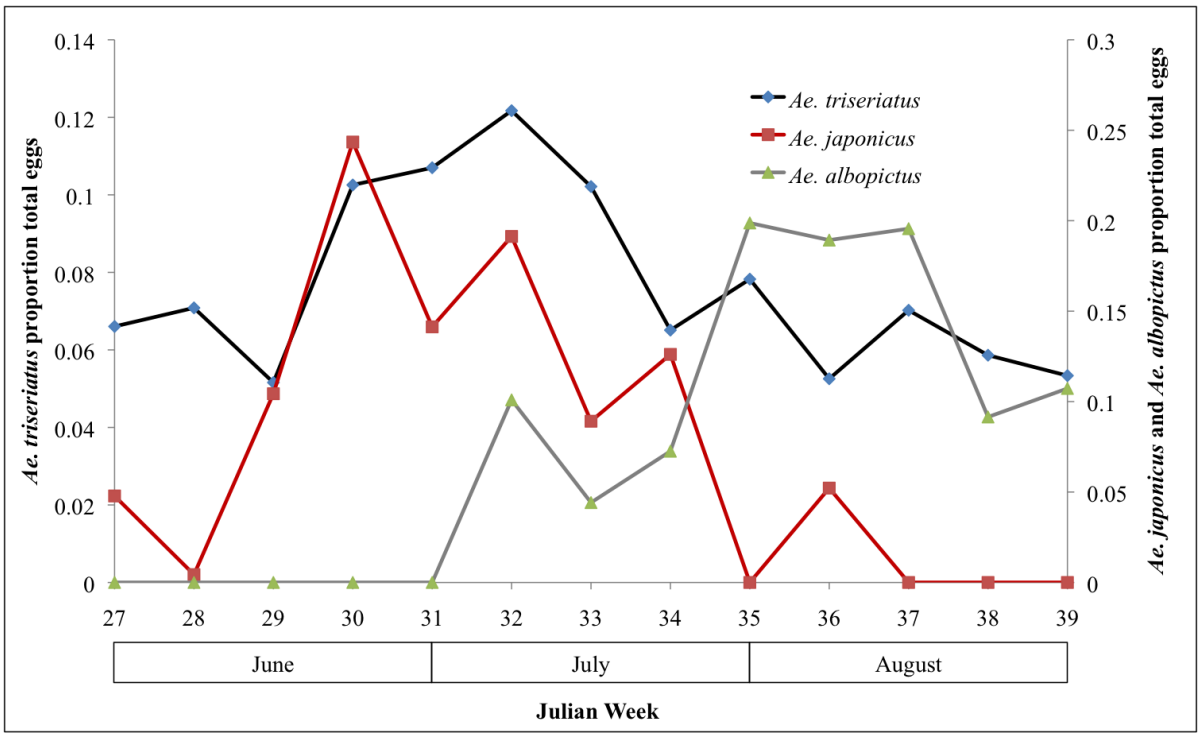

Fig. 3.6. Seasonal abundance of *Ae.* triseriatus, *Ae. japonicus*, and *Ae. albopictus* as measured by adults.

