DISSERTATION

ANALYZING THE ROLE OF THE *AEDES TRISERIATUS* INHIBITOR OF APOPTOSIS 1 GENE IN TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2007

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ABSTRACT OF DISSERTATION

ANALYZING THE ROLE OF THE *AEDES TRISERIATUS* INHIBITOR OF APOPTOSIS 1 GENE IN TRANSOVARIAL TRANSMISSION OF LA CROSSE VIRUS

Aedes triseriatus is the primary vector of La Crosse virus (LACV) in North America. The following studies were performed using field collections to elucidate the role of the *Ae. triseriatus* inhibitor of apoptosis 1 gene (AtIAP1) in conditioning TOT and to compare LACV ovarian titers in field collected mosquitoes with several laboratory *Ae. triseriatus* strains.

Mitochondrial DNA from field collected *Ae. triseriatus* mosquitoes showed few genetic differences and suggested that mosquitoes from within the collection area exist as a panmictic population. Most importantly, polymorphisms conditioning TOT should be distributed throughout the collection area.

The AtIAP1 gene was amplified from field collected LACV +/- *Ae. triseriatus* mosquitoes. Sequence analysis revealed that this gene is highly polymorphic, but that none of these polymorphisms appear to be involved in conditioning TOT of LACV. Besides being a highly polymorphic gene, AtIAP1 mRNAs contain variable sequences in the 5'UTR. Genomic analysis showed these variable sequences are followed by *Drosophila* splicing motifs suggesting that mRNA variants are produced by alternative

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splicing. Q-RT-PCR analysis revealed differences in mRNA variant expression profiles in *Ae. triseriatus* life stages and tissue types. Similar studies revealed that there are significantly lower levels of AtIAP1 mRNA variant 3 in TOT permissive and LACV infected *Ae. triseriatus* mosquitoes when compared to TOT refractory and LACV uninfected mosquitoes. These studies suggest that expression of AtIAP1 mRNA variant 3 may be involved in conditioning TOT.

Finally, LACV S-segment genome equivalents (GE) were quantified in the midguts and ovaries of transovarially infected TOT permissive, TOT refractory, AIDL colony, and field collected *Ae. triseriatus* mosquitoes. The most intriguing result is that a small proportion of the LACV infected field mosquitoes have LACV GE levels similar to laboratory strains. This result suggests that some females develop stabilized, high titered LACV infections and possibly play a more important role in LACV persistence in nature.

These studies have added to the short list of alternative splicing examples in arthropod disease vectors and suggest that AtIAP1 mRNA expression may play a role in conditioning TOT. These studies also lay the groundwork for determining the role of stably infected *Ae. triseriatus* mosquitoes in LACV persistence.

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ACKNOWLEDGEMENTS

I would like to thank my committee members: Dr. Barry J. Beaty, Dr. William C. Black IV, Dr. Carol D. Blair, Dr. Chester G. Moore, and Dr. Barry R. Miller for all of their help during my doctoral studies. In particular, I would like to thank Dr. Beaty for all of his guidance during the research process and for taking the time to help me when I was struggling. I am deeply appreciative that he allowed me to work on a project I found interesting and his support in allowing me to graduate in a timely manner. I would like to thank Dr. Black for teaching me more than I will ever remember about population genetics and statistics. He was incredibly patient with me while I struggled with different computer programs and analyses. I would also like to thank everyone who helped me with one experiment or another at AIDL. I couldn't have gotten through it without your help. I can't thank Chris Cirimotich, Doug Brackney, Steve Erb, Sara Reese, Ian Sutherland, and Jason Richardson enough for their friendship and for making it easy to come to work every day.

I would also like to thank my parents who have been incredibly supportive over the many, many years that I have been in school. They taught me to go after what I wanted and to never give up. Finally, I would like to thank my wife, Andrea. Without her love, support, and confidence in me I never could have completed this program.

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Chapter 1:

Literature Review

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1. Introduction

Vector-borne diseases are responsible for an incredible amount of morbidity and mortality throughout the world. These diseases are caused by infection with many types of pathogens including viruses, bacteria, and parasites; the most prevalent vector-borne disease is malaria. *Plasmodium* parasites cause between 300 and 500 million cases of human malaria per year (Campbell, 1997). This disease has the greatest effect on children, with roughly 75% of the more than one million yearly deaths occurring in children under five years old (Winstanley et al., 2004). There are currently treatments available for malaria infections as well as for control of malaria-transmitting anopheline mosquitoes; however, the parasites and the mosquitoes are capable of developing resistance to drugs and insecticides, respectively, increasing the necessity for continued research on these topics.

Arthropod-borne viruses (arboviruses) replicate in both the invertebrate vector and the vertebrate host. Viruses that only infect arthropods, and typically cause untoward effects, are not considered arboviruses. The arbovirus that causes the most morbidity and mortality is dengue virus, which causes dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Roughly 2.5 billion people are at risk from dengue virus infection and there may be as many as 100 million new dengue infections each year (Gubler, 2002). Currently, the only dengue virus prevention strategy is vector control (Gubler, 2006). While there are fewer arboviral infections in the United States than in the other parts of the world, several indigenous arboviruses cause serious human disease including West Nile virus, La Crosse virus, St. Louis encephalitis virus, and eastern equine encephalitis virus (Briese et al., 1999, Calisher, 1994).

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La Crosse virus (LACV) is an important cause of pediatric arboviral encephalitis in the United States (McJunkin et al., 1998, Rust et al., 1999, Thompson et al., 1965). LACV is most commonly transmitted by *Aedes triseriatus* mosquitoes and is found primarily in the upper Midwestern United States (Thompson et al., 1972, Watts et al., 1972). LACV was first isolated from the brain tissue of a 4-year-old girl in La Crosse, Wisconsin, who died of encephalitis (Thompson et al., 1965). The virus has the greatest impact on children younger than fifteen years of age where symptoms range from flu-like to encephalitis and death. There are on average 73 cases of LACV infection reported annually, however, this number is probably grossly underestimated due to misdiagnosis and subclinical infections (Jones et al., 1999). Throughout the United States estimates of LACV incidence, including subclinical cases, are as high as 300,000 human infections per year (Rust et al., 1999). The recent introduction of another efficient LACV vector, *Ae. albopictus*, into the United States will most likely serve to increase LACV incidence (Gerhardt et al., 2001, Sprenger & Wuithiranyagool, 1986).

