Overwintering Mechanisms of La Crosse Virus Vectors

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> Doctor of Philosophy In Entomology

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#### **Abstract (Academic)**

The La Crosse virus (LACV) is an emerging pathogen in the Appalachian region of the United States. The virus maintains a complex natural cycle through horizontal transmission with sciurid rodents and Aedes mosquitoes in Virginia. Transovarial transmission also occurs in this host-parasite system and has evolved for the virus to persist through winter. The virus perpetuates in diapause induced embryos and infects naive rodents the following spring. As global temperatures rise, it is imperative we evaluate how the virus and its vectors overwinter. We conducted experiments to evaluate and determine the diapause induction, or prediapause stage, in Aedes japonicus japonicus, and the ecophysiology and low temperature biology of how LACV affects Aedes triseriatus and Aedes albopictus embryos at low temperatures. We found that the prediapause stage of Ae. j. japonicus is the maternal stage, the developing adult female that lays diapause eggs. This more closely resembles Ae. albopictus and not Ae. triseriatus. As measured in the field and laboratory, LACV has a clear negative effect on the ability of Ae. triseriatus and Ae. albopictus to survive the winter. There was no major effect of LACV infection on the ability of these two species to enter diapause or their critical photoperiods, but there was a significant negative effect of LACV infection on survivorship of embryos that were placed in their natural habitat and in their susceptibility to low temperatures. LACV infection had a more significant negative effect on Ae. albopictus than on Ae. triseriatus and suggests that Ae. albopictus is a subordinate vector relative to Ae. triseriatus in the maintenance of the virus over the winter season. Our findings highlight the need for the increased surveillance of LACV as temperatures continue to rise due to climate change.

#### **Abstract (Public)**

In Virginia, mosquitoes may transmit the La Crosse virus (LACV) that can cause swelling of the brain in humans. This virus infects mosquitoes which is then transmitted to chipmunks. When a mosquito bites an infected chipmunk and then bites a human, the virus be transmitted to the human. Adult female mosquitoes can also pass the virus on to their developing offspring, which is the way it is maintained throughout the winter. Certain infected mosquitoes will spend the winter as eggs and the mosquitoes that hatch carry the virus and can infect chipmunks the following spring. The experiments conducted here looked at how one mosquito that can spread the LACV begins the process to survive the winter. We also did experiments to look at how the virus affects two other mosquito's ability to begin the same process and then if they can survive the winter and cold temperatures while infected. We found that the Asian Bush Mosquito enters diapause in a different way than we suspected by the female mosquito sensing shorter days at the end of the summer; at this point she will then lay eggs that survive the winter. We discovered that viral infection will hurt the chances of the Asian Tiger Mosquito and Eastern Tree-hole Mosquito to survive the winter. We also found that virus-infected mosquitoes cannot survive temperatures as lower than those that are not infected. This means that the risk of the virus to humans can be greater as temperatures rise due to global warming.

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#### Chapter 1

#### **Introduction and Literature Review**

Mosquitoes (Culicidae) are true flies capable of spreading debilitating diseases to humans, and their presence and associated pathogens have undoubtedly altered human history in immeasurable ways. Naive populations of humans, from colonists to armies, introduced to new tropical and subtropical regions have been disproportionately affected. Some scholars even largely credit mosquito-borne diseases to the formation of certain countries such as Haiti and Venezuela because of the large disease risk to armies needed to retain control of colonies, and they were an important factor in the American slave-trade of 1600s (McNeill 2010). Native western and central Africans were resistant to human malaria, a single-celled Plasmodium parasite spread by Anopheles mosquitoes that has been a major cause of human mortality. While this in and of itself did not foreordain or excuse the horrific nature of slavery in America, it did help establish the plantation-slavery model of the southern United States (Mann 2011). Because of the commerce of goods and humans, stowaway populations of African-native Aedes aegypti were introduced to the Americas. This mosquito, commonly called the Yellow Fever mosquito, brought viruses like dengue and yellow fever out of the jungles of Africa to new locations in the western hemisphere (Arnebeck 2008, Dick et al. 2012). These mosquito-borne viruses were common in the tropics, but outbreaks of yellow fever occurred as far north as Philadelphia, Pennsylvania in 1793 and Wilmington, North Carolina in 1862. Uunlike malaria, no human populations had any significant immunity to these new viruses (Moreno-Madriñá and Turell 2018).

In the late 1890's United States Army Medical Officer Walter Reed and Cuban Physician Carlos Finlay experimented and showed that *Ae. aegypti* was the vector of the yellow fever virus that was plaguing the construction of the Panama Canal (Reed 1911). Around the same time, Sir Ronald Ross a British medical doctor discovered *Anopheles* mosquitoes vectored malaria (Ross 1911). With this information, malaria was subsequently eliminated from the continental United States over the next several decades (CDC 2018) and a major reduction in mosquito-borne viruses occurred in more temperate climates. Viruses like the yellow fever virus and the dengue virus are now primarily restricted to tropical regions around the globe that support populations of *Ae. aegypti*, but more poleward encroachment is now a very real risk due to climate change.

As major well-studied anthropogenic viruses like dengue virus and yellow fever virus continue to stymie mitigation efforts in the tropics, a more temperate zoonotic virus, the La Crosse virus (LACV; family Bunyaviridae, genus Orthobunyavirus), is now the second most commonly reported mosquito-borne viruses in Virginia after West Nile virus (CDC 2018). The *Aedes*-vectored LACV which relies on complex natural cycle involving vertebrate reservoir hosts and accidental infection of humans can cause neurological sequelae and even death (Calisher 1994, Rust et al 1999). The overwintering mechanisms of this virus is of utmost importance in this region as temperatures rise in order to predict future dynamics of its disease ecology (Moulton and Thompson 1971, Gauld et al. 1974, Watts et al. 1974, Beaty and Thompson 1975, Miller et al. 1977, Thompson and Beaty 1977, Borucki et al. 2002, Hughes et al. 2006). As such, the research found herein focuses on winter hibernal diapause of accessory vectors and how LACV effects the ecophysiology and low temperature biology of the main vector and invasive, competent vectors established in this area.

The LACV was originally isolated from the brain tissue of a deceased four-year-old girl in La Crosse, Wisconsin in 1964 (Thompson et al. 1965). After its identification, a rapid elucidation of the viral natural cycle occurred. Humans are considered tangential or dead-end hosts and infections predominately occur in children younger than 15 years of age. The male to female ratio of humans infected is skewed toward males and may possibly indicate a higher rate of infected-mosquito exposure in boys (McJunkin et al., 1998, 2001). The main vector is the mosquito *Aedes triseriatus* (Say). The invasive mosquitoes *Aedes albopictus* and *Aedes japonicus japonicus* have emerged as accessory vectors in their sympatric ranges (Westby 2015, Harris et al. 2015, Bara et al. 2016).

Sciurid rodents have been suggested as the main reservoir hosts of LACV and the virus has been isolated from squirrels (*Sciurus carolinensis and Sciurus.niger*) and Eastern chipmunks (*Tamias striatus*). In addition to rodents, the Red fox (*Vulpes vulpes*) has also yielded LACV infected samples. Infections of naive chipmunks peak in late summer and early autumn and new infections cease upon the beginning of winter which indicates the lack of chronic infection and absence of overwintering maintenance of the virus in mammalian reservoir hosts (Moulton and Thompson, 1977, Issel et al., 1972; Gauld et al. 1975, Amundson and Yuill 1981, Amundson et al. 1985). Neutralizing antibodies to LACV and Jamestown Canyon virus, a closely related California virus, were identified in White-tailed deer (*Odocoileus virginianus*) and the Northern racoon (*Procyon lotor*) (Issel et al. 1972b, Amundson and Yuill 1981, Murphy 1989).

Amplification of LACV occurs via three transmission processes. Vertical or transovarial transmission (1) occurs when the LACV infects gravid female ovaries and some of the following progeny are infected. Transovarially-infected males can also sexually transmit (2) LACV to uninfected females. Horizontal transmission (3) is where a susceptible female mosquito feeds

upon a reservoir host with a sufficiently high viremia. Overwintering of LACV relies on transovarially infected *Aedes* embryos. In temperate areas *Aedes* mosquitoes primarily overwinter as pharate, first instar larvae within eggs. Field-collected *Ae. triseriatus, Ae. albopictus,* and *Ae. j. japonicus* larvae have tested positive for LACV (Westby et al. 2015) and infected mosquitoes that successfully survive winter temperatures can then transmit the virus to naïve reservoirs the following spring, therefore maintaining the virus.

The LACV is a negative-sense RNA virus with a tripartite genome. The three segments are the small which codes for nonstructural proteins, the medium which codes for G1 and G2 glycoproteins, and the large segment which codes for the polymerase. They were named due size of the nucleotide sequences of each segment. Transcription occurs in the cytoplasm and G1 and G2 glycosylation is initiated in the endoplasmic reticulum and terminates along with virus maturation in the Golgi apparatus (Elliot 1990, Bishop 1996).

Since the 1990's, Appalachia has emerged as a geographic area of concern for LACV, with the majority of human cases now being reported from this area (CDC 2018). This eastern shift in cases from the midwestern states coincided with the introduction of the invasive accessory vectors *Ae. albopictus* and *Ae. j. japonicus* (Jones et al. 1999, 2000, Nasci et al. 2000, Erwin et al. 2002, Lambert et al. 2010, Westby et al. 2014, Harris et al. 2015). Bewick et al. (2016) rejected the hypothesis that *Ae. albopictus* is responsible for the emergence of LACV in Appalachia through the use of epidemiological models. They suggest other factors including *Ae. j. japonicus*, climate change, and changes in reservoirs and wildlife as possible alternatives to the changes in the LACV disease dynamics. Though this model suggests other causative factors than *Ae. albopictus*, further data on this species and *Ae. j. japonicus* is needed. There is also a dearth of information on how these species impact the overwintering ecology of LACV.

#### A note on nomenclature:

Relatively recent revisions to the *Aedes* genus have occurred (Reinert et al. 2004, 2006, 2008, 2009) and there may be several different names for a species that are all used interchangeably in the literature depending on the laboratory in which the research was conducted or the journal in which it was published. In Clements (2013) the author provides an excellent justification of the new nomenclature; however, I used herein the nomenclature provided by the Walter Reed Biosystematics Unit. This unit agrees with the conclusion generated from the revised phylogenies for the tribe Aedini but argue utility and stability of prior nomenclature is more important to the public health working community (Wilkerson et al. 2015). Nevertheless, they criticize Reinert et al. (2009) for solely using morphology. The most recent molecular phylogeny can be found in Soghigian et al. (2017) showing that the *Aedes* genus is undoubtedly not monophyletic, but no proposed changes to nomenclature were made.

#### **1.1** Aedes triseriatus (= Ochlerotatus triseriatus)

The Eastern Tree-hole mosquito *Ae. triseriatus* (Say) is the most important vector of LACV in the North American *Aedes (Protomacleaya)* Triseriatus Group. This species group consists of four species: *Ae. triseriatus, Aedes hendersoni, Aedes brelandi,* and *Aedes zoosophus* (Zavortink 1972, Taylor 1990, Munsterman et al. 1982, Soghigian et al. 2017). These mosquitoes all have a black or brown thorax with a dark dorsal stripe and silver-white lateral scales and white lateral abdominal scales (Ward 2005). As the common name suggests, they exploit natural hardwood phytotelmata for oviposition and aquatic larval development. Most areas that report a high incidence of LACV contain hickory, oak, and maple trees, all of which provide the needed

microhabitat for these mosquito species to complete their lifecycles (Woodring et al. 1996). Although they are found primarily in heavily wooded areas, these mosquitoes regularly come in contact with humans (DeFoliart and Lisitza 1980). Where available, *Ae. triseriatus* also exploits artificial containers such as car tires (Thompson, 1983b, Nasci et al., 2000), and *Ae. triseriatus* and *Ae. hendersoni* are sympatric in much their North American range; however, *Ae. hendersoni* extends further westward whereas *Ae. triseriatus* populations are limited east of 100<sup>th</sup> meridian. Interestingly, *Ae. hendersoni* exhibits a salivary gland escape barrier, rendering this species an inconsequential vector of LACV (Paulson and Grimstad 1989). Distributions of these species in the United States can be found in studies by Ward (2005) and Farajollahi and Price (2012).

The main vector of the LACV is *Ae. triseriatus* and this was determined as such by identification of infected individuals in the field (Thompson et al. 1972, Berry et al. 1974, Pantuwatana et al. 1974, Balfour et al. 1975), its blood-feeding preference on amplifying reservoirs (Wright and DeFoliart 1970), population ranges that coincide with reported human cases (Berry et al. 1975, Balfour et al. 1976, Barker et al. 2003), and the evidence of vector competence in the laboratory (Bara et al 2016).

#### 1.2 Aedes albopictus; Synonym: Stegomyia albopicta

The Asian tiger mosquito *Aedes albopictus* (Skuse) is probably the most successful invasive mosquito species in the world and is capable of transmitting several diseases to humans, including the LACV (Bennedict 2007, Gratz 2004). Adults can be identified by a single white dorsal band on the thorax and white banding on the legs. This mosquito is native to Asia and was first identified in the continental United States in the mid 1980s. It was likely introduced in used tires (Hawley et al 1987), and since its introduction, human driven spread occurred (Moore and

Mitchell 1997), and *Ae. albopictus* has been collected in 34 states including the District of Columbia (Hahn et al. 2016). The key to the invasion success largely lies with rapid adaptation of diapausing populations in new areas and their range continues to expand (Urbanski et al. 2012).

Remarkably, laboratory studies indicate *Ae. albopictus* is a competent vector of at least 22 known arboviruses (Gratz 2004); however, their role in outbreaks and natural cycles of these viruses remains ambiguous. Most commonly considered an accessory vector, *Ae. albopictus* has been the main vector of a few outbreaks of chikungunya virus (Rezza et al. 2007, Tsetsarkin et al. 2007, de Lamballerie et al 2008, Grandadam et al 2011). Females have an aggressive daytime feeding behavior, targeting a number of different mammal species (Sullivan et al., 1971), but in urban and suburban areas, they can be highly anthropophilic (Faraji et al. 2014). Faraji et al. (2014) collected blooded *Ae. albopictus* via Biogents Sentinel traps and were able to identify 165 sources of vertebrate blood. Human bloodmeals accounted for 95%. Because of the outbreaks of arboviruses driven by *Ae. albopictus* and their preference for human blood in urban and suburban areas. Faraji et al. (2014) conclude the risk of *Ae. albopictus* being capable of initiating and maintaining an arbovirus outbreak in the United States is more than hypothetical. Field-collected *Ae. albopictus* have tested positive for LACV (Westby et al. 2015) and have been shown to be highly efficient vectors for this virus in the laboratory (Bara et al. 2016)

# 1.3 Aedes japonicus japonicus (= Ochlerotatus japonicus japonicus and Hulecoeteomyia japonica japonica)

The Asian rock pool or Asian bush mosquito *Aedes japonicus japonicus* (Theobald) is an invasive mosquito in the eastern United States and is competent vector of LACV in much of the

regions where the majority of human LACV cases are reported. It is part of a species group consisting of four subspecies: *Ae. j. japonicus, Aedes japonicus yaeyamensis, Ae. japonicus shintienensis* and *Aedes japonicus amamiensis*. There is very little morphological differentiation between the subspecies, and adult mosquitoes can be identified by white banding on their legs and a golden lyre shape on the dorsal side of their dark brown-black thorax. Some of these subspecies' ranges are expanding geographically but the most successful and widespread subspecies is *Ae. j. japonicus* (Kaufman et al. 2014). In 1998, the first reported collections of *Ae. j. japonicus* in the US came from New Jersey, New York, and Connecticut (Peyton et al. 1999, Munsterman and Andreadis 1999). However, additional reports were soon made due to enhanced trapping meant to capture *Culex* mosquitoes in initial phases of the United States West Nile virus epidemic of the late 1990s and early 2000's. Here, gravid traps were widely used and, fortuitously, these traps are very effective for trapping *Ae. j. japonicus* (Scott 2001). Since the discovery of *Ae. j. japonicus* in the northeastern United States, populations rapidly spread north to eastern Canada and south to Florida (Kaufman et al. 2014, Riles 2017).