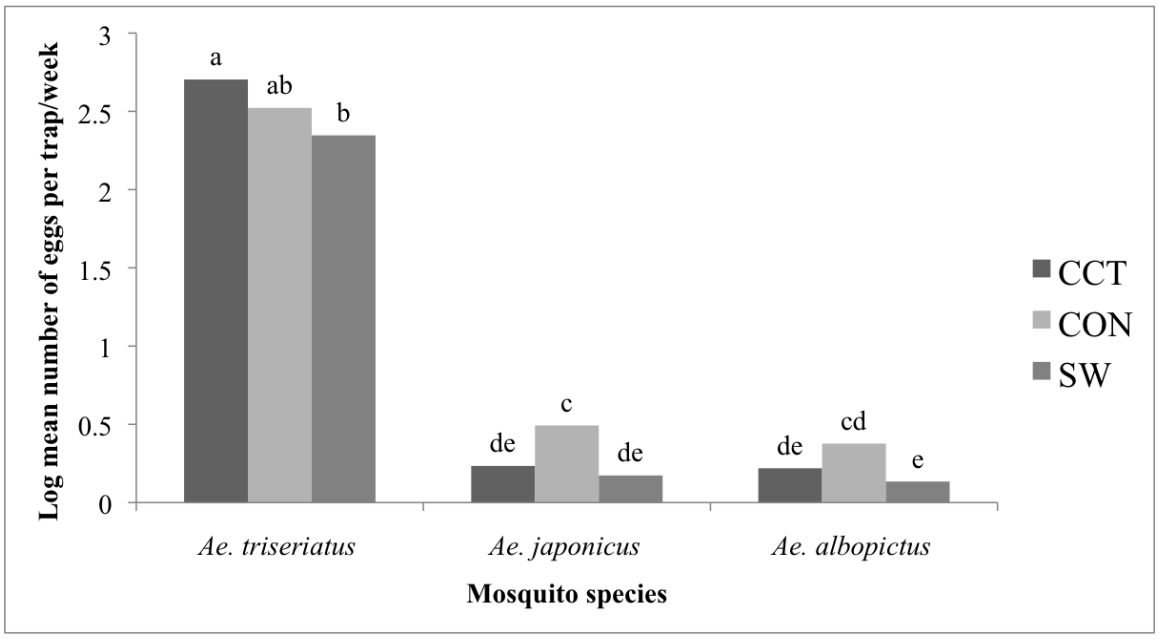

Fig. 3.7 Habitat preference as measured by eggs. Means with different letters are significantly different (p<0.001).

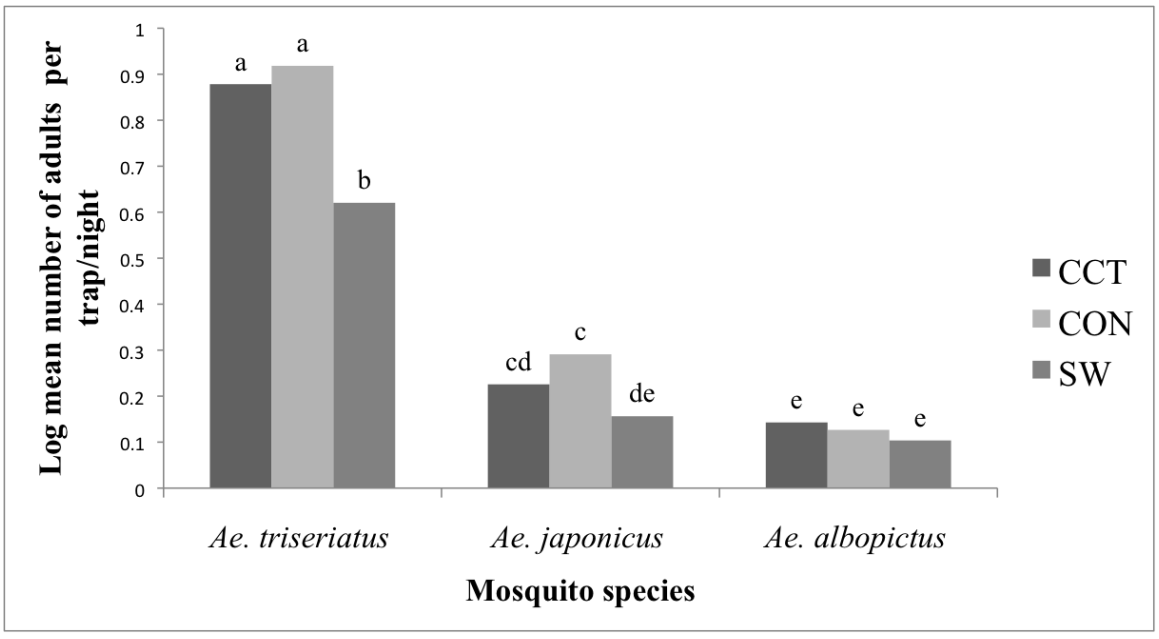

Fig. 3.8 Habitat preference as measured by adults. Means with different letters are significantly different (p<0.001).

Table 3.1. Total numbers of mosquitoes collected in ovitraps and gravid traps

Mosquito species	Ovitraps		Gravid Traps	
	# Eggs	% Total	# Adults	% Total
Ae. triseriatus	111601	99.31	1801	84.4
Ae. japonicus	460	.41	222	10.4
Ae. albopicus	317	.28	111	5.2
Total	112378	100	2134	100

Chapter 4

Description of the Diapause Egg of *Aedes albopicutus* Using Auto-Montage Technology

4.1 Introduction

Aedes albopictus (Skuse) mosquitoes are invasive in many temperate regions, and their expanding geographic range can, in part, be attributed to their efficiency at maintaining populations during unfavorable winter conditions through cold-tolerant diapause eggs (Benedict et al. 2007). These eggs are also desiccation resistant and thus are capable of surviving long periods of transport (Juliano and Lounibos 2005). The combination of desiccation resistant eggs, eggs capable of surviving temperate winters and human-aided dispersal have helped *Ae. albopictus* become nearly cosmopolitan in distribution (Lounibos 2002).