Arthropod-borne viruses are clearly very important causes of morbidity and mortality around the world, and some have also recently been classified as serious bioterrorism and emerging disease threats. In fact, the National Institutes of Health priority pathogens list contains viruses from the major arbovirus families (*Togaviridae*, *Flavivirdae*, and *Bunyaviridae*) including representatives from four of the five genera that make up the *Bunyaviridae* family (http://www3.niaid.nih.gov/biodefense/bandc_priority.htm). Continued research and better understanding of both vectors and viruses will help to alleviate the burden these viruses place on the people and animals of the world.

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2. The Bunyaviridae

A. Introduction and Medical Importance

Bunyamwera virus (BUNV) is the prototype virus of the *Bunyaviridae* family. It was originally isolated in 1943 from *Aedes* mosquitoes collected in the Bunyamwera region of the Semliki Forest in Uganda (Smithburn et al., 1946). This virus, along with several others, did not fit into the classic arbovirus serogroups (known as A and B, which later became the *Alphavirus* genus of the family *Togaviridae* and the *Flavivirus* genus of the family *Flaviviridae*, respectively) and so were classified as the Bunyamwera supergroup and eventually the family *Bunyaviridae* (Casals, 1963). There are currently over 300 members of the family Bunyaviridae (Calisher, 1996), which can be placed into five distinct genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (Schmaljohn & Hooper, 2001).

Members of the *Orthobunyavirus* genus are predominantly transmitted by mosquitoes, while *Nairoviruses* are transmitted by ticks, *Phleboviruses* by sandflies and ticks (some by mosquitoes), *Tospoviruses* by thrips, and *Hantaviruses* are transmitted in the excreta of rodents (Nichol, 2001).

Of the five *Bunyaviridae* genera, only the *Tospovirus* genus has no members that are pathogenic to humans. This genus contains plant viruses such as tomato spotted wilt virus for which the genus is named. The remaining four genera all have notable human pathogens that can be found on the NIH list of priority pathogens including La Crosse virus (genus *Orthobunyavirus*), Rift Valley fever virus (genus *Phlebovirus*), Crimean-Congo hemorrhagic fever virus (genus *Nairovirus*), and Sin Nombre virus (genus *Hantavirus*) (Nichol, 2001). Human disease, caused by members of the *Bunyaviridae*

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family, comes in a variety of forms including encephalitis, febrile illness, hemorrhagic fever, hantavirus pulmonary syndrome, and hemorrhagic fever with renal syndrome (Nichol, 2001).

B. Physical Characteristics of the *Bunyaviridae*

Viruses in the family *Bunyaviridae* are spherical particles that are 80-120 nm in diameter with 5-10 nm projections protruding from their surface (Schmaljohn & Hooper, 2001, Talmon et al., 1987). For one member of the family, La Crosse virus, there are approximately 650 spikes on each virion consisting of heterodimers of the G1 and G2 glycoproteins (Obijeski et al., 1976a, Schmaljohn & Hooper, 2001). The spherical virions have an envelope that is obtained from budding through the Golgi membrane (Bishop & Shope, 1979).

Each virion contains a tripartite, single-stranded, negative-sense, RNA genome made up of a large, medium, and small (L, M, and S) segment. One copy of each segment must be incorporated into the virion for it to be viable, however, it is possible to have multiple copies of any segment in the virion and maintain infectivity (Bishop, 1996). The nucleotides are complexed with the nucleocapsid and the polymerase proteins, and all three segments are circular due to complementary sequences at the 5' and 3' ends of the molecule (Hewlett et al., 1977, Obijeski et al., 1976b). These complementary sequences form a panhandle structure and are the same for all three RNA segments (Hewlett et al., 1977). The terminal sequences are highly conserved within a genus, but differ among genera (Schmaljohn & Hooper, 2001). However, the coding strategy and function of each segment is relatively conserved throughout the entire family.

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C. The Large (L) RNA Segment – Structure and Function

The *Bunyaviridae* L segment codes for an RNA-dependent RNA polymerase (RdRp) (Jin & Elliott, 1991). The L segment of Bunyamwera virus is 6875 nucleotides in length and encodes a polypeptide that is 2238 amino acids in length (Elliott, 1989). The La Crosse virus L segment has been completely sequenced and is of similar length (6980 nucleotides) to its Bunyamwera counterpart (Roberts et al., 1995). In addition to its function as an RdRp, the L protein also has endonuclease activity that is responsible for the "cap-scavenging" that allows bunyaviruses to use host mRNA caps to prime synthesis of viral mRNAs (Jin & Elliott, 1993). The RdRp is able to use either positive-sense or negative-sense RNA as a template, allowing it to function in both genomic replication and mRNA production (Jin & Elliott, 1993). The polymerase gene contains several RNA-dependent polymerase motifs, known as the "polymerase module," that are conserved among RNA-dependent polymerases of negative-sense RNA viruses. In addition, there are conserved sequences found only in the segmented, negative-sense RNA virus families (*Orthomyxoviridae, Arenaviridae,* and *Bunyaviridae*). These conserved regions may play a role in "cap-scavenging", which doesn't occur in the non-segmented negative-sense RNA viruses (Order *Mononegavirales*) (Muller et al., 1994).

D. The Medium (M) RNA Segment – Structure and Function

The medium (M) RNA segment of the bunyaviruses codes for either two or three proteins. In all five genera this segment codes for two glycoproteins referred to as G1 and G2 (or Gc and Gn based on their proximity to the carboxy or amino termini of the polyprotein formed upon translation). A third protein, NSm, is encoded by the M segment in the *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* genera, but not the

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Hantavirus or *Nairovirus* genera (Elliott, 1997). The M segment contains a large open reading frame that codes for a polyprotein consisting of the two glycoproteins (and NSm in *Orthobunyaviruses* and *Phleboviruses*) (Bouloy, 1991, Elliott, 1997). The *Tospovirus* genus is the only one of the three NSm producing genera to use an ambisense coding strategy to produce this protein (Kormelink et al., 1992). The polypeptide itself has not actually been found in infected cells, probably due to cotranslational processing (Bouloy, 1991). Generally, both of the glycoproteins have a signal sequence that likely leads to cleavage from the polyprotein by host signalases (Fazakerley & Ross, 1989).