There has been one Japanese encephalitis virus outbreak in which *Ae j. japonicus* was implicated as the main vector (Takashimi and Rosen 1989), but otherwise it is not considered a major vector of human pathogens (Kaufman et al. 2014). Despite this attribution, LACV infected individuals of *Ae. j. japonicus* have been identified in Appalachia (Westby et al. 2015, Harris et al. 2015) and lab studies confirmed its vector competence in the early 2000s (Sardelis 2002). Bara et al. (2016) orally challenged *Ae. j. japonicus*, *Ae. triseriatus*, *Ae. albopictus*, and *Ae. aegypti* with LACV and found *Ae. j. japonicus* to have the highest effective vector competence value. Surprisingly, *Ae. triseriatus* received the lowest vector value, which questions traditional

knowledge about this disease system. However, better information and more studies are needed to expound upon the epidemiology of this virus.

#### 1.4 Mosquito diapause and cold tolerance

Mosquito diapause is a growing area of interest to the entomological and public health communities and is critical to understand how these important vectors are reacting to a changing and warming climate. Detailed reviews on the subject have recently been published by Denlinger and Armbruster (2014, 2016); however, there remains critical gaps in our knowledge of different mosquito vectors in temperate areas.

Facultative diapause is a preprogramed state of dormancy that is hormonally regulated and causes the organisms metabolism and development to slow or even stop. In insects, it is often induced by shorter photoperiods in the autumn that are the harbingers of cold winter temperatures. Diapause differs from quiescence because of preprograming. An example in *Aedes* mosquitoes is when non-diapause eggs are glued above the waterline of a water source, the eggs are in a quiescent state and will not hatch until submerged into the favorable aquatic habitat. In contrast, diapause eggs will not hatch until they have undergone a variable duration of cold temperatures and photoperiod regardless of an immediate favorable environment like rising water. Another state of dormancy commonly utilized by mosquitoes is aestivation that in mosquito systems has become the term attributed to the similar hibernal process to bridge dry seasons instead of winter temperatures (Denlinger and Armbruster 2014, 2016).

There are two main diapause phases. The prediapause phase is sensitive to the environmental cues that induce diapause and the actual diapause phase. *Aedes* mosquitoes primarily utilize their egg life-stage as their diapause phase, or more specifically, the embryo

surrounded by the desiccation resistant chorion of the mosquito egg shell. In *Ae. triseriatus* the prediapause phase and diapause phase are the same, the embryo (Kappus and Venard 1967). This prediapause strategy is utilized by several different species mosquito species especially in more northern latitudes in North America. In contrast, in *Ae. albopictus* it is the maternal generation that is the prediapause stage (Mori et al. 1981). The prediapause stage is discussed further in Chapter 2.

Induction of diapause is often studied by evaluating the critical photoperiod (CPP) of a given population. The CPP is the photoperiod needed to induce diapause in half of a given population. An insect's CPP typically increases the more poleward a given population is located, thus triggering hibernal diapause earlier in the shorter growing season that coincides with longer winters (Andrewartha 1952; Danilevskii 1965; Sims 1982, Shroyer and Craig 1983, Tauber et al. 1986, Urbanski et al. 2012). A detailed study by Urbanksi et al. (2012) compared populations of *Ae. albopictus* at different latitudes in 2010 to those in 1989. There was a rapid evolution of diapause in this species that coincided with its northward invasion.

Along with diapause, how mosquitoes survive and react to cold temperatures is a significant aspect in understanding their overwintering ecology and ecophysiology and has significant implications for studying pathogen and disease transmission. *Aedes* mosquito embryos can regularly survive subfreezing temperatures, and diapause induced embryos are even more cold-tolerant. Cold acclimation plays an important physiological role in cold tolerance as well in *Ae. albopictus* and *Ae. triseriatus* (Hanson and Craig 1994 and 1995, Copeland and Craig 1990, Thomas et al. 2012). The LACV has been shown to reduce overwintering survival of *Ae. triseriatus* in the field as well (McGraw et al. 1998).

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#### Chapter 2

## The Prediapause Stage of *Aedes japonicus japonicus* and the Evolution of Embryonic Diapause in the Genus *Aedes*

#### 2.1 Abstract

*Aedes japonicus japonicus* is an invasive mosquito in the United States with very little known of its overwintering ecology and basic biology. The importance of this mosquito in disease systems like La Crosse virus remain uncertain. Embryonic diapause is used by *Ae. j. japonicus* to survive temperate winters, and persistence of this species in the Appalachian region is a result of overwintering has important implications for transmission of this virus to humans. The objective of this study was to identify the prediapause stage, or the stage sensitive to environmental cues needed to induce diapause in this mosquito. By exposing each *Ae. j. japonicus* life-stage independently to short-day photoperiods, we determined that the maternal life-stage is the prediapause stage. Using the most recent phylogeny and prior literature on the prediapause stages of *Aedes* mosquitoes that overwinter as eggs. We hypothesized the embryonic prediapause stage evolved from more poleward species that also undergo obligate diapause, while maternal prediapause stage may be the ancestral form due to distantly related species utilizing this method.

#### **2.2 Introduction**

Many mosquitoes are faced with adverse weather conditions depending on their geographic range, and a major survival strategy is to enter into a hibernal diapause to shield themselves from low temperature extremes. Multivoltine, temperate mosquitoes utilize a facultative diapause usually driven by shorter photoperiods and temperature. Most temperate

*Aedes* mosquitoes overwinter as the pharate first instar within their egg, but the life-cycle stage that is sensitive to environmental cues that induce diapause, referred to as the prediapause stage, can differ between *Aedes* species. Either the developing embryo or maternal generation is sensitive to light and temperature to induce embryonic diapause (Fig 1) (Armbruster and Denlinger 2014, Denlinger and Armbruster 2016, Diniz et al. 2017).

*Aedes japonicus japonicus* is an invasive mosquito in the United States and is capable of vectoring several viruses (Kaufman and Fonseca 2014). This mosquito is a cold-adapted species and has been known to overwinter predominantly as eggs, but some overwintering larvae have been identified (Andreadis et al. 2001). In the Appalachia region, La Crosse virus and Cache Valley virus infected pools of *Ae. j. japonicus* have been collected (Westby 2015, Harris 2015, Yang 2018). Its invasion biology is similar to that of the Asian tiger mosquito *Ae. albopictus*. While a common mosquito in the eastern United States, much remains to be discovered about its basic biology and its increasingly pertinent vector status makes this imperative to understanding future disease emergence related to this species.

The goal of this study was to identify the prediapause stage of *Ae. j. japonicus* to better understand the evolution of the diapause system by mapping the known phylogeny for the prediapause stage of *Aedes* mosquitoes that undergo embryonic diapause. Previous discussion on the evolution of diapause has centered around the diapause stage (Denlinger and Armbruster 2014); however, we show here the prediapause phenotype should be considered when evaluating the evolution of embryonic diapause in *Aedes spp*.

#### 2.3 Materials and Methods

The *Ae. j. japonicus* used in this study were collected using modified Reiter gravid traps from a forested area in Montgomery County (Yang et al. 2018). Adult mosquitoes were allowed

to oviposit upon seed germination paper and were then screened for possible viral infection by plaque assay. All mosquitoes were virus-negative (data not shown).

To elucidate the prediapause stage of Ae. j. japonicus, populations of a single life-stage of the F<sub>2</sub> generation were exposed to 8:16 (L:D) short-day photoperiod while the remainder F<sub>2</sub> lifestages were exposed to 16:8 (L:D) long-day photoperiod according to modified methods from Kappus and Venard 1967. As such, the egg, larval, pupal, and adult life-stages were all exclusively exposed to the short-day photoperiod while the additional life- stages from the same generation were exposed to long day photoperiods. In addition, groups were raised entirely under short-day photoperiod or entirely under long-day photoperiod for the control, so that all lifestages were exposed to either short or long days. All trials were conducted at 21°C with 75% relative humidity. Adult mosquitoes from the F<sub>2</sub> generation were force-mated (Gerberg et al. 1994), offered a human blood meal, and the eggs were collected upon seed germination paper (Anchor Paper Company, Saint Paul, MN). After 14 days, 200 eggs from the F<sub>3</sub> generation were exposed to a hatching stimulus of .5 grams of nutrient broth dissolved in 250 ml of deionized water for 24 hours. Following the first hatch attempt, the oviposition papers were allowed to dry for another 24 hours at an 8L:16D photoperiod and were then subjected to a second hatch attempt. Upon completion of the second hatch attempt, all unhatched eggs were then chemically cleared using acetic acid and sodium chlorite solution. Eggs were considered embryonated if the pharate first instar larvae eye spots, egg burster, and hatching spine were easily noticeable. Five replicates of F<sub>3</sub> eggs per F<sub>2</sub> life stage subjected to diapause conditions were used with the exception of the F<sub>2</sub> egg treatment. Percent of viable eggs was calculated as: (no. embryonated eggs/ by the no. of non-embryonated + the no. of embryonated eggs). Percent diapause was

calculated as: (no. of embryonated eggs not hatching after two hatching attempts/no. of viable eggs).

A molecular phylogeny from Soghigian et al. (2017) was used as a context to infer the evolutionary history of prediapause phenotypes of *Aedes spp*. that utilize embryonic hibernal diapause. The phylogeny we present here has reduced taxon coverage because of the limited available studies on temperate *Aedes spp*. overwintering strategies. Some mosquitoes overwinter in more than one stage, typically the larva and egg life-cycle stages. Only the prediapause stage responsible for embryonic diapause was listed. A full list of references for each species to create the phylogeny can be accessed under supplementary data Table 1.

Data were analyzed using Chi-square followed by pairwise comparisons of percent hatch for different life-stages by Fisher's exact test using Prism 7 for Mac OSX (GraphPad Software, Inc., 2017).

#### 2.4 Results

The life-stage of mosquito exposed to short-day photoperiods (8L:16D) had a significant effect of the hatch rate of F<sub>3</sub> eggs ( $X^2$ , P<0.01) (Table 1 and Figure 2). Exposure of the maternal adult F<sub>2</sub> generation to short-days resulted in F<sub>3</sub> egg diapause (no hatch) irrespective of all other F<sub>2</sub> life-stage treatments. The hatch rate of eggs produced from egg and larval stage exposure to short-day photoperiod was not different from the controls that had not been exposed to any shortday photoperiods (Fisher's exact test, P<0.01). Short-day treatments on F<sub>2</sub> pupae elicited a weak diapause response resulting in a hatch rate that was lower than that of the controls but higher that that of the adults that had been exposed to short-days (Fisher's exact test, P<0.01). All larvae maintained at a short-day photoperiod continued development to pupal ecdysis. Percent egg viability from force-mated pairs of *Ae. j. japonicus* ranged from 50.7 to 58.6% with a total mean of 54.2%, 95% CI (52.9; 55.4) (Table 2).

Groupings of similar prediapause phenotypes are noticeable in closely related species; however, not all (Fig. 3). Maternal prediapause is evident in distantly related species, while embryonic prediapause appears in several clusters of closely related species.

#### **2.5 Discussion**

*Aedes j. japonicus* undergoes embryonic diapause facilitated by the prediapause maternal generation that begins detecting environmental cues in the pupal life-cycle stage and extends into the adult life-cycle stage (Table 1 and Figure 2). This maternal prediapause stage is similar in the closely related *Aedes togoi* (Galka & Brust 1987), *Aedes epactius*, and *Aedes atropalpus* (Anderson 1968) and the more distantly related *Aedes albopictus* (Mori and Wada 1981) (Fig. 3). Low percentages of viable eggs from force-mated females highlight the challenges of rearing *Ae. j. japonicus* (Table 2). These percentages were consistent across the experiment. With several identifications of virus infected mosquitoes from the field, a better understanding of the biology of this mosquito is important to understand vector biology and disease transmission (Westby et al. 2014, Harris et al. 2015, Yang et al 2018).

Recent molecular phylogenetic analyses have made it possible to examine the evolution of the prediapause stages of *Aedes* mosquitoes that undergo hibernal, embryonic diapause. Embryonic diapause also occurs in *Psorophora ferox* and *Anopheles walker* (Hurlbut 1938, Pinger and Eldridge, 1977). These non-*Aedes* species both employ the maternal prediapause stage to induce embryonic diapause in the following generation. While *Aedes* are undoubtedly not monophyletic, the ancestral prediapause phenotype may be the maternal generation due to distantly related species also utilizing this approach (Denlinger and Armbruster 2016, Soghigian et al. 2017). The embryonic prediapause phenotype likely evolved multiple times from obligate hibernal diapause in extreme cold-acclimated species. As some species transitioned from obligate diapause populations to facultative diapause populations, the egg life-cycle stage might have remained photosensitive. In *Aedes campestris* (Tauthong & Brust1977), *Aedes geniculatis* (Sims & Munstermann 1983), and *Aedes canadensis* (Pinger & Eldrige1977) all species have individuals that undergo obligate embryonic diapause to survive northern winters, but more southern populations have individuals capable of facultative embryonic diapause. While more distantly related, this strategy appears to be convergent. A more exhaustive genetic analysis to determine the underpinnings of prediapause is needed.

With the addition of identifying the prediapause stage of *Ae. j. japonicus* and subsequent phylogenetic analysis, we add basic knowledge to the US invasive *Ae. j. japonicus*, and we present a more detailed understanding of the evolution of embryonic diapause in *Aedes* mosquitoes. The prediapause phenotype should be considered when investigating the evolution of mosquito diapause.

#### 2.6 References

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### 2.7 Figures and Tables



Fig 2.1. Two common diapause induction methods under a short photoperiod utilized by facultative embryonic diapause in the genus *Aedes*. Gradient arrows indicate the ability of the life-cycle stage to sense environmental cues needed to enter diapause either in the  $F_n$  generation or  $F_{n+1}$  generation.
Table 2.1. Effect of short-day photoperiod (8L:16D) on *Ae. j. japonicus*  $F_2$  life- stages on  $F_3$  percent hatch. All other  $F_2$  life-cycle stages were exposed to long-day photoperiod (14L:8D). \*= eggs of the  $F_2$  generation were tested for percent diapause. Percentages followed by different letters are significantly different (Fisher's exact test,  $P \le 0.01$ ).

Life-cycle Stage	Ν	% Hatch
All	521	99.81 A
Adults	532	100 A
Eggs	507*	2.96 B
Larvae	536	4.66 B
Pupae	586	20.82 C
None	568	2.82 B



Figure 2.2. Effect of short-day photoperiod (8L:16D) on *Ae. j. japonicus*  $F_2$  life- stages on  $F_3$  percent hatch. All other  $F_2$  life-cycle stages were exposed to long-day photoperiod (14L:8D). \*= eggs of the  $F_2$  generation were tested for percent diapause.