In addition to being a biting nuisance, *Ae. albopictus* mosquitoes are capable of transmitting at least 22 arboviruses, including West Nile , dengue, and La Crosse viruses, making them a major public health concern wherever they have become established (Gratz 2004). *Ae. albopictus* mosquitoes were thought to be transported over much of the world via the used tire trade and importation of lucky bamboo (Hawley et al. 1987, Scholte et al. 2008). The first breeding population in the United States was discovered in 1985 in Houston, Texas (Sprenger and Wuithiranyagool 1986). Since then, the range has expanded to include states as far north as Illinois and New Jersey and south to Florida. The ability of *Ae. albopictus* to produce diapause eggs is likely a reason it expanded its range so quickly and why the eggs survived transport (Hawley et al. 1987, Juliano and Lounibos 2005).

The native range of *Ae. albopictus* encompasses both temperate zones and tropical zones. *Ae. albopictus* populations endemic to temperate zones can produce photoperiod-induced diapause eggs while those endemic to tropical zones cannot (Hawley 1988). When the temperate population's pupae and adults are exposed to short-day (SD) lengths gravid females

will oviposit diapause eggs (Wang 1966, Mori et al. 1981). Enclosed in the chorion of the egg is a pharate larva capable of surviving increased desiccation and colder temperateures (Mori et al. 1981, Sota and Mogi 1992).

The molecular mechanism behind induction of diapause is polyphenetic and not well understood in insects. There is a multi-step process starting from a reduced critical photoperiod and resulting in the diapause phenotype; however, juvenile hormone, ecdysteroids, and insulin signaling play major roles (Sim and Denlinger 2013). Pre-diapause pharate larvae contain a greater amount of lipids than non-diapause pharate larvae and can conserve lipid uptake for a longer period of time (Reynolds et al. 2012). The resulting first instar pharate larvae housed in a diapause egg is able to conserve resources and survive the adverse weather conditions characteristic of temperate winters.

Herein we provide the first description of the external chorion of *Ae. albopictus* diapause eggs and provide color images using Auto-Montage technology.

4.2 Materials and methods

Mosquito rearing: *Aedes albopictus* mosquitoes were collected in Blacksburg, Virginia and reared at 24°C with a LD of 16:8 photoperiod cycle. First instar larvae were removed and reared at 24°C with a LD of 8:16 photoperiod cycle to induce prediapause in the adults (Hanson and Craig 1994). Three days after adult eclosion, females were allowed a blood meal from a human host. An oviposition site was provided by placing seed germination papers in 50 ml disposable beakers along with 25 ml of DI water. Seed germination papers bearing eggs were stored on moist paper towels in sealable plastic bags.

Imaging: Color images diapause non-diapause eggs were produced using aWild Photomakroskop M 400 microscope with a JVC KY-F75U 1/2" 3-CCD digital capture video camera and Syncroscopy Auto-Montage Pro imaging software (Syncroscopy, Frederick, MD). The seed germination papers containing the eggs were prepared for microscopy and imaging by completely soaking the papers then allowing them to dry for 8-10 minutes. This allows the eggs to partially dry, reducing reflection from excess water. Multiple source images were taken at different planes of focus and then the Auto-montage (AM) software creates one single in-focus image. Images were taken on the same day or day after oviposition.

Induction of diapause: After the eggs were imaged, the pharate larvae were allowed to complete development by storing them in a moist paper towel for 1week at 21 °C. They were then immersed in a 1g/L nutrient broth solution (Novak and Shroyer 1978) used as a hatching stimulus for 24 hrs. To assess viability, the unhatched eggs were then bleached with 9% sodium chlorite to dissolve the exochorion. The viable pharate larvae are visible through the transparent serosal cuticle that remains. If the pharate larvae were visible and the eggs did not react to the hatching stimulus it was concluded that they were, indeed, in diapause.