The M RNA segment of *Orthobunyaviruses* plays an important role in salivary transmission by mosquitoes, as well as dissemination from the infected midgut. One study using reassortant viruses containing a combination of snowshoe hare virus (SSHV) and La Crosse virus (LACV) RNA segments showed that the natural vector of LACV, *Ae. triseriatus*, is much more efficient at transmitting reassortant viruses with the LACV M segment. The viruses with the SSHV M RNA segment could still infect the salivary glands of *Ae. triseriatus*, however, they were much less likely to be transmitted to an uninfected host (Beaty et al., 1981a). Similarly, when *Ae. triseriatus* mosquitoes were given an infectious bloodmeal with SSHV and LACV reassortants, the midgut became infected greater than 90% of the time regardless of the origin of the M segment. However, disseminated infection occurred in about 98% of mosquitoes infected with a virus containing the LACV M segment and only about 26% of the time when infected with a virus containing SSHV M segment. The glycoproteins encoded by the M segment are an important determinant of viral dissemination from the midgut (Beaty et al., 1982).

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The G1 glycoprotein of *Bunyaviruses* is a class I membrane protein found in the viral envelope (Schmaljohn & Hooper, 2001). In LACV, G1 plays an important role in oral infection of the vector, *Ae. triseriatus*. LACV mutants resistant to neutralization by a specific G1 monoclonal antibody only infected 15% of orally challenged *Ae. triseriatus* midguts. In contrast, the wild type parent virus infected 89% of midguts (Sundin et al., 1987). The G1 glycoprotein is also necessary for infection of invertebrate cells in mosquitoes and in cell culture, as well as vertebrate cell culture (Hacker et al., 1995) and is thought to be the major cell-binding protein in vertebrates (Ludwig et al., 1989; 1991). Not only is this an important cell-binding protein, but G1 also functions as a type II fusion protein and undergoes a conformational change binding to allow fusion with the endosomal membrane and viral entry. The conformational change is triggered by a decrease in pH associated with the endosome (Pekosz & Gonzalez-Scarano, 1996, Plassmeyer et al., 2005). G1 is the most important immunogen during a LACV infection. When mice are vaccinated with both LACV glycoproteins, the majority of antibodies bind to the G1 rather than the G2 glycoprotein. Also, immunization of mice with a recombinant LACV G1 protein leads to complete protection from subsequent infection; however, the correct conformation of the protein is important to immune system stimulation (Pekosz et al., 1995).

The G2 glycoprotein is also a Class I membrane protein found in the viral envelope (Schmaljohn & Hooper, 2001). In LACV infection, the G2 protein is important in binding to the invertebrate midgut epithelium. Presumably there is proteolytic cleavage of the G1 glycoprotein, in the mosquito midgut, which reveals the G2 glycoprotein. Without this cleavage the virus cannot bind to the mosquito midgut

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(Ludwig et al., 1989; 1991). The G2 protein is also involved in trafficking the G1 protein to the Golgi body. The G1 protein by itself or with the NSm protein does not localize to the Golgi body, but when expressed simultaneously with the G2 glycoprotein, it will properly arrive in the Golgi body (Kamrud et al., 1998, Lappin et al., 1994).

The NSm protein is only made in members of the *Orthobunyavirus*, *Phlebovirus* (not all of them), and *Tospovirus* genera. This is the least well characterized protein produced by members of the *Bunyaviridae* family. In BUNV, the NSm protein can be found in the Golgi body of infected cells. The protein is not transported out of the cell with mature virions, but remains in the Golgi body (Nakitare & Elliott, 1993). Recently, the NSm protein of BUNV has been implicated in virus assembly (Shi et al., 2006). Using virus-like particles (VLPs) and reverse genetics the authors demonstrated that mutations in the NSm gene (specifically in transmembrane domains I and V and the luminal domain II) disrupted production of VLPs. The reverse genetics approach also failed to yield infectious virus if these domains were deleted (Shi et al., 2006). In the *Tospovirus* tomato spotted wilt virus (TSWV), the NSm protein is responsible for forming tubules in plant cells, as well as insect cells. These tubules are found near the plasmodesmata of plant cells (Storms et al., 1995). Evidence suggests that the NSm protein is the "movement" protein that transports viral ribonucleocapsids to adjacent cells through the plasmodesmata (Kormelink et al., 1994, Soellick et al., 2000).

E. The Small (S) RNA Segment -- Structure and Function

The S segment of the *Bunyaviridae* codes for either one or two proteins depending on the genus. All five genera code for the nucleocapsid protein while members of the *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus* genera also code for the

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non-structural protein NSs (Elliott, 1997). Similar to the M segment of the *Tospoviruses*, the non-structural protein NSs is transcribed in an ambisense manner in both *Phleboviruses* and *Tospoviruses* (Elliott, 1997). *Orthobunyaviruses* also code for NSs, but do so utilizing overlapping reading frames (the coding sequence for the NSs protein is found within the coding sequence of the N protein in a +1 reading frame). Two separate proteins are made since translation begins from two separate AUG start signals on the same mRNA (Fuller et al., 1983). Because leaky translation accounts for NSs production, this non-structural protein is produced at a much lower rate than the nucleocapsid protein (Schmaljohn & Hooper, 2001).

The most important role of the NSs protein, at least for *Orthobunyaviruses*, is to counteract the mammalian interferon response (Blakqori et al., 2007). When a BUNV reverse genetics system was used to knockout translation of the NSs gene, virus infection induced a strong interferon response in mammalian cells. In contrast, infection with wild type BUNV did not induce increased IFN mRNA or protein (Weber et al., 2002). Blocking the interferon response may increase BUNV virulence. Infection of mice with a BUNV lacking a functional NSs gene resulted in slower cell to cell viral spread in mice, lower titers, and a slower overall course of infection than wild type Bunyamwera virus (Bridgen et al., 2001).

The NSs protein of La Crosse virus has been implicated in inhibition of the RNAi pathway in mammalian cells (293T cells) (Soldan et al., 2005). However, a recent study showed that wt LACV has no fitness advantage over LACV without NSs in mosquito and IFN deficient mammalian cell lines. The same study shows that there is no difference in the RNAi response of mosquito cells when infecting with wt LACV or with LACV

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lacking the NSs gene. These results suggest that the most important role of NSs is to inhibit the IFN pathway in mammalian hosts and that NSs does not play a role in mosquito infection (Blakqori et al., 2007).