Definitions:

<u>Maternal prediapause stage</u>: the  $F_n$  maternal female is sensitive to environmental cues needed to induce diapause (Figure 1) while the  $F_{n+1}$  embryos enter diapause.

<u>Embryonic prediapause stage</u>: The embryo is sensitive to environmental cues needed to induce diapause and is the overwintering diapause stage

Obligate flood: These mosquitoes lay eggs that enter into an obligate aestivation to avoid dry seasons

<u>Obligate winter</u>: These mosquitoes lay eggs that enter into an obligate diapause to avoid harsh low temperatures and are located more poleward

<u>Temperature dependent</u>: Induction of diapause is dependent more upon sensing low temperatures and not photoperiods

Figure 2.3. Phylogeny of known prediapause stages in Aedes that undergo embryonic diapause.

Species with two prediapause stages have obligate univoltine and facultative multivoltine individuals. Some species utilize multiple life-cycle diapause stages and represented here is the prediapause stage solely to induce embryonic diapause. Temperature dependent diapause, not photoperiod, induction is denoted by †. The scale is in millions of years. Recently proposed genera are in parenthesis. Phylogeny generated from Soghigian et al. (2017).

Table 2.2. Percent viability of force-mated *Ae. j. japonicus* eggs by life-stage exposed to a shortday photoperiod (8L:16:D). Viability was determined as bleached eggs that display noticeable eye-spots and egg burster.*Ae. j. japonicus* males and females were force mated according to Gerberg et al. (1994).

% Viability of eggs
eggs
52.1
53.2
58.6
53.6
50.7
56.8

Supplemental Table 1. Description of species, prediapause stage to induce embryonic diapause, and references

# Aedes albopictusMaternalMori A, Oda T, Wada Y. 1981. Studies on the eggdiapause and overwintering of Aedesalbopictus in Nagasaki. Trop. Med. 23: 79-90

#### Aedes atropalpus Maternal

Anderson JF. 1968. Influence of photoperiod and temperature on the induction of diapause in *Aedes atropalpus* (Diptera, Culicidae). Entomol. Exp. Appl. 11: 321-30

Aedes campestris

#### Embryonic

Tauthong P, Brust RA. 1977. The effect of photoperiod on diapause induction, and temperature on diapause termination in embryos of *Aedes campestris* Dyar and Knab (Diptera: Culicidae). Can. J. Zool. 55: 129-34

#### Aedes canadensis

#### Embryonic

Pinger RR, Eldrige BF. 1977. The effect of photoperiod on diapause induction in Aedes canadensis and Psorophora ferox (Diptera: Culicidae). Ann. Entomol. Soc. Amer. 70: 437- 41

#### Aedes caspius

#### Maternal

Abdel-Rahman AM, Adham FK. 1983. The effect of photoperiod on diapause induction in *Aedes caspius* Pallus. J. Egyptian Soc. Parasitol. 13: 343-47

Vinogradova, E.B., 1975: Role of photoperiodism and temperature in the induction of diapause in the egg phase of *Aedes caspius caspius* Pallus. (Diptera, Culicidae). Parazitologiia 9(5): 385-392

# *Aedes dorsalis* Embryonic, Maternal = temperature dependent

Telford AD. 1958. The pasture *Aedes* of central and northern California. Seasonal history. Ann. Entomol. Soc. Amer. 51: 360-65

McHaffey, D. G., and R. F. Harwood. 1970. Photoperiod and temperature influence on diapause in eggs of floodwater mosquito, *Aedes dorsalis* (Meigen) (Diptera: Culicidae). J. Med. Entomol. 7: 631-44.

# Aedes epactius

#### Maternal

Anderson JF. 1968. Influence of photoperiod and temperature on the induction of diapause in *Aedes atropalpus* (Diptera, Culicidae). Entomol. Exp. Appl. 11: 321-30

#### Aedes fitchii

#### **Obligate**, Flood

Horsfall WR, Lum PTM, Henderson LM. 1958. Eggs of floodwater mosquito (Diptera: Culicidae) V. Effect of oxygen on hatching of intact eggs. Ann. Entomol. Soc. Amer. 51: 209-13

#### Aedes geniculatus

#### Embryonic and Obligate, Winter

Sims SRM, Munstermann LE. 1983. Egg and larval diapause in two populations of *Aedes geniculatus* (Diptera: Culicidae). J. Med. Entomol. 3: 263-71

#### Aedes hendersonii Embryonic

Gallaway WJ. 1985. Larval diapause of Aedes hendersoni and Aedes trisereatus from southern Manitoba. J. Am. Mosq. Contr. Assoc. 1: 92-93

Shroyer, D. A. 1979. Seasonal aspects of egg hatching in *Aedes triseriatus* (Say): sex ratio distortion and diapause. Ph.D. diss. Univ. of Notre Dame, Notre Dame, IN. 181 pp.

#### Aedes hexodontus

# **Obligate**, Winter

Beckel WE. 1958. Investigations of permeability, diapause, and hatching in eggs of the mosquito *Aedes hexodontus* Dyar. Can. J. Zool. 36: 541-55

#### Aedes impiger

#### **Obligate**, Winter

Corbet PS,Danks HV 1975. Egg-laying habits of mosquitoes in the high arctic. Mosq. News. 35:8-14 Corbet PS, Danks HV. 1973. Seasonal emergence and activity of mosquitoes (Diptera:Culicidae) in a high-artic locality. Can. Entomol.

105: 837-72

#### Aedes japonicus japonicus Maternal

This paper

#### Aedes mariae

# Maternal

Coluzzi M, Di Deco M, Gironi A. 1975. The influence of photoperiod on the selection of oviposition sites in *Aedes mariae* (Diptera: Culicidae). Parassitologia 17: 121-30

Aedes nigripes

**Obligate**, Winter

Corbet PS, Danks HV. 1975.	Egg-laying habits of mosqui	toes in the high arctic. Mosq.
News. 35:8-14		

Corbet PS, Danks HV. 1973. Seasonal emergence and activity of mosquitoes (Diptera:Culicidae) in a high-artic locality. Can. Entomol. 105: 837-72

#### *Aedes nigromaculis* Embryonic, temperature dependent

Telford AD. 1958. The pasture *Aedes* of central and northern California. Seasonal history. Ann. Entomol. Soc. Amer. 51: 360-65

#### Aedes sierrensis Embryonic, primarily L4 larvae

Jordan RG, Bradshaw WE. 1978. Geographic variation in the photoperiodic response of the western tree-hole mosquito, *Aedes sierrensis*. Ann. Entomol. Soc. Amer. 71: 787-90

Jordan RG. 1980. Embryonic diapause in three populations of the western tree-hole mosquito, *Aedes sierrensis*. Ann. Entomol. Soc. Amer. 73: 357-59

# Aedes sollicitans

#### Embryonic

Parker BM. 1988. Photoperiod-induced diapause in a North Carolina strain of *Aedes* sollicitans; photosensitivity of fully formed and developing embryos. J. Am. Mosq. Contr. Assoc. 4: 57-63

#### Aedes squamiger

#### **Obligate**, Flood

Telford AD. 1958. The pasture *Aedes* of central and northern California. Seasonal history. Ann. Entomol. Soc. Amer. 51: 360-65

#### Aedes sticticus

#### **Obligate**, Flood

Horsfall WR, Trpis M. 1967. Eggs of floodwater mosquitoes. X. Conditioning and hatching of winterized eggs of *Aedes sticticus* (Diptera: Culicidae). Ann. Entomol. Soc. Amer. 60: 1021-25

#### Aedes stimulans

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Horsfall WR, Fowler HWJ. 1961. Eggs of floodwater mosquitoes VIII. Effect of serial temperatures on conditioning of eggs of *Aedes stimulans* Walker (Diptera: Culicidae). Ann. Entomol. Soc. Amer. 54: 664-66

#### Aedes taeniorhynchus Embryonic

Parker BM. 1985. Effects of photoperiod on the induction of embryonic diapause in *Aedes taeniorhynchus* (Diptera:Culicidae). J. Med. Entomol. 22: 392-97

Aedes togoi	Maternal
Galka	, B. E., & Brust, R. A. 1987. The effect of temperature and photoperiod on
	the induction of larval diapause in the mosquito Aedes togoi (Theobald)(Diptera:
	Culicidae). Can. J of zool. 65(9), 2262-2265.
Aedes triseria	atus Embryonic
Каррі	as KD, Venard CE. 1967. The effects of photoperiod and temperature on the
	induction of diapause in Aedes triseriatus Say. J. Insect Physiol. 13: 1007-19
Aedes vexans	Obligate, Flood
Wilso	n GR, Horsfall WR. 1970. Eggs of floodwater mosquitoes XII. Installment hatching
	of Aedes vexans (Diptera: Culicidae). Ann. Entomol. Soc. Amer. 63: 1644-47
Psorophora f	erox Maternal
Pinger	r, R.R., Eldridge, B.F., 1977. The effect of photoperiod on diapause induction in
	Aedes canadensis and Psorophora ferox (Diptera:Culicidae). Ann. Entomol. Soc.
	Amer. 70, 437–441.

#### Chapter 3

# The effect of LACV on the ability of *Aedes triseriatus* and *Aedes albopictus* to enter diapause and their respective critical photoperiods

#### 3.1 Abstract

The La Crosse virus (LACV) has evolved to exploit diapausing *Aedes triseriatus* eggs to bridge winter months; however, the impact of the invasive *Aedes albopictus* on the overwintering disease ecology remains unclear. In this study, we examined the effect of LACV transovarial transmission (TOT) on the ability of *Ae. triseriatus* and *Ae. albopictus* to enter diapause and the critical photoperiod (CPP) of populations from Blacksburg, Virginia. Local *Ae. triseriatus* and *Ae. albopictus* eggs were intrathoracically challenged with LACV and their corresponding prediapause stages were exposed to different photoperiods. The critical photoperiods of LACV TOT infected *Ae. triseriatus* was 13.47:L and 13.54:L respectively while the critical photoperiods of LACV TOT infected *Ae. albopictus* was 13.22:L and 13.27L respectively. LACV TOT did not affect the ability of either mosquito species to enter diapause and had very little, if any effect on their critical photoperiods in the laboratory. Further studies are needed to determine how these findings relate to natural conditions.

#### **3.2 Introduction**

Container-inhabiting *Aedes* mosquitoes in temperate areas utilize a photoperiodic diapause to survive unfavorable weather conditions during winter months and overwinter as pharate first instar larvae within the protective chorion of eggs or infrequently as larvae (Denlinger and Armbruster 2014). These mosquito species and populations capable of facultative winter diapause are able to spread further into more poleward climes while species and

populations incapable of diapause are latitudinally restricted typically to the tropics (Diniz et al. 2017). In this embryonic or larval diapause state, development is arrested and metabolism slows allowing survival for several months (Denlinger and Armbruster 2016).

Embryonic and larval diapause in *Aedes* mosquitoes is exploited by the La Crosse virus (LACV; family Bunyaviridae, genus Orthobunyavirus) in the United States through a highly effective transovarial transmission (TOT) process as a way for the virus to bridge inimical winter conditions inside their mosquito hosts. The virus is then reintroduced to naïve populations of small mammals and other vertebrate hosts by horizontal transmission the following spring (Moulton and Thompson 1971, Gauld et al. 1974, Watts et al. 1974, Beaty and Thompson 1975, Miller et al. 1977, Thompson and Beaty 1977, Borucki et al. 2002, Hughes et al. 2006). The LACV is the leading cause of pediatric encephalitis in the United States and up to 300,000 cases are estimated to occur each year (Calisher 1994, Rust et al 1999). Appalachia, including southwestern Virginia, has emerged as an area of concern for this virus since the 1990s, and the majority of human cases are now being reported from this region (Jones et al 1999, Leinsham and Juliano 2012, McJunkin et al 2001, Nasci et al 2000, Bewick et al 2016).

Three sympatric *Aedes* mosquitoes commonly vector LACV in southwestern Virginia (Harris et al. 2015, Barker et al 2003). The principal vector *Aedes triseriatus* (Say) is endemic to the United States with a latitudinal range from Florida to Maine along the east coast (Grimstad et al. 1977, Farajollahi and Price 2013), while two invasive mosquitoes *Aedes albopictus* and *Aedes japonicus japonicus* are likely bridge vectors in this region (Westby et al. 2015, Harris et al. 2015). All three species have been shown to be competent vectors in the laboratory (Grimstad et al. 1989, Cully et al. 1992, Sardelis et al. 2002, Bara et al. 2016) and positive pools of each species have been found in the field (Gearhardt et al. 2001, Barker et al. 2003, Westby et al.

2014, Harris et al. 2015). However, the role of these two invasive mosquito species in the emergence and increased number of LACV human cases in new areas remains uncertain (Bewick et al 2016).

Since its introduction to the continental United States in the mid 1980's (Sprenger 1986), *Ae. albopictus* has shown remarkable plasticity in its invasion (Benedict et al 2007). First identified in Texas, this mosquito quickly spread over much of the United States typically through human intervention and along the established highway system (Moore and Mitchell 1997). From 1995 to 2016 individuals have been collected from 34 states and the District of Columbia (Hahn et al. 2017). While non-diapausing populations of *Ae. albopictus* thrive in tropical regions, the success of this mosquito in the United States can in large part be attributed to the swift adaptation of diapausing populations capable of surviving winters as far north as Connecticut (Nawrocki and Hawley 1987, Urbanski et al. 2012, Armstrong et al. 2017). In addition to vectoring LACV, *Ae. albopictus* is also capable of transmitting a number of different important arboviruses (Gratz 2004, Turell et al. 2005). A better understanding of the overwintering ecology of different diseases in relation to this species is needed and especially so in disease systems like LACV that utilize TOT and embryonic diapause in their maintenance.

Diapause in Culicidae undoubtedly evolved multiple times, but the robust chorion of *Aedes* spp. allowed embryonic diapause to emerge as the most common method to survive extreme low temperatures and humidity in this genus (Denlinger and Armbruster 2014, Diniz et al. 2017), and larval diapause likely evolved as a failsafe mechanism for mosquitoes prematurely hatching into unfavorable lower environmental temperatures (Holzapfel and Bradshaw 1980). While most *Aedes spp.* overwinter as pharate first instar larvae within their eggs, they employ two common prediapause life stages that are sensitive to the environmental cues needed to

induce facultative diapause. The prediapause stage in *Ae. triseriatus* is the embryo itself that overwinters (Kappus and Venard 1967), while it is maternal generation in *Ae. albopictus* (Mori et al. 1981). To estimate the transition of a non-diapausing population to a diapausing population within a local species, critical photoperiods (CPP) are commonly used. The CPP of a certain population is calculated as the number of light-hours sensed by the prediapause stage needed to induce diapause in 50% of the population. The CPP of many diapausing insects, including *Ae. triseriatus* and *Ae. albopictus*, in the Northern Hemisphere decreases from north to south to appropriately anticipate the arrival of winter at different geographic foci (Andrewartha 1952; Danilevskii 1965; Sims 1982, Shroyer and Craig 1983, Tauber et al. 1986, Urbanski et al. 2012).

McGraw et al (1998) showed the ability of LACV TOT-infected *Ae. triseriatus* embryos to enter diapause but noted higher mortality rates during winter months than in uninfected *Ae. triseriatus* embryos; however, this is study is the first time the effect of an arbovirus on the ability to induce diapause and effects on CPP in *Ae. triseriatus* and *Ae. albopictus* has been evaluated.