4.3 Results and Discussion

Temperate zone winters present a significant challenge to the survival of insects. Many insects surmount this challenge by entering a development arrest induced by photoperiod, i.e., diapause (Tauber et al. 1986). Photoperiodic diapause is a physiologically dynamic state rather than just a simple physiological shutdown (Denlinger 2002). Cold and desiccation resistance are key components of the diapause response (Yoder and Denlinger 1991; Benoit and Denlinger 2007; Rinehart et al. 2007). The main response of North American *Ae. albopictus* to unfavorable

winter climates has been shown to be the life history strategy of producing diapausing eggs, rather than quantitative variation in reproduction (Leisnham et al. 2011). A key component to diapause in *Ae. albopictus* is enhanced drought resistance compared to non-diapause eggs (Juliano et al. 2002; Lounibos et al. 2010).

Urbanski et al. (2010) demonstrated that diapause eggs had one-third more surface hydrocarbons and one-half the water loss rates of non-diapause eggs. This may be due to increased wax and lipids on the exterior and possibly in the serosal cuticle of the egg. Using AM technology, I have been able to show a visible difference in the diapause eggs of *Ae. albopictus* compared to non-diapause eggs. Diapause eggs have deeper chorionic cells and an overall rougher exochorion when compared to the non-diapause eggs (Fig. 1). Overall, the rough, matt black appearance of the diapause egg is more similar to the egg of *Ae. triseriatus* (see Chapter 2) than the shiny black non-diapause egg. It is interesting to note that the method of diapause is different in *Ae. triseriatus*. In this species, the female is not sensitive to photoperiod and lays only 1 type of egg. Although the environmental cue, photoperiod, is the same for both species, it is the pharate larva inside the egg that is photosensitive in *Ae. triseriatus* rather than the adult female as in *Ae. albopictus*.

In addition to providing additional information about the biology of *Ae. albopictus*, this work has an applied use as well. Using egg morphology to recognize diapause eggs can greatly enhance laboratory studies by allowing researchers to gather data without needing to wait for embryonation and hatching of the eggs, reducing time and labor.

4.4 Literature Cited

Benedict, M. Q., R. S. Levine, W. A. Hawley, and L. P. Lounibos. 2007. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne and Zoonotic Diseases 7: 76-85.

Benoit, J. B. & Denlinger, D. L. 2007. Suppression of water loss during adult diapause in the northern house mosquito Culex pipiens. J. Exp. Biol. 210, 217–226.

Gratz, N. G. 2004. Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology 18: 215-227.

Hanson, S. M., and G. B. Craig, Jr. 1994. Cold acclimation, diapause, and geographic origin affect cold hardiness in eggs of *Aedes albopictus* (Diptera: Culicidae). J Med Entomo.l 31: 192-201.

Hawley, W. A. 1988. The biology of *Aedes albopictus*. 4: 1-40.

Hawley, W. A., P. Reiter, R. S. Copeland, C. B. Pumpuni, and G. B. Craig, Jr. 1987. *Aedes albopictus* in North America: probable introduction in used tires from northern Asia. Science, USA 236: 1114-1116.

Juliano, S. A., and L. P. Lounibos. 2005. Ecology of invasive mosquitoes: effects on resident species and on human health. Ecology Letters 8: 558-574.

P. T. Leisnham , L. Towler and S. A. Juliano. 2011. Geographic variation of photoperiodic diapause but not adult survival or reproduction of the invasive mosquito *Aedes albopictus* (Diptera: Culicidae) in North America. Ann Entomol Soc Am104(6):1309-1318.

Lounibos LP, O'Meara GF, Juliano SA, Nishimura N, Escher RL, Reiskind MH, Cutwa MM, Greene K. 2010. Differential survivorship of invasive mosquito species in south Florida cemeteries: do site-specific microclimates explain patterns of coexistence and exclusion? Ann Entomol Soc Am. 2010;103:754–770.

Mori, A., T. Oda, and Y. Wada. 1981. Studies on the egg diapause and overwintering of *Aedes albopictus* in Nagasaki. Tropical Medicine 23: 79-90.

Novak, R.J. & Shroyer. D.A. 1978. Eggs of Aedes triserialus and Aedes hendersoni: & method to stimulate optimal hatch. Mosquito News, 38, 515-521.

Scholte, E. J., E. Dijkstra, H. Blok, A. d. Vries, W. Takken, A. Hofhuis, M. Koopmans, A. d. Boer, and C. B. E. M. Reusken. 2008. Accidental importation of the mosquito *Aedes*

albopictus into the Netherlands: a survey of mosquito distribution and the presence of dengue virus. Medical and Veterinary Entomology 22: 352-358.

Sim, C., and D. L. Denlinger. 2013. Insulin signaling and the regulation of insect diapause. Front Physiol 4: 189.