Finally, the Bunyavirus NSs protein has a similar amino acid sequence to the *Drosophila* pro-apoptotic protein, Reaper. NSs lacks the N-terminal region of the *Drosophila* Reaper protein, which codes for an inhibitor of apoptosis protein-binding domain; however, the C-terminal region of the protein is similar to that of the reaper protein. Even though the NSs protein lacks the N-terminal portion of Reaper, it inhibits translation in a manner similar to Reaper (Colon-Ramos et al., 2003). Also, the NSs protein can induce apoptosis through caspase activation (Colon-Ramos et al., 2003).

The nucleocapsid (N) protein is associated with genomic RNA and full-length complementary RNA (Obijeski et al., 1976b). When genomic RNA lacking the N protein is presented to the L protein, no transcription occurs (Dunn et al., 1995, Lopez et al., 1995). Therefore, the N protein is required for transcription of the ribonucleocapsids by the L protein in *Orthobunyaviruses* and *Phleboviruses* and most likely for the other three genera as well. The N protein may also initiate a functional change in the L protein. Accumulation of the N protein may signal the viral polymerase to switch from cap scavenging and mRNA production to anti-sense genomic RNA production so that replication can occur (Schmaljohn & Hooper, 2001). The nucleocapsid protein is used to serologically categorize viruses as members of the family *Bunyaviridae*. Originally, the complement fixation test was used to group these viruses into the family *Bunyaviridae* with the nucleocapsid being the principal viral antigen recognized in this test (Shope & Causey, 1962).

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F. Viral Replication in the Family *Bunyaviridae*

The first step in viral replication is attachment to the host cell. This process is mediated by the G1 and G2 glycoproteins. As mentioned previously, LACV is believed to utilize the G1 protein for attachment to mammalian cells and the G2 glycoprotein for mosquito midgut cells (Ludwig et al., 1991), but specific cellular receptors have not yet been identified. The next step in the replication process is entry into the cytoplasm followed by uncoating of the ribonucleocapsids; however little is known about these steps.

Attachment presumably leads to receptor-mediated endocytosis. Inside the endosome, pH change may cause the glycoproteins to undergo a conformational change leading to fusion of the viral envelope to the endosomal membrane mediated by one or both of the viral glycoproteins. This is followed by release of the ribonucleocapsids into the cytoplasm (Schmaljohn & Hooper, 2001).

Upon entry into the cytoplasm, the ribonucleocapsids undergo primary transcription, where viral RNA is transcribed into mRNA. This step is mediated by the viral RNA polymerase that is attached to each RNP (Schmaljohn & Hooper, 2001). The L protein functions both as an RNA dependent RNA polymerase and as an endonuclease to remove the cap from host mRNAs in the cytoplasm (Bishop et al., 1983, Patterson et al., 1984). The polymerase uses these host-derived mRNA caps to prime transcription of viral mRNAs. Transcription of the L, M, and S segments generally leads to the production of one mRNA from each. mRNAs are made of positive-sense RNA that are subgenomic and have a 5' cap that has been removed from a host mRNA. Additionally, the mRNAs are not encpasidated by the nucleocapsid protein (Schmaljohn & Hooper,

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2001). In all five *Bunyaviridae* genera the L segment creates an mRNA that codes solely for the viral polymerase. The M segment of the genome produces one mRNA for all genera, except for the *Tospoviruses*, which also produces an ambisense mRNA. The S segment produces one mRNA for *Orthobunyaviruses*, *Nairoviruses*, and *Hantaviruses*. In the *Tospovirus* and *Phlebovirus* genera two mRNAs are produced using an ambisense expression pattern (Elliott, 1997). Once the mRNAs are produced they are translated. The S and L mRNAs are translated on free ribosomes in the cytoplasm, while the M mRNA is translated as a polyprotein on membrane bound ribosomes (Schmaljohn & Hooper, 2001).

Because the genomes of the *Bunyaviridae* are negative-sense, a positive-sense genome complement, which differs from the mRNAs, must be made as a template to create more negative-sense genomic RNA. Unlike mRNAs the genome complement lacks a host-derived 5' cap and is a full-length complement of the negative-sense genome that is encapsidated by the nucleocapsid protein (Schmaljohn & Hooper, 2001). The factors initiating the switch from mRNA production to genome-complement RNA production to genomic RNA production remain unknown (Schmaljohn & Hooper, 2001). It is possible that accumulation of the nucleocapsid protein initiates this switch, as it does in other negative-sense viruses like vesicular stomatitis virus and Sendai virus (Arnheiter et al., 1985, Blumberg & Kolakofsky, 1981, Blumberg et al., 1981, Lamb & Kolakofsky, 2001). Proteins encoded by the M segment localize to the Golgi apparatus, where virus assembly takes place (Nakitare & Elliott, 1993, Ruusala et al., 1992, Wasmoen et al., 1988). The C-termini of the glycoproteins are found on the cytoplasmic side of the Golgi membrane (with the remainder of the protein found in the cisternae of the Golgi

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apparatus) and are thought to associate with the nucleocapsid protein. The N protein is assembled into nucleocapsids with the genomic RNA segments (Smith & Pifat, 1982). Virions are formed by budding into the cisternae of the Golgi apparatus and are transported to the surface of the cell in vesicles and released through exocytosis (Schmaljohn & Hooper, 2001).

G. Evolutionary Potential of the *Bunyaviridae*

Members of this family evolve through two major processes, mutation and reassortment, and a less studied process, recombination (Beaty & Bishop, 1988, Lukashev, 2005). Spontaneous mutation or genetic drift can lead to point mutations, deletions, or insertions. Error rates range from 1 error every 10^3 to 10^6 nucleotides. These errors cannot be repaired because viral RNA dependent RNA polymerases lack proofreading capabilities (Holland et al., 1992). For *Orthobunyaviruses*, which have genomes of ~12-19 kb in total length, this translates to between 0.019 and 12 point mutations per genomic replication. Neutral point mutations can accumulate over time. For this reason, negative sense RNA viruses tend to exist as quasispecies, (i.e. a group of closely related viruses infecting the same organism whose genomic sequences vary slightly due to low fidelity RNA replication) (Holland et al., 1992). This high mutation rate allows RNA virus evolution to occur rapidly because there are constantly new viruses being produced for selection to act upon. Indeed, of 30 La Crosse virus isolates analyzed (collected as near together as eggs from the same tree hole, and as far apart as several states), none had identical oligonucleotide fingerprints for all three complete genomic segments, indicating sequence variation between all isolates (El Said et al., 1979, Klimas et al., 1981).