#### **3.3 Materials and Methods**

#### Aedes triseriatus and Ae. albopictus mosquito colonies

*Aedes triseriatus* mosquitoes were collected during the 2016 summer via gravid traps and were allowed to oviposit eggs in the lab. *Aedes albopictus* mosquitoes were collected by using a human attractant and aspirator and were provided a sheep blood meal from an artificial membrane feeder in the laboratory to promote egg production. The  $F_{2-4}$  generation of *Ae*. *albopictus* mosquitoes and  $F_{2-3}$  generation of *Ae*. *triseriatus* were used in this study. To ensure embryonated eggs, *Ae*. *triseriatus* mosquitoes were force-mated (Gerberg et al. 1994). All adult mosquitoes collected from the field were screened for virus by plaque assay after laying eggs. No eggs from virus infected female mosquitoes were used to start colonies.

Mosquitoes were reared according to Kauffman et al. (2017) at 24°C with a 75% relative humidity.

#### Virus strain and injections

The LACV strain was originally harvested from field-collected adults and larval mosquitoes by Barker et al. (2003) in Wise County. Virus stock was prepared by inoculating confluent Vero cells in a 75-cm<sup>2</sup> flask and incubated for 36 hours in M199 cell medium (500 mL M199, 10 mL penicillin/streptomycin (5000 I.U./ml), 27 mL fetal bovine serum, 2 mL Amphotericin B (250  $\mu$ L/mL) and 0.5 ml gentamycin (50 mg/mL)) The cell supernatant with a virus titer of 6.75 log x 10<sup>7</sup> pfu/mL was stored at -80 °C. Adult female F<sub>2</sub> *Ae. triseriatus* and F<sub>2-3</sub> *Ae. albopictus* were injected with up to 0.2  $\mu$ L of prepared LACV stock following the methods of Rosen and Gubler (1974).

#### **Construction of photoperiod chambers**

Photoperiod exposure chambers were constructed from 2.0 L HDPE plastic buckets covered with Reynolds Wrap Heavy Duty Aluminum Foil (Reynolds Consumer Products, Lincolnshire, Illinois) and Gorilla 6035180 Black Duck Tape (Gorilla Glue Company, Sharonville, OH). A Meridian LED Night Light Base (Meridian Lighting, Maryland Heights, MO) connected to a Woods Indoor 24-Hour Digital Timer (Coleman Cable, Inc, Waukegan, II) was used as a light source attached to the chamber. There was no difference between the temperature of the photoperiod exposure chamber and the environmental chamber as measured by an infrared thermometer. Transparent, sealable plastic bags containing *Ae. triseriatus* TOT+/eggs and 250 mL plastic cups containing a LACV infected and uninfected *Ae. albopictus* adult

females were placed inside individual photoperiod exposure chambers to subject the correct prediapause stage of respective mosquito species to differing 24-hour photoperiods. A 45ml plastic cup lined with seed germination paper (Anchor Paper Company, Saint Paul, MN) and partially filled with distilled water was positioned inside the 250ml cups to provide an oviposition site for *Ae. albopictus* mosquitoes.

#### **Determination of CPP and diapause rates**

To determine if LACV has an effect on CPP, adult and pupa *Ae. albopictus* and *Ae. triseriatus* eggs were subjected to the following light cycles: 10L:14D, 12L:12D, 12.5L:11.5D, 13L:11D, 13.25L:10.75D, 13.5L:10.5D, 13.75L:10.5D, 14L:10D. Adult and pupal *Ae. albopictus* females were housed in small plastic buckets with a water source and exposed to the photoperiod regimen previously listed. After 10 days and every seven days thereafter of the treatment they were offered a bloodmeal to produce eggs. No twilight or crepuscular period was used between scotophase and photophase. Oviposition papers containing the eggs were fully submerged in a broth solution twice. The second attempt at hatching exposure occurred 48 hours after the first hatch. The oviposition paper were stored at the respective photoperiods between hatch attempts. Unhatched eggs were cleared by a bleaching solution and those not embryonated were excluded from the study. Critical photoperiod is defined as the number of light hours at which 50% of the exposed and viable mosquito eggs are in diapause. Percent diapause was determined using the following formula: (no. unhatched embryonated eggs/ no. hatched eggs + no. unhatched eggs embryonated).

#### Infectivity assay

Modified methods from Gortez et a. (2017) were used to screen larvae from 2<sup>nd</sup> -4<sup>th</sup> gonotrophic cycles for LACV infection. Larvae from the second gonotrophic cycle were pooled

by maternal individual and tested for CPE to determine the presence of TOT. Pools consisted of 15 larvae or less. Only eggs from the 3<sup>rd</sup>-4<sup>th</sup> gonotrophic cycles from individuals that yielded infected larvae from their 2<sup>nd</sup> gonotrophic were used to calculate percent diapause of each photoperiod for the LACV TOT infected colony.

Vertical transmission rates were estimated by testing thirty whole body L<sub>4</sub> mosquito larvae from the 3<sup>rd</sup> to 4<sup>th</sup> gonotrophic cycle of individuals that produced positive pools from the 2<sup>nd</sup> gonotrophic cycle. Larvae were homogenized in M199 complete using two BBs until sufficiently disrupted and the supernatant was inoculated onto Vero cells on a 96-well plate. If the cells showed signs of viral CPE, the larva or pool was considered to be positive for LACV.

#### Statistical analysis

The CPPs for each colony were calculated using a logistic fit and inverse prediction in JMP® Pro 13.0.0 (SAS Institute, Cary, North Carolina, U.S). Values without overlapping confidence intervals were considered to be statistically significant.

#### 3.4 Results

The mean vertical transmission estimate for *Ae. triseriatus* was 66.3%, 95% CI [62.39, 70.95] and 48.25, 95% CI [44.11, 52.39] for *Ae. albopictus* (Table1). The vertical transmission rate was significantly higher in *Ae. triseriatus* than in *Ae. albopictus* ( $X^2$  (1, N=480) = 16.5, p< 0.001).

All colonies of mosquitoes were able to enter diapause regardless of LACV infection. The CPP of *Ae. albopictus* LACV TOT+/- did not differ significantly at 13.22:L and 13.27:L respectively while the CPP of *Ae. triseriatus* LACV+/- did differ significantly at 13.47:L and 13.54:L respectively (Table 2 and Figure 1). Both LACV infected and uninfected *Ae. triseriatus*  colonies had a higher CPP than both LACV infected and uninfected *Ae. albopictus* colonies (Table 2).

#### **3.5 Discussion**

The natural cycle of LACV relies on TOT into winter diapausing *Aedes* embryos to span winter months, and thus it is not surprising LACV did not have an effect on the ability of infected mosquitoes to enter diapause in the principal vector, *Ae. triseriatus* nor the accessory vector *Ae. albopictus*. The critical photoperiod of uninfected *Ae. triseriatus* was 13.47:L and LACV TOT infected was 13:54:L which is consonant with a previous study (Shroyer et al. 1983). Although our study yielded significant differences in the CPP of LACV infected and uninfected *Ae. triseriatus* embryos, we do not believe there is a biological significance because this difference would result in LACV infected *Ae. triseriatus* populations entering diapause four days before LACV uninfected *Ae. triseriatus* populations (Table 4). The critical photoperiod of uninfected *Ae. albopictus* was 13.22:L and LACV infected was 13.27:L, which corresponds with previous studies (Urbanski et al. 2012).

Interestingly, while other insect and mosquito species have shown changes in CPP due to climate change (Bradshaw and Holzapfel 2001, Urbanski et al. 2012), our data obtained from *Ae. triseriatus* collected from Blacksburg, Virginia (Montgomery County) corresponds with those from 1983 at 37°N (Shroyer et al. 1983). The Blacksburg *Ae. triseriatus* colony's CPP falls between the South Bend (Kramer and Burdette) colony and TOPSY colonies tested by Shroyer et al. (1983, Growing seasons, decrease from south to north in the United States and an earlier entrance into diapause in more northern climes ensures overwinter survival. Further studies on

*Ae. triseriatus* populations along a latitudinal gradient are needed to determine if there have also been changes to their CPPs in recent years.

In addition to latitudinal gradient effects on CPP, altitude should also be considered (Focks 1994). Air masses expand, rise, and cool when faced with an increase in altitude due to mountain ranges (MacArthur 1972), and freeze-free days decrease by 94 days with every 1000 m of elevation gain (Bradshaw 1976, Bradshaw and Holzapfel 2007). Using the Pitcher-plant mosquito Wyeomyia smithii, Bradshaw and Lounibos (1977) provide an altitude-corrected latitude (ACL) equation to account for the direct relationship between an increase in altitude and an increase in CPP: ACL = latitude (°N) + altitude (m) \* ( $b_{ALTITUDE} \div b_{LATITUDE}$ ) where b is the regression coefficients of CPP on altitude and latitude. The altitude of Blacksburg, Virginia is 634m above sea-level which may result in a greater CPP and an earlier seasonal entrance into diapause of Ae. triseriatus and Ae. albopictus at this location than populations of these respective species occupying similar latitudes at lower altitudes. Urbanski et al. (2012) noted the evolutionary changes in CPP of Ae. albopictus from 1988 to 2008 as populations at similar temperate latitudes enter diapause later in the season, but they did not include altitude as a variable in their analysis. Also, changes in Wyeomyia smithi populations' ACL and photoperiodism have been reported in as little time as five years (Bradshaw & Holzapfel 2001, 2006). The only latitudinal and altitude gradient studies for these mosquitoes in the United States used data collected over 30 years ago for Ae. albopictus (Pumpuni 1989, Focks et al, 1994) and over 38 years ago for Ae. triseriatus larval diapause (Holzapfel and Bradshaw 1981, Shroyer et al 1979). It is also unclear as to the effects of altitude on embryonic diapause in Ae. triseriatus (Shroyer et al. 1983). Due to the rapid changes in mosquito photoperiodism most likely caused by climate change and lack of data on altitude's influence on Ae. triseriatus CPP of embryonic

diapause, we cannot estimate the relative impacts of climate and altitude on CPP of our Blacksburg populations of *Ae. triseriatus* and *Ae. albopictus*.

While information gleaned from laboratory CPP studies like this one that use an artificial environment with constant temperatures, humidity, and no crepuscular period can be incredibly beneficial, applying them to field populations can be difficult. The wavelength and intensity of light needed to induce diapause in Ae. albopictus and Ae. triseriatus remains unstudied, and we did not consider this in our experiment. In addition, it is unclear as to what measurement of daylength to use in order to relate our laboratory study to field populations. Civil twilight of dawn and dusk, when the solar altitude is 6° below a given horizon (Smithsonian institution 1939), is often used in biological systems as the beginning and end of organism's ability to sense daylight (Hut et al. 2013), but it may be appropriate to only use a percentage of civil twilight in models and comparisons of laboratory studies of CPP to field populations (Beck and Apple 1961, Bean et al. 2014, Grevstad and Coop 2015) (Table 4.). Lacour et al. (2015) compared laboratory CPPs to field CPPs of a population of French-Mediterranean Ae. albopictus and found the laboratory CPP not using a crepuscular period was 0.8 hours greater, or CPP occurred 17 days earlier in the season, than the CPP of field-collected eggs. Also, they (Lacour et al. 2015) found the photoperiods that correspond with dates of given daylight necessary to induce diapause in the laboratory only persisted 20 days while it took over 40 days in the field. This is likely due to the selectivity of diapause insensitive phenotypes in some locations to remain active through end of the egg-laying season.

The temperature used in this study of 24 °C was higher than the optimal temperature of 21 °C needed to induce diapause in *Ae. triseriatus* and *Ae. albopictus* (Shroyer and Craig 1980, Pumpuni 1989; Pumpuni et al.1992). These studies indicate 21 °C as an optimal diapause

inducing temperature when they tested the effect of temperatures on CPPs; however, 24 °C was not evaluated in these prior studies and summer day-time temperatures around the time of diapause induction that corresponds to the field are almost always above 24 °C. This may have had an effect on CPPs for these species, but it is unlikely. Nevertheless, all colonies were able to enter diapause at 24 °C under the correct photoperiod.

We conclude that LACV infection of *Ae. triseriatus* and *Ae. albopictus* embryos has no observable effects on diapause induction and very little effect on CPP in the laboratory. However, further studies are needed to clarify how LACV TOT affects *Ae. triseriatus* and *Ae. albopictus* diapause induction in their natural environments under field conditions.

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# **3.7 Tables and Figures**

Table 3.1. Estimated vertical transmission rates of LACV in *Ae. triseriatus* and *Ae. albopictus* mosquitoes at different photoperiods. Thirty whole-body L4 larvae were individually tested for virus from each species at each photoperiod. Only eggs from the third and fourth gonotrophic cycle were used from maternal mosquitoes whose pooled second gonotrophic larvae tested positive for LACV.

Photoperiod (L:D)	Species	Vertical Transmission Rate Estimates %
10.14	Ae. triseriatus	77
10:14	Ae. albopictus	50
12.12	Ae. triseriatus	63
12:12	Ae. albopictus	43
10 5.11 5	Ae. triseriatus	67
12.3.11.3	Ae. albopictus	57
12.11	Ae. triseriatus	60
15:11	Ae. albopictus	53
12 25.10 75	Ae. triseriatus	73
15.25.10.75	Ae. albopictus	40
12 5.10 5	Ae. triseriatus	70
15.5:10.5	Ae. albopictus	47
12 75.10 25	Ae. triseriatus	63
15./5:10.25	Ae. albopictus	53
14.10	Ae. triseriatus	60
14:10	Ae. albopictus	43

Table 3.2. Percent diapause of LACV infected and uninfected *Ae. triseriatus* and *Ae. albopictus* mosquitoes exposed to different photoperiods. Percent diapause was defined as: (no. unhatched embryonated eggs/ no. hatched eggs + no. unhatched eggs embryonated). Nonviable eggs were excluded.

Photoperiod (L:D)	Colony	Ν	% Diapause (SE)
10:14	Ae. triseriatus LACV +	577	100 (0)
	Ae. triseriatus LACV -	579	99.31 (0.34)
	Ae. albopictus LACV+	564	98.94 (0.43)
	Ae. albopictus LACV -	569	99.3 (0.35)
10.10	Ae. triseriatus LACV +	568	99.47 (0.30)
	Ae. triseriatus LACV -	587	100 (0)
12:12	Ae. albopictus LACV+	570	94.39 (0.96)
	Ae. albopictus LACV -	579	94.99 (0.91)
	Ae. triseriatus LACV +	587	92.22 (1.1)
12 5.11 5	Ae. triseriatus LACV -	584	93.99 (0.98)
12.3.11.3	Ae. albopictus LACV+	572	85.59 (1.5)
	Ae. albopictus LACV -	569	88.32 (1.3)
	Ae. triseriatus LACV +	577	84.06 (1.5)
12.11	Ae. triseriatus LACV -	590	86.27 (1.4)
13.11	Ae. albopictus LACV+	570	78.77 (1.7)
	Ae. albopictus LACV -	584	76.88 (1.7)
12 25.10 75	Ae. triseriatus LACV +	579	84.80 (1.5)
	Ae. triseriatus LACV -	585	84.62 (1.5)
13.23.10.75	Ae. albopictus LACV+	553	69.98 (1.9)
	Ae. albopictus LACV -	568	74.12 (1.8)
	Ae. triseriatus LACV +	585	65.47 (2)
13 5.10 5	Ae. triseriatus LACV -	579	81.17 (1.6)
15.5.10.5	Ae. albopictus LACV+	577	16.64 (1.6)
	Ae. albopictus LACV -	562	22.24 (1.8)
	Ae. triseriatus LACV +	584	14.90 (1.5)
13 75.11 25	Ae. triseriatus LACV -	587	18.23 (1.6)
15./5.11.25	Ae. albopictus LACV+	564	12.77 (1.4)
	Ae. albopictus LACV -	582	14.26 (1.4)
	Ae. triseriatus LACV +	593	1.18 (0.44)
14.10	Ae. triseriatus LACV -	589	2.38 (0.63)
1-1.10	Ae. albopictus LACV+	570	1.053 (0.43)
	Ae. albopictus LACV -	567	2.47 (0.65)



# 🔆 = CPP

Figure 3.1. Diapause response curves of LACV infected *Ae. triseriatus* and *Ae. albopictus*. The  $F_{2-4}$  generation of *Ae. albopictus* mosquitoes and  $F_{2-3}$  generation of *Ae. triseriatus* were used in this study. All mosquitoes were collected locally in Montgomery county, Virginia in the summer of 2017.