Sota, T., and M. Mogi. 1992. Survival time and resistance to desiccation of diapause and non-diapause eggs of temperate *Aedes* (*Stegomyia*) mosquitoes. Entomologia Experimentalis et Applicata 63: 155-161.

Sprenger, D., and T. Wuithiranyagool. 1986. The discovery and distribution of *Aedes albopictus* in Harris County, Texas. Journal of the American Mosquito Control Association 2: 217-219.

Tauber, M. J., Tauber, C. A. & Masaki, S. 1986. Seasonal adaptations of insects. New York, NY: Oxford University Press.

Urbanski, J. M., J. B. Benoit, M. R. Michaud, D. L. Denlinger, and P. Armbruster. 2010. The molecular physiology of increased egg desiccation resistance during diapause in the invasive mosquito, *Aedes albopictus*. Proc Biol Sci 277: 2683-2692.

Wang, R. L. 1966. Observations on the influence of photoperiod on egg diapause in *Aedes albopictus* Skuse. . Acta Entomologica Sinica 15: 75-77.

Yoder, J. A. & Denlinger, D. L. 1991. Water balance in flesh fly pupae and water absorption associated with diapause. J. Exp. Biol. 157, 273–286.

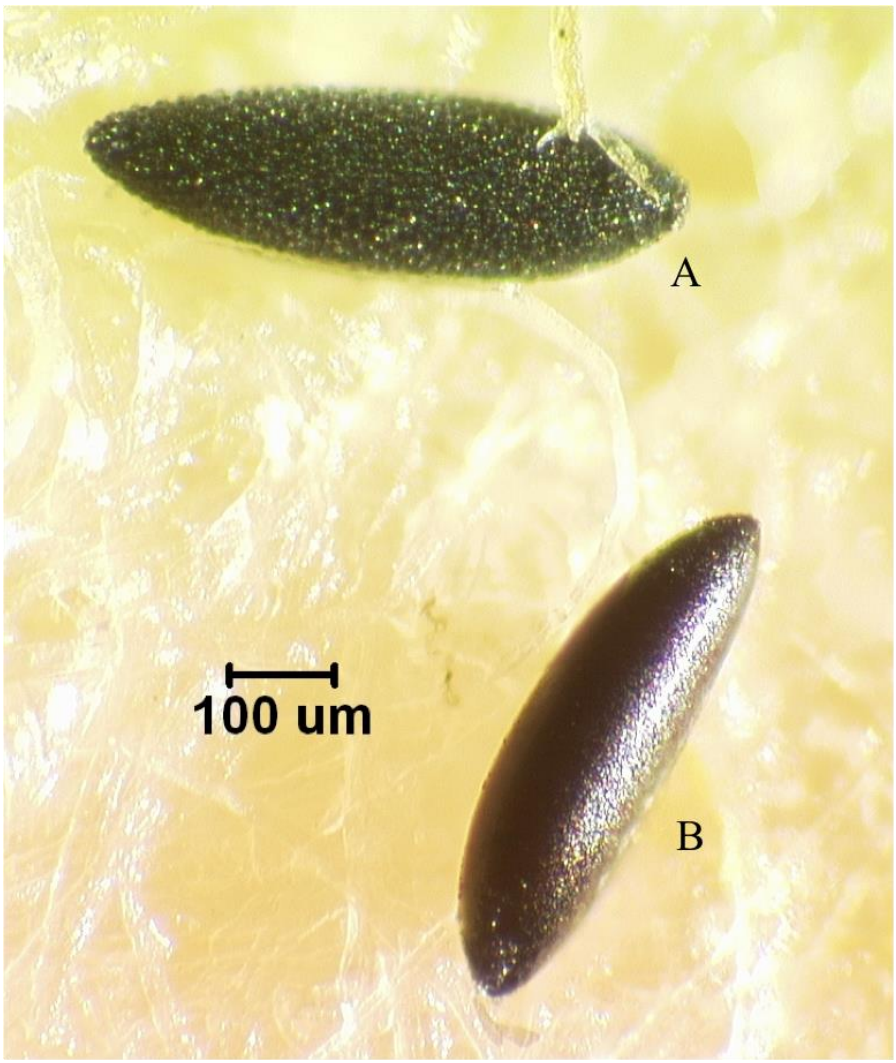

Fig. 4.1. Auto-montage image of diapause (A) and non-diapause (B) eggs of *Ae. albopictus.*