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While point mutations are the most common means of viral RNA evolution, genomes of the *Bunyaviridae* family can also undergo segment reassortment and evolve even more rapidly. Dual infection of host cells can lead to the creation of new viruses with one or two segments from either parent virus. For the *Orthobunyaviruses* SSHV and LACV, reassortment of viral segments has been shown to occur both in cell culture and in mosquitoes (Beaty et al., 1981b, Rozhon et al., 1981). Segment reassortments that form viable progeny may be restricted to members of the same serogroup (Bishop, 1985, Pringle et al., 1984). In naive mosquitoes, there is only a short opportunity for dual infection to occur. Mosquitoes infected with one virus become resistant to superinfection with a related *Orthobunyavirus* within 72 hours (Beaty et al., 1983, Sundin & Beaty, 1988). However, when mosquitoes ingest multiple viruses simultaneously or within this 72 hour time period, then reassortant viruses are very likely to be produced (Beaty et al., 1985). In the case of the mosquito-borne *Orthobunyaviruses*, approximately 20% of transovarially infected mosquitoes (mosquitoes infected as embryos by an infected mother) can become superinfected when ingesting an infectious bloodmeal containing a different virus. Furthermore, high frequency reassortment can occur in these superinfected mosquitoes (Borucki et al., 1999).

Not only have reassortant *Bunyaviridae* been generated in the laboratory setting, but several examples have been found in nature. La Crosse virus has been categorized into 3 different viral genotypes (A, B, and C) by oligonucleotide fingerprinting. A LACV isolate from an *Ae. triseriatus* mosquito collected in Rochester, MN contained the S segment from a genotype B virus and the L and M segments from a genotype A virus (Klimas et al., 1981).

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Another example of natural reassortment can be found in Caraparu virus (CARV), which is a group C *Orthobunyavirus*. Sequence analysis of one CARV strain revealed that the S-segment is nearly identical to another group C virus, Oriboca virus (ORIV), while the M-segment is nearly identical to that of the prototype CARV (Nunes et al., 2005). Bruconha virus (BRCV) is another group C *Orthobunyavirus*. The virus was initially isolated from Brazil and was characterized as a new member of group C (Calisher et al., 1983). However, recent studies have reported that the S-segment from this virus is nearly identical to that of CARV and the virus may actually be a reassortant between two viruses rather than a separate virus. Most of the Group C viruses have been associated with a self-limited human disease with symptoms of fever, headache, myalgia, nausea, weakness, etc. that can last for 2-5 days (Nunes et al., 2005). Reassortment in group C viruses may create more virulent viruses, increase vectorial capacity, change host range, etc.

Another example of natural reassortment was seen with Ngari and Garissa viruses. Ngari and Garissa viruses (possibly the same virus) are members of the genus *Orthobunyavirus* that were isolated from hemorrhagic fever cases in Africa. They appear to be reassortants containing the S and L segments of Bunyamwera virus (Gerrard et al., 2004) and the M segment from Batai virus another member of the *Orthobunyaviruses* (Yanase et al., 2006).

Bunyaviridae reassortment isn't restricted to members of the *Orthobunyavirus* genus. It is also possible to generate reassortant *Phleboviruses*. When *Culex pipiens* mosquitoes were inoculated intrathoracically with two strains of Rift Valley fever virus (RVFV) or provided infectious bloodmeals containing two different strains of RVFV,

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reassortant RVFV could be isolated. Similar to LACV, secondary infection needed to occur within 48 hours of primary infection for superinfection and reassortment to occur (Turell et al., 1990). Reassortant RVFV has also been isolated from nature. The RNA of several RVFV isolates have been sequenced and natural reassortants have been detected in both mosquito vectors and mammalian hosts (Sall et al., 1999). Reassortant bunyaviruses have the potential to be dangerous because reassortment may lead to a shift in vertebrate host range, vector range, or virulence.

There is also evidence for intramolecular recombination in several negative-sense RNA viruses including members of the *Bunyaviridae* (Chare et al., 2003, Lukashev, 2005). Crimean-Congo hemorrhagic fever virus is one such example. There is good evidence for recombination in the S segment; however, studies of the L and M segments haven't revealed clear evidence of recombination (Lukashev, 2005). A similar study using Tula hantavirus demonstrated that recombination has likely occurred in at least two points of the S segment between two different strains of the virus (Sibold et al., 1999).

The *Bunyaviridae* are very versatile viruses that can be transmitted by a wide variety of vectors. These viruses are well adapted to their environment and maintain the ability to adapt quickly. Four of the five *Bunyaviridae* genera contain major human pathogens that can be found on the National Institute of Allergy and Infectious Diseases priority pathogens list of potential bioterrorism and emerging disease threats. These viruses are responsible for a significant amount of morbidity and mortality around the world and can potentially be modified for use as bioterrorism weapons. In terms of human disease in the United States, the most important member of the *Bunyaviridae* is La Crosse virus.

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3. Natural History and Epidemiology of La Crosse Virus

A. Introduction

La Crosse virus (LACV) is a member of the Family *Bunyaviridae,* genus *Orthobunyavirus*, species *California encephalitis virus* (ICTV Database). LACV was originally isolated in La Crosse, WI, from the brain tissue of a four-year-old girl who died of meningo-encephalitis. LACV was the first isolate of a *California encephalitis virus* from a human in the United States (Thompson et al., 1965). It has subsequently been isolated from many North American mosquitoes including the primary vector, *Aedes triseriatus* (Thompson et al., 1972, Watts et al., 1972) and the recently introduced mosquito *Aedes albopictus* (Sprenger & Wuithiranyagool, 1986).

Ae. albopictus, the Asian tiger mosquito, was recently introduced into the United States through a port in Texas (Sprenger & Wuithiranyagool, 1986) and has gradually moved north and east. This mosquito can now be found throughout the southeastern United States and in isolated areas as far north as Illinois and Indiana (Darsie & Ward, 2005, Kitron et al., 1998, Moore, 1999). *Ae. albopictus* is an efficient vector of LACV and also possesses the ability to transovarially transmit (TOT) LACV to its progeny (Grimstad et al., 1989, Hughes et al., 2006, Tesh & Gubler, 1975). However, the catholic feeding habits of this mosquito decrease the likelihood that LACV will be horizontally transmitted to a host capable of developing a high titer viremia (Sullivan et al., 1971), TOT allows any bloodmeal to serve as an opportunity for an infected female to maintain or even amplify LACV. TOT of LACV has been demonstrated in the field by LACV isolation from *Ae. albopictus* mosquitoes collected as eggs and reared to adults in the laboratory (Gerhardt et al., 2001). The presence of another successful LACV vector

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could potentially increase the incidence of LACV infections in United States (Grimstad et al., 1989).