Table 3.3 Critical photoperiod of LACV infected (+) and uninfected (-) *Ae. triseriatus* and *Ae. albopictus* as calculated by logistic fit and inverse prediction in JMP® Pro 13.0.0. CPPs with differing letters indicate statistical significance by non-overlapping confidence intervals.

Colony	Critical Photoperiod (h:m)	CI Lower Limit .95	CI Upper Limit .95
Ae. triseriatus LACV+	13:28.2 A	13:29.4	13:27.0
Ae. triseriatus LACV-	13:32.4 B	13:31.2	13:33.6
Ae. albopictus LACV+	13:13.2 C	13:12.0	13:15.0
Ae. albopictus LACV-	13:16.2 C	13:15.0	13:18.0

	August		September		
Day	Sunrise-Sunset	Civil Dawn-Civil Twilight	Sunrise-Sunset	Civil Dawn-Civil Twilight	
1	14:03	15:03	12:58	13:58	
2	14.01	15:01	12:56	13:56	
3	13:59	14:59	12:53	13:53	
4	13:57	14:57	12:51	13:51	
5	13:55	14:55	12:49	13:49	
6	13:53	14:53	12:47	13:47	
7	13:51	14:51	12:44	13:44	
8	13:49	14:49	12:42	13:42	
9	13:48	14:48	12:40	13:40	
10	13:46	14:46	12:37	13:37	
11	13:44	14:44	12:35	13:35	
12	13:42	14:42	12:33	13:33	
13	13:39	14:39	12:30	13:30	
14	13:37	14:37	12:28	13:28	
15	13:35	14:35	12:26	13:26	
16	13:33	14:33	12:23	13:23	
17	13:31	14:31	12:21	13:21	
18	13:29	14:29	12:18	13:18	
19	13:27	14:27	12:16	13:16	
20	13:25	14:25	12:14	13:14	
21	13:23	14:23	12:11	13:11	
22	13:20	14:20	12:09	13:09	
23	13:18	14:18	12:07	13:07	
24	13:16	14:16	12:04	13:04	
25	13:14	14:14	12:02	13:02	
26	13:12	14:12	12:00	13:00	
27	13:09	14:09	11:57	12:57	
28	13:07	14:07	11:55	12:55	
29	13:05	14:05	11:52	12:52	
30	13:03	14:03	11:50	12:50	
31	13:00	14:00			

Table 3.4. Hours of light as measured by sunset to sunrise and civil dawn to civil twilight (hours:minutes). Data were retrieved from the Astronomical Applications Department of the United States Naval Observatory for the year 2017.

#### **Chapter 4**

# The effect of La Crosse Virus Infection on Overwintering Mortality of *Aedes triseriatus* and *Aedes albopictus* in Southwestern Virginia

#### 4.1 Abstract

The La Crosse virus (LACV) is an important arbovirus in the United States and maintains its natural cycle over the winter in transovarially infected (TOT+) *Aedes* spp. mosquito eggs. LACV TOT+/- *Aedes triseriatus* and *Aedes albopictus* eggs were subjected to natural temperatures in Montgomery County, Virginia during the winter months of 2016-2017. Random eggs were brought to the laboratory and assayed for embryo and larval mortality and diapause rates. Eggs missing due to detachment from the oviposition papers were also counted but not used in embryo mortality calculations. The LACV had a clear adverse effect on embryo and larval mortality for both species but not on breaking diapause. The LACV also had an adverse effect on the ability of eggs to remain adhered to the oviposition substrates. We present here evidence of the ability of LACV TOT+ *Ae. triseriatus* and *Ae. albopictus* mosquitoes to survive adverse winter temperatures and possibly impact the epidemiology of this virus as global temperatures continue to rise.

#### **4.2 Introduction**

The La Crosse virus (LACV; family Bunyaviridae, genus Orthobunyavirus) is a mosquito-borne arbovirus and one of the leading causes of pediatric encephalitis in the United States (Jones et al. 1999). Estimated annual cases could be as high as 300,000 but only 30-180 cases are reported each year due to the mild nature of the majority of infections (Calisher 1994, Rust et al 1999). The virus primarily affects children but may be important in adults as both mild and severe cases can be misdiagnosed (Calisher 1994, McJunkin et al. 2001, Haddow et al. 2011, Leisnham and Juliano 2012, Teleron et al. 2016).

Since its identification in the early 1960s in Wisconsin, LACV cases have been reported from 30 different states and the majority of cases are now being reported from the Appalachian region. The introduction of the two invasive mosquitoes, *Ae. albopictus* and *Ae. j. japonicus* (Theobald), correlate with the emergence of this virus at these new regional foci (Jones et al. 1999, 2000; Nasci et al. 2000; Erwin et al. 2002; Haddow et al. 2009, 2011; Lambert et al. 2010), but a statistical model by Bewick et al. (2016) hypothesizes *Ae. albopictus* is not entirely responsible and different variables, including changing climate, other vectors like *Ae. j. japonicus*, and wildlife densities, should also be considered.

The sylvatic cycle of LACV in hardwood forests includes primarily horizontal transmission between *Aedes spp.* mosquito vectors and small mammal reservoirs such as the eastern chipmunk (*Tamias striatus griseus*) and gray squirrel (*Sciurus carolinensis*) (Calisher 1994). The native *Ae. triseriatus* is considered the principal vector while the two introduced species *Aedes albopictus* (Skuse) and *Aedes japonicus japonicus* (Theobald) could act as bridge vectors. All three species are sympatric in southwestern Virginia and LACV has recently been isolated from field-collected specimens in this region (Grimstad et al. 1989, Gerhardt et al. 2001, Westby et al. 2015, Harris et al. 2015). All of these mosquitoes have been shown to be competent, and indeed efficient, vectors of LACV in the laboratory (Bara et al. 2016); however, the role of the two invasive mosquitoes in the epidemiology of LACV remains speculative.

Vertical or transovarial transmission is another key process in which the LACV is maintained and is how it remains viable over temperate winters. *Aedes spp.* primarily utilize embryonic diapause to overwinter (Watts et al. 1974, Beaty and Thompson, 1975, Borucki et al.

2002, Hughes et al. 2005, Denlinger and Armbruster 2014). Facultative diapause is photoperioddriven in *Aedes spp.* and induced in the maternal generation of *Ae. albopictus* and in *Ae. triseriatus* eggs (Kappus and Venard 1967, Shroyer and Craig 1983). The diapausing embryos are protected by a desiccation-resistant chorion (Urbanski et al. 2012) and development is depressed allowing the pharate first instar larva to survive long periods of low temperatures (Sota and Mogi 1992, Urbanski et al. 2010, Denlinger and Armbruster 2014, Denlinger and Armbuster 2016).

Laboratory studies indicate there is no effect of LACV infection on *Ae. triseriatus* fecundity and survivorship (Miller et al. 1977, Patrican et al. 1985). Lower metabolic activities during embryonic diapause limit the resources, including macromolecules and mRNA caps, required for LACV replication. However, in overwintering LACV-infected *Ae. triseriatus* eggs, sequences of host-derived viral mRNA primers undergo time-dependent changes throughout winter months indicating some level of host transcription and viral replication occur (Dobie et al. 1997, McGraw et al. 1998). As such, McGraw et al. (1998) reported lower survivability of LACV-infected *Ae. triseriatus* eggs in the field but the majority of infected eggs survived the winter.

We sought to further our understanding of the impact LACV has on overwintering *Ae*. *triseriatus* and *Ae*. *albopictus* mosquitoes by exposing LACV infected and noninfected embryos induced into diapause to natural overwintering temperatures in Montgomery County, Virginia.

#### 4.3 Materials and Methods

Aedes triseriatus and Ae. albopictus Mosquito Colonies

During the summer of 2016, gravid adult *Ae. triseriatus* mosquitoes were collected via gravid trap, and host-seeking *Ae. albopictus* females were collected using a human attractant and aspiration in Montgomery County, Virginia. Adult *Ae. albopictus* were offered a sheep blood meal via artificial membrane feeder. Both species were provided with an oviposition substrate of seed germination paper (Anchor Paper Company, Saint Paul, MN) for egg collection. To avoid contamination from natural virus-infected mosquitoes, adult mosquitoes were screened for virus by plaque assay post oviposition, and only eggs from uninfected adults were used to start *Ae. triseriatus* and *Ae. albopictus* colonies. Mosquitoes were reared as described by Kauffman et al. (2017). To ensure fertilized eggs in the *Ae. triseriatus* colony only eggs collected from force-mated (Gerberg et al. 1994) females were included in this study.

#### Virus strain

The LACV strain originally isolated from *Ae. triseriatus* larvae and adults collected from Wise County, VA (Barker et al. 2003), was used. To prepare the virus stock, 1 ml of LACV in Vero cells and M199 cell medium (500 ml M199, 10 ml penicillin/streptomycin (5000 I.U./ml), 27 ml fetal bovine serum, 2 ml Amphotericin B ( $250 \mu$ l/ml) and 0.5 ml gentamycin (50 mg/ml)) was inoculated onto confluent Vero cells in a 75cm<sup>2</sup> flask and incubated at 37 °C and 5% CO<sub>2</sub> for one hour and agitated ever 15 minutes. Additional M199 complete media was then added to the flask for a total volume of 20 ml. The flask was incubated for 36 hours at 37 °C and 5% CO<sub>2</sub> and was then frozen at -80 °C for 24 hours. Upon thawing, the cell supernatant was harvested and stored at -80 °C. The stock virus used in this study had undergone 13 passages as described above. The virus titer was calculated using an end point dilution assay on Vero cells.

#### Intrathoracic injections

The stock LACV with a titer of log  $5.75 \times 10^7$  pfu/ml was intrathoracically injected into three to five days old *Ae. albopictus* and *Ae. triseriatus* female mosquitoes. Mosquitoes were briefly placed on ice until a noticeable chill coma was observed and were then injected with up to .2µl of the prepared LACV stock according to the methods of Jackson et al. (2012). Cessation of injection occurred if the thorax and abdomen of the mosquito appeared about to rupture. Uninfected mosquitoes were injected with solely M199 cell media. The adult mosquitoes were maintained at 25 °C, 75% relative humidity, and 16L:8D photoperiod with a constant 10% sucrose solution. Sheep blood meals were offered every 10 days via artificial membrane feeder and the mosquitoes were sugar starved 24 hours prior. To help ensure LACV transovarial transmission (TOT), F<sub>2</sub> eggs from the second, third, and fourth gonotrophic cycles were used in this study.

#### **Induction of diapause**

Upon oviposition,  $F_3$  LACV-infected and uninfected *Ae. triseriatus* eggs were subjected to a short-day length for two weeks at 21°C and 8L:16D photoperiod. After pupation,  $F_2$  *Ae. albopictus* males and females were reared under short-day diapause inducing conditions and female *Ae. albopictus* were intrathoracically injected with LACV during this time. The  $F_3$  eggs of LACV-infected and uninfected *Ae. albopictus* were then stored in the same conditions until relocation to the field. Time 0 was designated at the time the eggs were relocated from the laboratory to the field site.

## Egg measurements

Length and width measurements of *Ae. triseriatus* and *Ae. albopictus* eggs were used in this study (Bova 2014) to evaluate if LACV infection affects egg size. Thirty randomly selected

eggs from each colony were selected for measurement prior to being subjected to field conditions.

#### **Field study**

Oviposition papers (OPs) containing the F<sub>3</sub> diapause eggs of LACV-infected and uninfected *Ae. triseriatus* and *Ae. albopictus* colonies were placed in sealable, clear plastic bags along with four layers of diH20 saturated 5cm x 10cm seed germination sheets to prevent desiccation. Small holes in the plastic bags were made to allow air into the bags and to better mimic natural conditions. Initial discolored or irregular eggs were removed and not included in the study. The OPs were placed above the saturated seed germination paper, not touching, to avoid the possibility of contact freezing. The plastic bags were then attached to the side of an Igloo® 28 Quart Quantum Wheeled Cooler (Igloo Product Corporation, Katy, TX). To allow natural sunlight to reach the mosquito eggs, a 35cm x 28cm aperture was cut in the lid of the cooler with a 45cm x 61cm x 0.24cm clear acrylic sheet glass attached, covering the aperture. A cooler was used because both of these mosquitoes have been found in artificial containers.

To measure the effect of LACV on overwintering eggs, the cooler containing the eggs was placed in a forested area near the campus of Virginia Tech (37.210211, -80.435174) where Cache Valley and La Crosse virus infected mosquitoes have been previously collected (Yang et al. 2018). Around the same number of eggs were collected after two weeks and every five weeks thereafter. The eggs were subjected to winter temperatures from November 21, 2016 to April 21, 2017.

Temperatures were obtained from the National Weather Service in Blacksburg, Virginia every month and photoperiod data were obtained from the United States Naval Observatory.
Because of the extreme photosensitivity of mosquitoes, total daylight hours were calculated from morning civil twilight to evening civil twilight.

# **Determination of diapause rates**

Modified methods of McGraw et al. (1998) were used to establish diapause rates for each time point. Eggs collected from the field were submerged in hatching medium consisting of .5 grams of nutrient broth dissolved in 250 ml of deionized water four times at two-week intervals. After 24 hours larvae were enumerated and sorted into optimal rearing volumes of 4ml of water per larvae with a minimum larval rearing water volume of 10 ml. All unhatched eggs after the first hatch attempt were kept in non-diapause conditions of 25 °C, 75% relative humidity, and 16L:8D photoperiod to promote the termination of diapause. To determine diapause rates, the number of larvae from each hatch attempt was divided by the total number of larvae and the number of viable eggs remaining in diapause after the fourth hatch attempt.

#### **Determination of larval and egg mortality**

Egg mortality was calculated as the number of nonviable eggs following the 4<sup>th</sup> hatch attempt divided by total number of eggs retained throughout the study. The number of eggs were counted after each hatch attempt. Succeeding the fourth hatch attempt, eggs were immersed in a bleach solution (Trpis 1970) and viability was determined according to McGaw et al. (1998).

Larvae were fed ground Kaytee Koi's Choice Premium Fish Food (Kaytee Products, Inc., Chilton, WI) and reared according to Kaufman et al. (2017). Larval mortality was calculated as the number of larvae not surviving to the L4 instar divided by the total number of larvae.

### Missing eggs

After each hatch attempt, some eggs became detached from the oviposition substrate did not hatch and were not accounted for. These missing eggs were quantified but not used in the egg mortality calculations. Hatching stimuli and larval rearing water were not reused throughout the experiment.

#### Infectivity assay

Modified methods from Goertz et al (2017) were used to determine LACV infection of *Ae. triseriatus* and *Ae. albopioctus* larvae. Whole body L4 larvae were stored at -80C until the assay was conducted. They were homogenized in M199 cell media with an autoclaved BB until the sample was adequately disrupted and then centrifuged for 1 minute at 10,000 rpm. Thirty µl of each homogenate sample was inoculated onto VERO cell monolayers in 96 well plates. The inoculum was removed after 4 hours and replaced with M199 cell medium and cytopathic effect (CPE) was scored 2-3 days post infection (DPI). Samples were considered to be positive for LACV by visual CPE. Vertical transmission rates were determined by dividing the number of infected mosquito larvae by the total number tested at each time point PDI.

#### Statistical analysis

Statistical analyses were conducted using JMP® Pro 13.0.0 (SAS Institute, Cary, North Carolina, U.S) at the 0.05 statistical level. Egg measurements were compared using a one-way analysis of variance and Tukey's HSD test within species. Contingency tables and pairwise Chi Squared with Fisher's exact test were conducted for percent diapause, percent egg and larva mortality, and eggs missing by week.

# 4.4 Results

# **Environmental conditions**

The 2016-2017 winter in Montgomery County, Virginia was relatively mild with average low temperatures reaching below 0°C infrequently. The lowest temperatures were recorded

during weeks 3-7 PDI with the lowest temperature recorded of -15°C. The highest temperatures were recorded during weeks 7-22 PDI. Civil daylight decreased until the winter solstice on December 21, 2016 and then progressively increased until culmination of the study (Table 1). **Egg size** 

LACV did not have an intraspecific effect on the size of *Ae. triseriatus* and *Ae. albopictus* eggs as measured by length and width. The average length and width of *Ae triseriatus* LACV TOT+/- eggs was 700.07 µm L by 220 µm W and 699 µm L by 221.6 µm W respectively. The average length and width of *Ae. albopictus* TOT +/- were 597.9µm L by 185.3µm W and 598.1µm L by 184.9 µm W respectively (Table 2). The eggs of *Ae. albopictus* are normally smaller than the eggs of *Ae. triseriatus* (Bova et al. 2014).

# **Embryo mortality**

With the exceptions of week 0 and 12 PDI, there were significant intraspecific differences in embryo mortality rates between LACV-infected and uninfected *Ae. triseriatus* and LACV-infected and uninfected *Ae. albopictus* with LACV-infected colonies experiencing higher mortality. Interspecific comparisons between LACV-infected and uninfected colonies yielded statistical differences only during weeks 17 and 22 with greater embryo mortality in LACV-infected and uninfected *Ae. albopictus* colonies respectively (Table 3 and Figure 1). LACV-infected *Ae. albopictus* showed the highest total embryo mortality while noninfected *Ae. triseriatus* eggs displayed the lowest total embryo mortality.

# Diapause

There was not a clear intraspecific effect of LACV-infection on termination of diapause, but *Ae. triseriatus* colonies exited diapause before *Ae. albopictus* colonies (Table 4). *Aedes triseriatus* eggs began to terminate diapause during the week 17 PDI time point where the civil daylight ranged from 11:50 - 13:09 hours and *Ae. albopictus* began to terminate diapause during the week 22 time point where civil daylight ranged from 13:12 to 14:30 hours (Table 1 and Table 4).

#### Larval mortality

Intraspecific larval mortality was higher in LACV-infected mosquitoes for weeks 12, 17, and 22 PDI for *Ae. triseriatus* and weeks 12 and 17 for *Ae.* albopictus. LACV-infection did have an effect on total larval mortality within species. Higher total mortality rates were observed in LACV-infected colonies of both species (Table 3 and Figure 2).

# Missing eggs

A greater number of LACV-infected eggs were not accounted for in both species when compared to noninfected eggs within species (Figure 3). The number of missing eggs did not show a trend throughout the duration of the study as measured by individual time points PDI.

# 4.5 Discussion

We determined LACV infection did have an adverse effect on embryo and larval survivability of *Ae. triseriatus* and *Ae. albopictus* mosquitoes subjected to natural conditions in Montgomery County, Virginia. The LACV is known to affect the blood-feeding behavior of *Ae. triseriatus* and *Ae. albopictus* with infected females taking smaller blood-meals than uninfected females. LACV infection also increases the chance of refeeding within one gonotrophic cycle in *Ae. triseriatus* but not in *Ae. albopictus* (Jackson et al. 2012). The effect of LACV infection on nectar-feeding has not been studied. A constant sugar source was available to all adult mosquitoes throughout the duration of the study, but frequency and quantity of blood or nectar-feeding was not calculated. LACV infected mosquitoes might have taken smaller bloodmeals and fed on sugar less frequently resulting in depressed energy reserves in the subsequent

diapause generation; however, the size of the eggs did not differ intraspecifically. Lipid content, a metric in which to measure energy reserves in diapausing insects (Hahn and Denlinger 2010), was not quantified for LACV-infected or uninfected mosquito eggs. Possible smaller bloodmeals taken by infected individuals may explain the higher embryo and larval mortality rates due to depleted energy reserves needed to survive overwinter and eventually develop into adult mosquitoes. Despite rearing conditions being the same, fewer LACV-infected larvae for both *Ae. triseriatus* and *Ae. albopictus* survived until the fourth instar indicating the adverse effects of LACV infection progress into the larval life stage. This contradicts previous laboratory studies (Patrican and DeFoliart 1985). In addition to *Ae. triseriatus* being more cold-acclimated and having coevolved with LACV as opposed to *Ae. albopictus*, the tendency of LACV-infected *Ae. triseriatus* to refeed during one gonotrophic cycle could also have increased their survivability overwinter (Jackson et al. 2012).

Installment hatching, or the process where eggs hatch over a number of different exposures to hatching stimuli, is an important mechanism in the maintenance of LACV. This enables virus-infected individuals to emerge at different times. However, our results did not indicate a major difference in the termination of diapause based upon LACV infection or show a clear trend for hatching attempts per time point. Termination of diapause occurred sooner in *Ae. triseriatus* when compared to *Ae. albopictus* which coincides with their respective phenologies (Barker et al 2003) (Tables 2 and 4).

Vertical transmission rates were higher in *Ae. triseriatus* than in *Ae. albopictus* which concurs with previous studies, but due to the injection of LACV directly into the hemolymph bypassing the midgut barriers, vertical transmission rates were higher than in studies where mosquitoes were orally infected (Hughes et al. 2006). Also, Woodring et al. (1998) reported

different filial infection rates of LACV and *Ae. triseriatus* based upon geographic locations of populations and reduced diapause phenotypes reduced filial infection rates. We used a local strain of LACV and locally collected mosquitoes to more accurately evaluate the natural cycle in this area. Vertical transmission rates did not increase or decrease with embryo mortality. The reason is unknown (Table 3).

LACV-infected colonies of both *Ae. triseriatus* and *Ae. albopictus* exhibited an intraspecific increase in missing eggs as opposed to uninfected colonies. Missing eggs from hatching treatments could indicate an underestimation of mortality as these embryos could have died and were not used in embryo mortality calculations or possibly, these eggs were viable and would have hatched at a later hatching treatment. The natural adhesive used to attach the eggs to the oviposition substrate may have been weaker in LACV-infected colonies. This could affect the ability of LACV-infected eggs to remain attached to different substrates and subsequently be exposed to hatching stimuli more frequently and earlier than uninfected eggs by becoming detached from the intended oviposition location. While the seed germination paper used in this experiment is commonly used in the laboratory, differing oviposition sites, from treeholes to artificial containers, may be more or less beneficial to overwintering LACV-infected mosquitoes as egg adherence and ability to remain in place through adverse winter conditions and repeated premature submersion may vary.

Humidity was not used as a factor in this study as the four layers of seed germination papers maintained their moisture content in each sealable plastic bag through the duration of the experiment without the need for additional water. Due to the hole punctured in each plastic bag, these conditions are similar to some artificial overwintering habitats that contain a water-source directly below oviposited *Aedes* mosquito eggs. Desiccation is a major limiting effect for

mosquito egg survival overwinter (Sota and Mogi 1992, Hanson and Craig 1994, Urbanski et al. 2010), and its impact on LACV transovarially infected competent vectors should be evaluated in future studies.

Our results display a greater percent embryo mortality in both infected and uninfected Ae. triseriatus compared to McGraw et al. (1998) while winter temperatures were lower in their experiment. This may be due to intrathoracic injections performed in our experiment while McGraw et al. (1998) orally infected their mosquitoes. How intrathoracic injections affect fitness of the following generation should be studied to rule this out as a factor. Conversely, our study used the same F<sub>2</sub> generation of eggs for both LACV-infected and uninfected colonies, and due to injections of noninfected mosquitoes with cell media we conclude the differences noted in this study are due to LACV infection and not the treatment.

While we documented adverse effects of LACV infection on the embryonic and larval survivability in *Ae. triseriatus* and *Ae. albopictus* overwintering mosquitoes, the majority of LACV-infected *Ae. triseriatus* survived the winter, and 42.33% of LACV-infected *Ae. albopictus* embryos survived the winter. *Aedes triseriatus* is more efficient at LACV overwinter maintenance than *Ae. albopictus*, but the presence of *Ae. albopictus* in temperate areas may provide an additional amplification and survival mechanism for LACV through winter months. Due to the method of injection, these estimates of survival are likely underestimates of true survivability of naturally infected mosquito embryos. Further studies should be conducted to better understand the importance of the ability of *Ae. albopictus* to overwinter with LACV. As temperate winters influencing the disease ecology and epidemiology of this virus. Our study indicates the importance of both vertical and horizontal transmission in the natural cycle of

LACV. With a fewer number of LACV infected individuals surviving the winter, summer horizontal amplification is likely needed.

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# 4.7 Figures and Tables

Table 4.1. Environmental Conditions in Blacksburg,	Virginia from November 21, 2016 to April
24, 2017.	

Weeks PDI	Date Range	Average High (C°)	High Range (C°)	Average Low (C°)	Low Range (C°)	Average Civil Daylight (hrs)	Civil Daylight Range (hrs)
0-2	Nov. 21, 2016 - Dec. 5, 2016	11	2 - 19	0	-5 - 8	10:51	10:44 - 11:01
3-7	Dec. 6, 2016 - Jan. 9, 2017	6	-8 - 15	-3	-15 - 6	10:39	10:36 - 10:46
8-12	Jan. 10, 2017 - Feb. 13, 2017	11	0 - 22	0	-11 - 8	11:14	10:47 - 11:48
13-17	Feb. 14, 2017 - Mar. 20, 2017	11	9 - 17	-1	-12 - 8	12:29	11:50 - 13:09
17-22	Mar 21, 2017 - Apr. 24, 2017	19	6-26	6	-5 - 13	13:51	13:12 - 14:30

Table 4.2. Length and width of La Crosse virus infected (TOT+) via transovarial transmission and uninfected (TOT-) eggs *Aedes triseriatus* and *Aedes albopictus*. Data were analyzed by one-way ANOVA and Tukey's HSD.

Colony	Average Length (µm)	p value	Average Width (µm)	p value
Ae. triseriatus TOT+ Ae. triseriatus TOT-	700.07 699	0.6780	220 221.6	0.4457
Ae. albopictus TOT+ Ae. albopictus TOT-	597.9 598.1	0.8936	185.3 184.9	0.8887

	LACV TOT+*										LACV TOT-		
Week	Species	N Eggs ‡	% Egg Mortality	N Larvae †	% Larval Mortality	%Diapause	Vertical Transmission Rate		N Eggs ‡	% Egg Mortality	N Larvae †	% Larval Mortality	% Diapause
0	Aedes triseriatus Aedes albopictus	381 435	0 0.46	97 28	6.19 10.71	100 100	23.1 68		622 543	0.64 0	12 63	0 1.59	100 100
2	Aedes triseriatus Aedes albopictus	473 353	6.55 9.63	180 187	1.67 1.07	100 100	50.3 22.7		770 882	2.21 1.25	350 359	0.57 0.56	100 100
7	Aedes triseriatus Aedes albopictus	398 410	20.1 18.78	293 330	4.44 3.94	100 100	55.7 33.8		474 838	7.17 5.85	397 597	2.02 1.01	100 100
12	Aedes triseriatus Aedes albopictus	530 565	5.09 9.56	449 494	3.56 16.4	100 100	46 10.9		695 708	6.91 5.65	635 605	0.63 3.67	100 100
17	Aedes triseriatus Aedes albopictus	504 574	38.89 32.23	359 376	18.11 22.07	83.9 100	35 68.9		386 734	11.4 17.3	337 604	2.37 7.95	90.7 100
22	Aedes triseriatus Aedes albopictus	456 521	23.46 57.77	346 232	10.98 14.22	42.4 44.8	47.7 9.5		839 707	18.71 53.18	682 331	3.08 15.71	53.1 35.1
Total §	Aedes triseriatus Aedes albopictus	2742 2858	16.08 22.845	1724 1647	8.18 13.05		45.2 30.2		3786 4412	8.03 13.67	2413 2559	1.78 5.12	

Table 4.3. Effect of La Crosse virus (LACV) infection on overwintering *Aedes triseriatus* and *Aedes albopictus*.

# \* TOT+ transovarially transmitted

- † Number of larvae per sample at a timepoint
- ‡ Number of eggs per sample at a timepoint
- § Total week 0 through week 22

Table 4.4. Percent eggs hatching at each hatch attempt relative to the total hatched for each time point including viable eggs that failed to hatch after four attempts. Differing letters at each time point denote statistical significance at  $\alpha$ =0.05 using pairwise comparisons of  $\chi^2$  and Fischer's exact test statistics.

Week	Colony	LACV +/-	Hatch 1%	Hatch 2%	Hatch 3%	Hatch 4%	Diapause Eggs*
2	Aedes triseriatus	+	0 A	0 A	4.76 A	36.51 A	58.73 A
	neues iniser iaras	-	0 A	0 A	7.57 A	38.91 A	53.52 A
	Andre alle enisters	+	0 A	0 A	56.11 B	2.51 B	41.38 B
	Aedes aldopictus	-	0 A	0 A	37.54 C	3.67 B	58.78 A
	Aedes triseriatus	+	0 A	63.69 A	25.85 A	0.31 AB	10.15 A
7		-	0 A	59.50 A	15.24 B	8.14 C	17.12 B
/	Andra all mintur	+	0 A	79.88 B	19.22 AB	0.00 A	0.90 C
	Aedes albopicius	-	0 A	65.15 A	9.51 C	1.01 B	24.33 D
	1 a dag tuig ani atug	+	0 A	79.45 A	8.70 A	0.59 A	11.26 A
10	Aeues iniseriulus	-	0 A	93.20 B	4.95 B	0 A	1.85 B
12	Aedes albopictus	+	0 A	94.32 B	2.35 C	0 A	3.33 B
		-	0 A	93.95 B	2.39 C	0 A	3.66 B
	Aedes triseriatus	+	16.09 A	70.54 A	0.00 A	2.23 A	11.14 A
17		-	9.37 A	56.73 B	32.46 B	0 B	1.46 B
1 /		+	0 B	97.94 C	1.80 A	0 B	0.26 B
	Aedes albopictus	-	0 B	99.67 C	0.33 A	0 B	0 B
	1 a dag tuig anigtug	+	57.59 A	32.95 A	8.60 A	0 A	0.86 A
	Aeaes iriseriatus	-	46.92 B	48.68 B	4.40 A	0 A	0 A
22		+	55.17 AB	43.97 AB	0.86 B	0 A	0 A
	Aedes albopictus	-	64.95 A	35.05 A	0.00 B	0 A	0 A

\*Viable eggs that remained in diapause after four hatching attempts



Figure 4.1. Embryo mortality in overwintering *Aedes triseriatus* and *Aedes albopictus* eggs. La Crosse virus infected transovarially transmitted (LACV+) and uninfected (LACV-) eggs were subjected to field conditions in Montgomery County, Virginia during the winter of 2016-2017. (A) Random samples of eggs were assayed for embryo mortality at specified weeks post diapause induction. (B) Total cumulative embryo mortality from week 0-22 PDI. Differing letters at each time point denote statistical significance at  $\alpha$ =0.05 using pairwise comparisons of  $\chi^2$  and Fischer's exact test statistics.