La Crosse virus cases occur most frequently in the late summer and early fall. This is likely due to increased mosquito populations, increased numbers of susceptible vertebrate hosts, and increased time humans spend outdoors (Cramblet et al., 1966, Gauld et al., 1974). Generally, infected individuals live in rural areas, or suburban villages where there are more trees and increased mosquito breeding sites (Balfour et al., 1973, Balkhy & Schreiber, 2000, Cramblet et al., 1966). Illness generally occurs in individuals younger than 15 years of age (Kappus et al., 1983). There are roughly 70 cases of LACV infection reported in humans each year (Rust et al., 1999). Most of these are found in the upper Midwestern United States; however there is an increasing incidence in the southeastern United States. Perhaps this is due to the previously mentioned introduction of *Ae. albopictus*, which has displaced *Ae. aegypti*, a very incompetent vector of LACV, from most of its southeastern United States distribution (Black et al., 1989, Hughes et al., 2006).

B. Clinical Manifestations of La Crosse Virus Infection

LACV encephalitis presents initially with flu-like symptoms including headache, fever, malaise, and gastrointestinal symptoms. Generally, the symptoms resolve in 5 – 8 days and the patient recovers. In severe forms of LACV encephalitis, fever and headache are followed shortly thereafter by seizures (Balkhy & Schreiber, 2000). One study of 66 children with LACV meningo-encephalitis revealed that 40% of the children had severe symptomology including seizures, coma, and altered consciousness. Of these children, 15% experienced neurologic sequelae after the infection was cleared including ataxia,

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aggressive behavior, dysarthia (a speech disorder), bilateral Babinski sign, arm weakness, and memory loss (Balfour et al., 1973). A similar study revealed that 66% of patients presenting with severe meningo-encephalitis had neurological sequelae. The sequelae included recurrent seizures and a diagnosis of attention deficit disorder with hyperactivity (ADDH). However, ADDH may have been present prior to LACV infection, but remained undiagnosed until in-depth neurological analyses following LACV infection were performed (Balkhy & Schreiber, 2000). These sequelae are not only a physical burden to the afflicted individuals, but the required care for affected individuals can be a very expensive undertaking. A recent study projects the disability adjusted life years (DALY; representing the number of productive years of life lost) for individuals suffering from neurologic sequelae following LACV encephalitis to be 12.90 to 72.37 and the related healthcare costs to be $48,775 to $3,090,798 (n = 5 patients) (Utz et al., 2003).

C. Natural Life Cycle of La Crosse Virus

La Crosse virus naturally circulates between the primary vector mosquito, *Ae. triseriatus* (Watts et al., 1972), and natural mammalian hosts, gray squirrels (*Sciuris carolinensis*) and chipmunks (*Tamias striatus*) (Ksiazek & Yuill, 1977). These mammalian hosts develop a high-titer viremia allowing for virus transmission to uninfected *Ae. triseriatus* mosquitoes when they take a bloodmeal. A viremia titer of 1.8 ($\log_{10}$) $TCID_{50}$ per 0.1 mL is sufficient to infect ~90% of *Ae. triseriatus* mosquitoes (Watts et al., 1972). Humans do not develop a high titer viremia and thus are incidental or dead end hosts (McJunkin et al., 1998). For horizontal transmission of LACV, once ingested by a mosquito the virus must infect and escape from the midgut and enter the

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salivary glands. Then it can be transmitted to uninfected animals during subsequent bloodmeals (Pantuwatana et al., 1972).

Chipmunks and squirrels do not maintain high titer viremias for longer than 6 days and while these short-term viremias may contribute to LACV amplification during the spring and summer months, they preclude small mammals as overwintering hosts in the northern range of LACV (Pantuwatana et al., 1972). In addition to horizontal transmission of LACV from an infected mammal to an uninfected mosquito during a bloodmeal, LACV can also be transmitted transovarially (TOT) from an infected female to her offspring (Pantuwatana et al., 1974, Watts et al., 1973). When a female *Ae. triseriatus* mosquito becomes infected after feeding on a viremic mammal, she becomes persistently infected for the duration of her life. Generally, the first egg batch laid after a viremic bloodmeal will not contain LACV infected progeny; however subsequent egg batches will contain transovarially infected offspring (Miller et al., 1979). Landmark studies in arbovirology revealed that TOT is the overwintering mechanism of LACV in temperate climates. During the spring, virus isolations can be made from *Ae. triseriatus* larvae before adults have emerged. These larvae hatch from LACV infected eggs that survive the winter (Watts et al., 1973; 1974). Laboratory studies showed that after feeding *Ae. triseriatus* mosquitoes a LACV infectious bloodmeal, the offspring retained the ability to infect a mammalian host with LACV for at least eight generations (Miller et al., 1977). These studies clearly suggest that TOT is very important in allowing LACV to persist in nature and that it is likely the most important overwintering mechanism of LACV in the northern range of *Ae. triseriatus* mosquitoes.

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The TOT rate (the number of LACV infected females that transmit virus to at least one offspring) and the filial infection rate (the percent of an infected female's offspring that are LACV infected) can both exceed 70% (Woodring et al., 1998). The TOT rates are similar in mosquitoes from the northern range of *Ae. triseriatus* (from Wisconsin) and the southern range (from Florida). However, the filial infection rate differs significantly between these strains with *Ae. triseriatus* mosquitoes from Wisconsin having a greater filial infection rate than those from Florida (Woodring et al., 1998). TOT seems to be the most important overwintering mechanism of LACV in temperate climates and is less important in warm climates where horizontal transmission can continue year round. Higher filial infection rates in temperate regions may be necessary to offset mortality associated with LACV infected eggs emerging from diapause. There is significantly greater mortality in TOT infected *Ae. triseriatus* eggs than in uninfected eggs after emerging from diapause, but high FIRs still make TOT a very successful overwintering strategy for LACV (McGaw et al., 1998). Another possible explanation for the discrepancy in filial infection rates is that the northern mosquitoes go into deep diapause to overwinter while southern *Ae. triseriatus* mosquitoes do not. The reduced metabolic activity of diapausing eggs may modulate virus infection in such a way that TOT is more successful in diapausing eggs.

LACV infected females can transmit LACV to both male and female progeny transovarially, which adds another facet to the LACV transmission cycle that can increase LACV prevalence in nature (Beaty & Thompson, 1976, Thompson & Beaty, 1978, Watts et al., 1973). TOT infected males have sufficient virus titers in the accessory sex gland fluids (no virus is detected in the sperm itself) to infect females venereally

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during copulation. When venereal transmission of LACV occurs, disseminated infection occurs in about 3.4% of non-bloodfed females (Thompson & Beaty, 1977), but can occur at rates up to 53% in previously bloodfed females (Schopen et al., 1991). Venereally infected females with disseminated infections are capable of transmitting the virus both horizontally (through an infectious bite) and vertically (through TOT) (Thompson & Beaty, 1978). LACV has a very complex life cycle and has evolved numerous ways to persist and amplify in nature. This complex life cycle takes advantage of all facets of the *Ae. triseriatus* life cycle and makes eradication of LACV a very difficult undertaking.

4. *Aedes triseriatus* Biology

A. Life Cycle of *Aedes triseriatus*

Aedes triseriatus (Say), the eastern treehole mosquito, is found throughout the eastern United States (Darsie & Ward, 2005). *Ae. triseriatus* females lay eggs in basal treeholes (Sinsko & Grimstad, 1977). This mosquito has also become quite well adapted to ovipositing in artificial containers such as discarded tires. After oviposition, eggs may hatch or enter a state of quiescence known as diapause, which is conditioned by the embryo primarily in response to the photoperiod. The photoperiod experienced by the mother has no bearing on whether or not the embryo will enter diapause (Clay & Venard, 1972). Eggs that do not enter diapause will hatch and emerge as 1st instar larvae. The larvae go through four instars, the duration of which are affected by several factors including the amount of food and the larval density in the treehole. These factors also have an effect on mosquito size (Mahmood et al., 1997). After the four larval stages, the mosquito pupates and eventually emerges as an adult. Adult female *Ae. triseriatus* mosquitoes require about 8 days post-emergence to take a sugar meal, mate, and search

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for a bloodmeal (as measured by mark-release-recapture studies) (Haramis & Foster, 1990). Following a bloodmeal, eggs are laid in a treehole or suitable container, and the cycle starts anew. Each gonotrophic cycle requires a bloodmeal, but mating only needs to occur before or during the first cycle. About 14 days are required for the completion of a natural gonotrophic cycle (Haramis & Foster, 1990).

B. Bloodfeeding Behavior of *Aedes triseriatus*

According to studies by Nasci and Burkot, the major blood sources for *Ae. triseriatus* mosquitoes are chipmunks (*Tamias striatus*), deer (*Oedocoileus virginianus*), and gray squirrels (*Sciuris carolensis*). While the numbers from these two studies differed slightly, possibly because collection locations differed, both indicated that chipmunks, deer, and gray squirrels are the top three bloodmeal sources for *Ae. triseriatus* females. Both studies showed a very low level of feeding on humans, which plays an important role in determining the human infection potential of LACV (Burkot & DeFoliart, 1982, Nasci, 1982). Another important observation about *Ae. triseriatus* feeding behavior is that females predominantly feed at ground level during the day. Almost half of the mosquitoes collected during landing catches were caught at ground level, while the remaining samples were caught in roughly equal amounts at five, eight, and eleven meters off the ground. *Ae. triseriatus* seems to feed at lower levels during the middle hours of the day and at higher levels near dawn and dusk (Scholl et al., 1979).

C. Mating Behavior in *Aedes triseriatus*

Laboratory and field studies have provided insight into the *Ae. triseriatus* mating ritual. The beginning stage is swarming, which can be initiated by a single male that changes his flight pattern from straight, short-distance flights to longer lasting zigzagging

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patterns that resemble figure eights (Wright et al., 1966). After one male begins the swarming process more will join, making the flight patterns even more erratic as individuals avoid collision. Swarming generally begins about 2 hours before total darkness and lasts until darkness. This behavior starts roughly 4 days post emergence and persists for life, with mating occurring only during swarming. Females fly through the swarm where a male clasps her legs and eventually lands in a tail-to-tail position. Copulation generally lasts about 55 seconds for blood-fed females and only about 25 seconds for non-blood-fed females (Wright et al., 1966). Although there is a difference in the copulation time, both blood-fed and non-blood-fed females mate and become inseminated at the same rate (Wright & Venard, 1967). Interestingly, LACV infection can alter the insemination rate. There is a significant increase in the insemination rate of *Ae. triseriatus* mosquitoes that have taken a LACV infectious bloodmeal compared to those that have taken an uninfectious meal (Gabitzsch et al., 2006). Increased insemination rates may help to offset deleterious effects that are seen in TOT infected *Ae. triseriatus* eggs when they break diapause (Gabitzsch et al., 2006, McGaw et al., 1998).

D. Diapause in *Aedes triseriatus*

As previously mentioned, *Ae. triseriatus* mosquitoes have the ability to enter a dormant state known as diapause. This condition allows mosquito eggs to survive the winter and to hatch in the spring or summer when conditions are appropriate. The two major factors contributing to whether an egg will hatch or enter diapause are photoperiod and temperature (Kappus & Venard, 1967, Shroyer & Craig, 1983). The response to diminished photoperiod is faster than the response to cooler temperature; however both have the same long-term effect, i.e. eggs do not hatch until diapause is broken. Embryos

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held at a short photoperiod of 10L:14D and a temperature of 21° C, diapause after only 6 days while some embryos held at a long photoperiod (16L:8D) and a low temperature (10° C), remain hatchable until day 21. Not only does decreased photoperiod cause a more rapid diapause response, but photoperiod-induced diapause is also much harder to break than temperature-induced diapause (Shroyer & Craig, 1980). Diapause can be terminated by several different stimuli. The most rapid breaking of diapause occurs when eggs are stored at a cold temperature (4° C) and a short light cycle (10L:14D) for 60 days and then temperature is increased (21° C). Diapause is broken in about 7 days under these conditions. Eggs can also break diapause when stored at 21° C and a short photoperiod (10L:14D) by increasing the photoperiod (16L:8D). Under these conditions it takes more than 60 days to break diapause. Finally, prolonged exposure to low temperatures (4°C) and short photoperiod (10L:14D) seems to help break diapause when conditions are optimal. Increased temperatures and photoperiods are the primary reasons that diapause is broken in *Ae. triseriatus* mosquitoes in the field (Shroyer & Craig, 1983).