Figure 4.2. Larval mortality in overwintering *Aedes triseriatus* and *Aedes albopictus* eggs. La Crosse virus infected transovarially transmitted (LACV+) and uninfected (LACV-) eggs were subjected to field conditions in Montgomery County, Virginia during the winter of 2016-2017. (A) Random samples of eggs were assayed for embryo mortality at specified weeks post diapause induction (PDI). Larva mortality was calculated as larvae that failed to survive until the fourth instar divided by total eclosed larvae for each week PDI. (B) Total cumulative larval mortality from week 0-22 PDI. Differing letters at each time point denote statistical significance at  $\alpha$ =0.05 using pairwise comparisons of  $\chi^2$  and Fischer's exact test statistics.



Figure 4.3. La Crosse virus infected (LACV+) via transovarially transmission and uninfected (LACV-) eggs were subjected to field conditions in Montgomery County, Virginia during the winter of 2016-2017. Eggs at specified time points were subjected to four hatching attempts at each time interval. Eggs were counted after each attempt and those that did not hatch and failed to remain attached to oviposition substrates were considered missing. Differing letters at each time point denote statistical significance at  $\alpha$ =0.05 using pairwise comparisons of  $\chi^2$  and Fischer's exact test statistics.

#### Chapter 5

# Effect of La Crosse virus infection on median lethal temperatures in Aedes triseriatus and Ae. albopictus embryos

# 5.1 Abstract

The relationship between low temperatures and La Crosse virus (LACV) infection in *Aedes triseriatus* and *Aedes albopictus* embryos is of great interest as winter temperatures continue to rise due to climate change. Transovarially-infected and uninfected embryos were exposed to a series of different temperatures and durations to establish lower lethal limits and median lethal temperatures (LT<sub>50</sub>) for 1, 4, 8, 12, and 24 hours. The LACV had a clear effect on *Ae. albopictus* at all durations while LACV only had a clear effect at longer durations in *Ae. triseriatus*; in this case the LT<sub>50</sub>s of LACV infected embryos were higher than those of uninfected. This study has implications for the increased risk of LACV infections after milder winters in temperate regions.

# **5.2 Introduction**

Temperature is a major limiting constraint on insect and mosquito populations, restricting their geographic distributions and seasonal activities (Chown and Nicolson 2004, Thompson et al. 2012). A better understanding of the changes to disease dynamics of arboviruses due to climate change is essential as winter temperatures rise (Epstein et al. 1998, Lafferty et al. 2009, Gale et al. 2010, Mills et al. 2010, Leisnham and Juliano 2012). The La Crosse virus (LACV; family Bunyaviridae, genus Orthobunyavirus) is an emerging disease in the Appalachia region of the United States (Westby et al. 2015, Harris et al. 2015) and is a leading cause of pediatric

encephalitis (Calisher 1994, Rust et al 1999). It utilizes diapausing container-inhabiting *Aedes* embryos to bridge temperate winters through transovarial transmission (Moulton and Thompson 1971, Watts et al. 1974, Beaty and Thompson 1975, Miller et al. 1977, Thompson and Beaty 1977, Borucki et al. 2002, Hughes et al. 2006).

The eastern tree-hole mosquito, *Aedes* triseriatus is the principal vector of LACV and *Aedes albopictus* has emerged as an accessory vector. Populations of overwintering *Ae. triseriatus* eggs regularly survive temperate winters as far north as eastern Canada (Grimstad et al. 1977, Ward 2005, Farajollahi and Price 2013). In laboratory studies, eggs readily survive temperatures as low as -15 °C and -20 °C (Copeland and Craig 1990) but LACV infection does negatively affect overwintering embryos and infected eggs showed higher mortality rates overwinter than uninfected eggs (McGraw et al. 1998). The Asian tiger mosquito, *Aedes albopictus* is a competent vector of LACV and infected individuals have been collected from the field (Gerhardt et al. 2001, Gratz 2004, Westby et al. 2014). It was first introduced to the United States in the mid 1980's and due to a rapid adaptation of overwintering populations, this mosquito has encroached further and further north (Urbanski et al. 2012). While likely an accessory vector of LACV, the exact role of this mosquito in the emergence of LACV in Appalachia remains unclear (Bewick et al 2016).

Vertically or transovarially infected eggs laid in the fall will generate infected adults in the following spring, ready to infect sciurid reservoir hosts commencing amplification to maintain its natural cycle. As such, understanding how LACV infection affects TOT infected embryos exposure to low temperatures is critical to predicting future trends in this disease system.

# **5.3 Materials and Methods**

# Aedes triseriatus and Ae. albopictus mosquito colonies

Both *Ae. triseriatus* and *Ae. albopictus* colonies were collected from Montgomery County, Virginia during the summer of 2017. Reiter gravid traps were used to collect adult *Ae. triseriatus* females which were then allowed to lay eggs onto seed germination paper. The F<sub>4</sub> generation of *Ae. triseriatus* was used in this study and forced copulation was used to ensure fertilization of embryos (Gerberg et al. 1994). A human attractant and aspirator were used to collect adult *Ae. albopictus* that were then offered a sheep blood-meal and oviposition substrate. The F<sub>3-4</sub> generations of *Ae. albopictus* were used in this study. All mosquitoes collected from the field tested negative for natural virus infection by plaque assay.

All mosquitoes were reared according to methods outlined by Kaufman et al. (2017) unless otherwise noted. Eggs were measured according to Bova (2014).

#### Virus strain, assays, and injections

The LACV strain used in this study was originally harvested in Wise County, Virginia from adult and larval *Ae. triseriatus* by Baker et al. (2003). To prepare virus stock, confluent Vero cells were inoculated with virus in M199 complete cell medium (500 ml M199, 27 ml fetal bovine serum, 0.5 ml gentamycin (50 mg/ml), 2 ml Amphotericin B (250  $\mu$ l/ml), and 10 ml penicillin/streptomycin (5000 I.U./ml)) and incubated for 36 hours before harvest and was stored at -80 °C. Approximately .22 $\mu$ l of supernatant with a virus titer of 6.75 log x 10<sup>7</sup> pfu/ml was intrathoracically injected into 3-5-day old adult female F<sub>2</sub> *Ae. triseriatus* and F<sub>3</sub> *Ae. albopictus* mosquitoes (Rosen and Gubler 1974). The same amount of cell medium without virus was injected into control mosquitoes. Termination of injection occurred if the abdomen or thorax appeared to be about to rupture. *Aedes triseriatus* and *Ae. albopictus* L<sub>4</sub> Larvae from the 2<sup>nd</sup> - 4<sup>th</sup> gonotrophic cycles were screened for LACV infection by using modified methods from Goertez et al. (2017). Individual and pools of L<sub>4</sub> larvae were homogenized in M199 complete cell media by two sterilized BBs until sufficiently emulsified and the supernatant was introduced to a Vero cell monolayer in a 96well plate. Any pools or individuals that generated viral CPE were considered positive for LACV infection. Pools of up to 15 L<sub>4</sub> larvae of the second gonotrophic cycle from maternal individuals were tested, and only those individuals that yielded positive pools were allowed to blood-feed again. Their subsequent larvae from their 3-4 gonotrophic cycles were used for low-temperature treatment. Vertical transmission rates were estimated by testing 100 randomly selected individual larvae from each of the 3-4 gonotrophic cycles of each LACV-injected colony.

#### Induction of diapause and cold acclimation

Diapause induction (DIAI) was achieved by exposing LACV TOT infected and uninfected  $F_3$  *Ae. triseriatus* eggs to a short-day photoperiod of 8L:16D at 21°C for 14 days to complete embryo development. The  $F_3$  *Aedes albopictus* pupae and adults were subjected to the same short-day photoperiod and temperature, and the  $F_4$  eggs were also allowed to complete embryo development in these conditions for 14 days. All oviposition papers were then transferred to 10°C under the same photoperiod for 60-70 days for cold acclimation (CA). With 10-20 days remaining before low-temperature treatment, the photoperiod was transitioned to a 16L:8D to enhance hatching.

#### Low-temperature treatment

Eggs from LACV positive and negative, cold acclimated and non-cold acclimated *Ae*. *triseriatus* and *Ae. albopictus* colonies were exposed to the following temperatures: 0 °C, -2 °C, - 5 °C, -7 °C, 10 °C, -12 °C, -15 °C, and -20 °C. Duration of exposure for each temperature treatment was 1, 4, 8, 12, and 24 hours.

A Lauda-Thermostat K-2/R refrigerated circulating water bath (Brinkmann Instruments Inc, Westbury, New York) attached to a Stir Kool Model SK 12 (Thermoelectrics Unlimited Inc., Wilmington, Deleware) was used to lower and maintain temperatures. Temperatures were calculated by Omega thermocouples (OMEGA Engineering, Stamford, Connecticut). To drop the temperature at a rate ≤0.5 °C per minute, 2mm-thick pieces of compressed corrugated cardboard and 5mm-thick aluminum squares were arranged atop the Stir Kool plate depending on the final temperature desired. An insulating arena was constructed from layers of polypropylene thermal insulating sheets and duct tape to encompass eggs and cardboard/aluminum. Each replicate consisting of 20 eggs were placed in 30mm glass petri dishes and covered by parafilm to prevent desiccation. The petri dishes were then placed atop the aluminum and cardboard and enclosed by the insulating arena. A thermocouple was placed directly on the seed germination with eggs to ensure accurate temperatures in which the eggs were exposed (Image 1).

After each treatment, the eggs were allowed to return to room temperatures and were then transferred to a sealable plastic bag with a humidity source and stored under a 16L:8D photoperiod for seven days. They were then introduced to a hatching stimulus consisting of 0.3 grams of nutrient broth dissolved in 100 ml of deionized water for 24 hours. The nutrient broth solution was then strained using fine mesh to retain any detached and unhatched eggs. The mesh and seed germination papers were allowed to dry for 24 hours before a second hatch attempt. The eggs that failed to hatch were then bleached (Trpis 1970) and the number of deceased viable embryos were counted according to Copeland and Craig (1990).

# Statistical analysis

To measure the interaction between low-temperature exposure and duration on mortality, the median lethal temperature ( $LT_{50}$ ) was calculated for each colony at each time point. Interspecific and intraspecific comparisons of  $LT_{50}$ s were calculated using an inverse prediction and logistic fit. Any non-overlapping confidence interval values were considered to be statistically significant. An ANOVA statistical analysis and Tukey's HSD were used to compare means of egg measurements. JMP® Pro 13.0.0 (SAS Institute, Cary, North Carolina, U.S) was used to conduct all statistical analyses.

#### 5.4 Results

There was no significant difference in DIAI/CA and LACV infection on the intraspecific size of *Ae. triseriatus* and *Ae. albopictus* eggs. All *Ae. triseriatus* colony eggs were larger than *Ae. albopictus* colony eggs ( $P \le 0.05$ ) (Table 1).

Duration of low-temperature exposure did have a significant effect on all colonies with each exhibiting higher LT<sub>50</sub>s at 24 hours when compared to one hour (Table 2 and Figure 1). DIA/CA had a significant effect on all *Ae. triseriatus* colonies, regardless of LACV infection, while only a significant effect on not LACV infected *Ae. albopictus* and a less pronounced effect on LACV infected *Ae. albopictus* colonies (Table 2 and Figure 1).

LACV infection had a significant effect on  $LT_{50}$  DIAI/CA *Ae. albopictus* for all time points, but only for hours 4 and 8 in non DIAI/CA *Ae. albopictus* colonies. LACV infection had a significant effect on  $LT_{50}$  DIAI/CA *Ae. triseriatus* only at time points at 8 hours and 24 hours, while LACV infection had very little effect on non DIAI/CA *Ae. triseriatus* colonies. With the exception of DIAI/CA non LACV infected *Ae. albopictus*, all *Ae. triseriatus* colonies have lower LT<sub>50</sub>s than their respective *Ae. albopictus* colonies. (Table 2 and Figure 1).

No *Ae. albopictus* colonies yielded larvae at any duration of exposure to -15 °C while only seven out of the 100 *Ae. triseriatus* DIAI/CA LACV infected eggs hatched and eight out of 100 *Ae. triseriatus* DIAI/CA not LACV infected eggs hatched at -15 °C (Figures 2 and 3). A small but more consistent number of DIAI/CA not infected *Ae. triseriatus* survived -12 °C treatment while small but consistent number of DIAI/CA not LACV infected *Ae. albopictus* survived -10 °C treatment for all time points (Figures 2 and 3). Egg conditioning was more important for survival than infection status.

## **5.5 Discussion**

LACV infection and DIAI/CA does have a negative effect on the ability of *Ae. triseriatus* and *Ae. albopictus* to survive lower temperatures. Previous field studies indicate LACV has an effect on the ability of *Ae. triseriatus* to overwinter (McGraw et al. 1998) but this is the first laboratory study on low temperature thresholds and LT<sub>50</sub>s of any embryos.

Temperature and duration had significant effects on all colonies. Thompson et al. (2012) and Hanson and Craig (1995) reported very little impact of duration of low-temperature exposure on hatch rates of DIAI *Ae. albopictus*, but our results indicate a stronger effect of egg conditioning on survivability of *Ae. triseriatus* and *Ae. albopictus* in this area (Table 1 and Figure 1). Also, our data for uninfected *Ae. albopictus* corresponds to previous studies, but not for uninfected *Ae. triseriatus* (Copeland and Craig 1990, Hanson and Craig 1994, Thomas et al. 2012). In a previous study on tree-hole mosquitoes in the Great Lakes region, *Ae. triseriatus* embryos easily survived 24-hour exposure to -15 C and over 80% survived exposure to -20 C for

24-hours (Copeland and Craig 1990). However, Copeland and Craig (1990) lowered the temperature at a slower rate, 0.7 °C per 10 minutes for their slow-cooled experiment, than in our study; however, when they fast-cooled *Ae. triseriatus* eggs by plunging test tubes containing the eggs in water, 77 percent hatched at -15 °C exposure for 24-hours. Regional differences in populations and method of low-temperature treatment could account for the difference in cold-tolerance between their study and ours. Mosquito populations form northern Indiana are acclimated to much more severe winters than southwestern Virginia. Prolonged and fluctuating low-temperature exposures more accurately reflecting field conditions and are needed to predict the role LACV infection impacts lower lethal limits and  $LT_{50}$ s of *Ae. triseriatus* and *Ae. albopictus* in their natural overwintering habitats.

The LACV infection has a stronger negative effect on the ability of *Ae. albopictus* to survive lower temperatures than *Ae. triseriatus*. Transovarial transmission in *Ae. albopictus* was also lower than *Ae. triseriatus* (Table 3). This indicates the role of *Ae. albopictus* in the overwintering ecology of the LACV remains subordinate to *Ae. triseriatus* in this location. In climate models of certain species, absolute minimum temperatures are relevant (Kreyling 2010); however, further research is needed to reveal what number of individuals of a given mosquito population capable of surviving a given temperature is needed to accurately predict future trends. While previous studies have indicated diapause *Ae. albopictus* eggs being larger than non-diapause eggs. Size of the eggs in this study was not a factor. It is also important to note; the TOT rate was not 100% with *Ae. triseriatus* or with *Ae. albopictus* and no larvae after cold treatment were tested for the presence of virus.

Longer periods of low temperature exposure (up to 8 hours) were needed to see a significant effect of LACV infection on LT<sub>50</sub>s in the principal vector *Ae. triseriatus* but not on *Ae. albopictus*. Furthermore, LACV infection has a stronger effect on *Ae. albopictus* than *Ae. triseriatus* embryos to survive low-temperatures. However, this study did not elucidate the mechanism behind which the LACV has a negative effect on cold-tolerance. Energetic costs of viral replication, primarily translation, could attribute to this effect (Mahmoudabadi et al. 2018), and any factor attributing to cold tolerance during diapause could be affected (Hahn and Denlinger 2011, Denlinger and Armbruster 2016). With temperate areas experiencing fewer prolonged periods of low-temperatures our study adds to the evidence of an increased risk of emergence of LACV after winter months. Current cases of LACV-positive pools of mosquitoes and their reservoir hosts peak in late summer (Szumlas 1996). With more LACV-positive mosquitoes surviving temperate winters the risk to humans could occur earlier in the growing season and be more severe as the virus is amplified through its natural cycle.

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# 5.7 Figures and Tables



Image 5.1. Low temperature exposure apparatus. Oviposition papers containing eggs were folded to retain eggs and placed in glass petri dishes. In addition to using the controls on the Stir-kool plate, corrugated cardboard and aluminum blocks were placed under and on top of the glass petri dishes to achieve the desired temperatures (right). Parafilm was used to seal the glass petri (not pictured) dishes before placing the insulating arena on top of the eggs (left).

Table 5.1. Length and width of La Crosse virus infected and uninfected eggs *Aedes triseriatus* and *Aedes albopictus* that have been induced into diapause and cold acclimated and those not induced into diapause and not cold acclimated. An ANOVA statistical analysis and Tukey's HSD were used to compare means. Numbers with different letters indicate statistical significance at  $P \le 0.05$ .

Colony	N	Average Length (µm)	CI Lower 95%	CI Upper 95%	Average Width (µm)	CI Lower .95	CI Upper .95
Ae. triseriatus ++	20	713.60 A	710.34	716.86	224.55 A	221.56	227.54
Ae. triseriatus +-	20	715.45 A	712.69	719.21	227.25 A	224.26	230.24
Ae. triseriatus -+	20	714.90 A	711.64	718.16	225.20 A	222.21	228.19
Ae. triseriatus	20	718.31 A	715.04	721.56	226.40 A	223.41	229.39
Ae. albopictus ++	20	610.25 B	606.83	613.67	194.52 B	191.35	197.65
Ae. albopictus +-	20	607.80 B	604.38	611.22	193.92 B	190.75	197.05
Ae. albopictus -+	20	604.45 B	601.03	607.87	189.65 B	186.50	192.80
Ae. albopictus	20	606.90 B	603.48	610.32	192.00 B	188.85	195.15

++ = diapause induced and cold acclimated and LACV TOT infected

+- = diapause induced and cold acclimated and not LACV TOT infected

-+ = not diapause induced and not cold acclimated and LACV TOT infected

-- = not diapause induced and not cold acclimated and not LACV TOT infected





-+ = not diapause induced and not cold acclimated and LACV TOT infected

-- = not diapause induced and not cold acclimated and not LACV TOT infected

Figure 5.1. Lethal median temperatures (LT<sub>50</sub>) of cold acclimated/not cold acclimated and diapause/non-diapause induced LACV TOT+/- *Ae. triseriatus* (A) and *Ae. albopictus* (B) colonies at different durations. LT<sub>50</sub>s were calculated by an inverse prediction and logistic fit.
Table 5.2. Lethal median temperatures (LT<sub>50</sub>) of cold acclimated/not cold acclimated and diapause/non-diapause induced LACV TOT+/- *Ae. triseriatus* and *Ae. albopictus* colonies at different durations. LT<sub>50</sub>s were calculated by an inverse prediction and logistic fit and any non-overlapping confidence interval values were considered to be statistically significant.

Time (Hr)	Species	Diapause*	LACV†	LT <sub>50</sub> §	CI Lower 95%	CI Upper 95%
1	Ae. triseriatus	Diapause	TOT +	-10.44 A	-11.01	-9.91
			TOT-	-10.42 A	-11.15	-9.76
		Non-diapause	TOT+	-6.22 B	-6.84	-5.58
			TOT-	-7.43 C	-7.93	-6.95
	Ae. albopictus	Diapause	TOT +	-6.55 D	-7.04	-6.05
			TOT-	-10.61 A	-11.57	-9.79
		Non-Diapause	TOT+	-5.47 D	-6.17	-4.71
			TOT-	-6.13 D	-6.75	-5.49
Δ	Ae. triseriatus	Diapause	TOT +	-9.05 A	-9.63	-8.50
			TOT-	-9.06 A	-9.73	-8.43
		Non-diapause	TOT+	-4.99 B	-5.46	-4.51
			TOT-	-6.16 C	-6.64	-5.69
7	Ae. albopictus	Diapause	TOT +	-5.61 C	-6.09	-5.12
			TOT-	-7.32 D	-7.90	-6.73
		Non-diapause	TOT+	-3.30 E	-3.97	-2.54
			TOT-	-0.96 F	-1.98	0.38
8	Ae. triseriatus	Diapause	TOT +	-6.71 A	-7.26	-6.16
			TOT-	-8.24 B	-8.90	-7.60
		Non-diapause	TOT+	-5.44 C	-5.91	-4.97
			TOT-	-5.31 C	-5.72	-4.90
	Ae. albopictus	Diapause	TOT +	-3.66 D	-4.08	-3.24
			TOT-	-6.64 A	-7.17	-6.11
		Non-diapause	TOT+	-2.81 E	-3.37	-2.19
			TOT-	-1.53 F	-2.36	-0.48
	Ae. triseriatus	Diapause	TOT +	-6.52 A	-7.12	-5.92
12			TOT-	-7.20 B	-7.87	-6.53
		Non-diapause	TOT+	-4.16 C	-4.61	-3.69
			TOT-	-4.56 C	-4.98	-4.14
	Ae. albopictus	Diapause	TOT +	-3.28 D	-3.70	-2.83
			TOT-	-5.43 E	-6.03	-4.80
		Non-diapause	TOT+	-2.49 F	-2.94	-2.01
			TOT-	-1.70 G	-2.16	-1.19
24	Ae. triseriatus	Diapause	TOT +	-5.54 A	-6.12	-4.93
			TOT-	-7.17 B	-7.73	-6.60
		Non-diapause	TOT+	-3.36 C	-3.76	-2.94
			TOT-	-3.97 C	-4.30	-3.61
	Ae. albopictus	Diapause	TOT +	-3.18 C	-3.53	-2.81
			TOT-	-5.26 A	-5.72	-4.78
		Non dianausa	TOT+	-0.61 E	-1.01	-0.13
		non-utapause	TOT-	-1 39 F	-1 74	-1.02

\* Diapause = diapause induced and cold acclimated

Non-diapause = non-diapause induced and not cold acclimated

† TOT+ = Transovarially transmitted

TOT- = Not transovarially transmitted

 $LT_{50}$  = Lethal median temperature



Figure 5.2. Hatching success of diapause induced and cold acclimated (DIAI/CA) LACV positive and negative (+/-) *Ae. triseriatus* mosquitoes after exposure to low-temperatures over a 24-hour period. Five replicates of twenty mosquito eggs were exposed to temperatures that ranged from 0°C to -20°C. No mosquitoes survived any duration of exposure to -20°C and were not included in the figure.



Figure 5.3. Hatching success of diapause induced and cold acclimated (DIAI/CA) LACV positive and negative (+/-) *Ae. albopictus* mosquitoes after exposure to low-temperatures over a 24-hour period. Five replicates of twenty mosquito eggs were exposed to temperatures that ranged from 0°C to -20°C. No mosquitoes survived any duration of exposure to -20°C and were not included in the figure.

Table 5.3. Estimated vertical transmission rates of LACV in diapause induced and cold acclimated *Ae. triseriatus* and *Ae. albopictus* mosquitoes and non-diapause induced and not cold acclimated *Ae. triseriatus* and *Ae. albopictus* mosquitoes. One hundred whole-body L<sub>4</sub> larvae were individually tested for virus from each colony. Only eggs from the third and fourth gonotrophic cycles were used from maternal mosquitoes whose pooled second gonotrophic larvae tested positive for LACV.

Species	Diapause*	Vertical Transmission Estimate (%)
Ae. triseriatus	Diapause Non-diapause	69 74
Ae. albopictus	Diapause Non-diapause	53 51

\* Diapause = diapause induced and cold acclimated Non-diapause = not diapause induced and not cold acclimated

#### **Chapter 6**

## **Summary and Conclusions**

#### 6.1 Summary and Conclusions

The La Crosse virus overwinters by exploiting diapause induced embryos of *Aedes* mosquitoes. In recent years, this virus has emerged in a new geographic area, the Appalachian region. Why this has occurred remains uncertain. Only one study is available that suggests LACV negatively affects the overwintering abilities of *Ae. triseriatus* (McGaw et al. 1998). Based upon this study we designed experiments in attempt to answer the following questions:

## What is the prediapause stage of Aedes japonicus japonicus?

The prediapause stage of *Ae. j. japonicus* is the maternal life-stage. We exposed different *Ae. j. japonicus* life stages to short-day photoperiods and established the maternal generation is sensitive to the environmental cues needed to induce diapause. The female will subsequently lay eggs with diapausing pharate first instar larvae that will survive temperate winters. Using these data and those from previously published literature we used the phylogeny from Soghigian et al. (2017) to track the evolution of the prediapause stages of embryonic diapause across the phylogeny of the *Aedes* genus. We hypothesize the prediapause embryo evolved in mosquitoes that displayed obligate diapause in their evolution to survival in more poleward climates. As the populations migrated into more temperate areas or as temperatures increased in certain areas, these mosquito embryos remained photosensitive. This is the case in species like *Ae. canadensis*, *Aedes campestris*, and *Aedes geniculatis* where they demonstrate both obligate and facultative diapause populations. The maternal prediapause stage, as in *Ae. j. japonicus*, may possibly be

the ancestral form of diapause because distantly related species utilize this strategy. Or it may be the strategy utilized by tropical species migrating into more temperate areas. Future directions and research should look at the molecular mechanisms driving prediapause in *Aedes* to tease apart how this fascinating system evolved. Further basic research on previously undescribed prediapause stages of certain species should also be evaluated. Much of the prior literature on the prediapause stages of *Aedes* mosquitoes is several decades old. Populations of certain species have undoubtedly been affected by climate change and how this may have influenced their diapause induction should be assessed. Our study adds to the evidence that diapause evolved multiple times in this genus and adds basic knowledge to the increasingly relevant vector *Ae. j. japonicus* (Chapter 1).

# Does LACV infection affect the ability of Aedes triseriatus and Aedes albopictus to enter diapause and their critical photoperiods?

The LACV does not affect the ability of *Ae. triseriatus* or *Ae. albopictus* to enter diapause and has a minor but probably biologically negligible effect on *Ae. triseriatus* and *Ae. albopictus* critical photoperiods (CPP). We exposed the prediapause stages of LACV infected and uninfected *Ae. triseriatus* and *Ae. albopictus* to different short-day photoperiods to calculate CPPs in these species. We based this study on methods from Shroyer and Craig (1983) and Urbanski et al. (2012). Because overwintering success of LACV relies on the overwintering success of their mosquito host, it is unsurprising there is very little effect on the integral first stage of this process. Future research should focus on how climate change and time may have changed populations of *Ae. triseriatus*' CPP and also on how exactly these mosquitoes sense light to better translate laboratory experiments to the mosquito's natural environment. The effect of altitude on CPPs in these species should also be studied (Chapter 3).

Does LACV infection affect the ability of Aedes triseriatus and Aedes albopictus to overwinter in Blacksburg, Virginia?

The LACV inhibits the ability of Ae. triseriatus and Ae. albopictus to overwinter in natural conditions of Blacksburg, Virginia. We infected and induced diapause in these species and placed eggs in the field to be subjected to natural temperatures over the winter months. At set time intervals we collected eggs from the field and assessed their mortality rates and also the subsequent larval mortality rates. We also were able to calculate how many eggs were lost over the successive hatching attempts each week. The LACV had a negative effect on embryo mortality rates, larval mortality rates, and lost eggs in Ae. triseriatus and Ae. albopictus. There was a more severe negative effect on Ae. albopictus than on Ae. triseriatus. This study was based upon McGaw et al (1998). The data generated adds to our current understanding of the vector status of these two species and supports Ae. triseriatus as a more important host for LACV overwinter; however, Ae. albopictus could have important impacts on the overwintering ecology of LACV. Prospective research should scrutinize Ae. j. japonicus in this system and conduct extensive field research to better understand how the two invasive species could overwinter with the LACV. Our study was the first to show evidence of a negative impact on larval mortality due to LACV infection. As to why mortality was higher in LACV embryos and larvae remains a mystery. The effect of LACV on energetics should be scrutinized, along with the presence of cryoprotectants and macromolecules needed for survival. A more focused study should clarify

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these results along with the ability of LACV-infected *Aedes* egg's ability to remain attached to the oviposition substrate (Chapter 4).

# *Does LACV infection affect the ability of* Aedes triseriatus *and* Aedes albopictus *to survive lowtemperature exposures in the laboratory*?

The LACV does have a negative effect on the ability of Ae. triseriatus and Ae. albopictus to survive low-temperatures in the laboratory. We exposed diapause induced and cold acclimated, LACV infected and uninfected Ae. triseriatus and Ae. albopictus embryos to different durations of increasingly low temperatures. We were able to establish median lethal temperatures (LT<sub>50</sub>) at different time points for the two species. We based our study on Thomas et al. (2012) and Hanson and Craig (1994,1995). Cold acclimated and diapause induced embryos of both species regardless of LACV infection survived lower temperatures than those not cold acclimated or diapause induced. In diapause induced and cold acclimated colonies LACV had an increasing negative effect on Ae. triseriatus the longer the exposure, while it had an immediate negative effect on Ae. albopictus regardless of duration of exposure. This laboratory study adds evidence to Chapter 4 as to how these mosquito species respond to low-temperatures and highlights the critical role of both horizontal and vertical transmission. With less than 100% vertical transmission rates and a negative effect on overwinter survival, horizontal transmission is likely needed to increase infections to maintain the virus in summer months. Future research to be conducted is similar to those suggested from Chapter 4. Physiological effects of LACV infection should be evaluated as they pertain to low-temperature exposures. Energetics, cryoprotectants, and other factors influencing diapausing mosquito eggs ability to survive low

temperatures should all be assessed as they pertain to LACV infection. This is important in predicting future trends of the overwintering ecology of this virus (Chapter 5).

## 6.2 References

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