Stimuli controlling egg diapause vary with changes in latitude. Over the range of *Ae. triseriatus* (~28° - 50° N latitude) the critical photoperiod required to prevent diapause increased one hour for each 4.2° increase in latitude. While there is a good correlation between critical photoperiod and latitude from 30° - 46° N, *Ae. triseriatus* eggs from the southernmost range (28° - 30° N latitude) fail to enter diapause altogether. To determine the genetic basis of diapause *Ae. triseriatus* mosquitoes from 46° N latitude were crossed with non-diapausing *Ae. triseriatus* mosquitoes from 28° N latitude and progeny were characterized phenotypically. These studies suggested that diapause is controlled by a single recessive locus in the *Ae. triseriatus* genome (Shroyer & Craig, 1983).

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Ae. triseriatus mosquitoes can also enter diapause during their fourth larval instar. Larval diapause occurs when mosquitoes are held at low temperatures with small amounts of food. Diapausing larvae are much less active and need much less food, allowing them to survive for long periods until nutrition is available (Clay & Venard, 1972). An increase in the molting hormone ecdysterone is capable of terminating diapause in 4th instar *Ae. triseriatus* larvae by increasing feeding activity, which in turn leads to pupation (Clay & Venard, 1971). Larval diapause is more likely to be important in the southern portion of the *Ae. triseriatus* range where temperatures can get cold, but not cold enough to freeze the water in the treehole. In the northern United States unpredictable spring weather may also lead to larval diapause (Sims, 1982).

E. Population Genetics of *Aedes triseriatus*

The first study of *Ae. triseriatus* population structure involved allozyme analysis of the esterase-6 locus. This study showed a significant difference in the frequency of esterase-6 allozymes between mosquitoes from two tree stands, one consisting mainly of beech trees and the other mainly of oak trees. The results suggested that esterase-6 alleles are linked to habitat type and that the esterase-6 alleles might influence habitat preference in *Ae. triseriatus* mosquitoes (Saul et al., 1978). However, better sampling techniques and genetic analysis refuted this claim that the esterase-6 locus has any involvement in habitat preference (Matthews & Munstermann, 1983).

Another early study of *Ae. triseriatus* population dynamics suggested that *Ae. triseriatus* mosquitoes exist in "ecological islands" (Sinsko & Craig, 1979). The authors used a mark-release-recapture study to determine vector movement in adjacent wood lots (about 300 m between lots). *Ae. triseriatus* mosquitoes released in one woodlot were

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never recaptured in the adjacent wood lot, nor were they captured in the open fields around and between the wood lots. The lack of recaptured mosquitoes between or in opposite wood lots suggested that there is very little migration occurring between separate woodlots (Sinsko & Craig, 1979).

A subsequent study using genetic markers suggested that *Ae. triseriatus* does not exist in ecologically isolated islands, but rather as a panmictic population (Matthews & Craig, 1980). The authors looked at estimates of heterozygosity over 14 different loci using PAGE and histochemical staining to detect protein differences for 10 different populations of *Ae. triseriatus* mosquitoes. The results showed that the populations shared more than 99% of the genetic variability. In a study of this nature, the less variability shared among individual populations the greater likelihood the populations are isolated. These data suggested that these populations are one panmictic population rather than individual populations existing as ecological islands (Matthews & Craig, 1980).

A final *Ae. triseriatus* population genetics study confirmed that *Ae. triseriatus* mosquitoes exist as a panmictic population and explained previous discrepancies to this theory. This study was performed using a modified and more accurate version of the esterase-6 electrophoresis assay used by Saul et al. (1978). The genetic results revealed no correlation between the esterase-6 locus and habitat and also demonstrated that there was gene flow between separate tree stands, refuting the "ecological islands" hypothesis. The authors postulated that the previous mark-release-recapture studies were not sensitive enough to detect the small numbers of migrants required to maintain homogeneity between woodlots. This study also revealed that mosquito population structures can be

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determined more accurately using genetic analyses rather than mark-release-recapture studies (Matthews & Munstermann, 1983).

F. Selection of Transovarial Transmission Permissive and Refractory Strains of *Aedes triseriatus* and Quantitative Trait Loci Mapping

Transovarial transmission of LACV from a female *Ae. triseriatus* mosquito to her offspring is, at least partially, a genetically-controlled trait. Strains of *Ae. triseriatus* mosquitoes were selected that were LACV TOT permissive and refractory (Graham et al., 1999). The mosquitoes showed a response in both directions. Selection of mosquito strains that have a high percentage of females with the ability to transmit LACV to at least one of their progeny and those with a low percentage of females with this ability was possible. However, when selection for filial infection rate was attempted, there was a response only to selection for a low filial infection rate. Selection for high filial infection rate was unsuccessful. This suggested that the ability of a female mosquito to transovarially transmit LACV to her progeny is controlled by a dominant allele at a single locus. Results indicated that while TOT rate is controlled by genetic factors, filial infection rate is not genetic in nature, but rather is a combination of stochastic factors involving both the mosquito and virus (Graham et al., 1999).

To investigate the genetic basis of TOT, permissive and refractory *Ae. triseriatus* mosquitoes were crossed and progeny were genotyped at 56 random amplified polymorphic DNA (RAPD) loci. Also, 10 cDNA loci were genotyped using single strand conformation polymorphism (SSCP). The results were used to construct a linkage map of the *Ae. triseriatus* genome that could then be used to determine which loci are involved in TOT of LACV. Three quantitative trait loci (QTL) (it is possible that there

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are more) were identified that contribute to the ability of a female mosquito to TOT LACV to her offspring. One QTL was located on chromosome two and two were found on chromosome three, further indicating that there is a genetic component to TOT of LACV by *Ae. triseriatus* mosquitoes (Graham et al., 2003).

G. *Aedes hendersoni*

Aedes hendersoni Cockerell and *Ae. triseriatus* mosquitoes are sibling species that are sympatric over most of their distribution, however the *Ae. hendersoni* distribution extends farther into the western United States than *Ae. triseriatus* (Darsie & Ward, 2005). Crosses between female *Ae. hendersoni* and male *Ae. triseriatus* mosquitoes produces fertile male and female offspring, however the reciprocal cross only produces fertile females (Truman & Craig, 1968). Hybrids formed by these two species have been found in the field and generally exhibit intermediate phenotypic characteristics between the two parents (Grimstad et al., 1974).

While these two species are similar they have some important differences. One of these differences is that *Ae. triseriatus* mosquitoes generally oviposit around ground level while *Ae. hendersoni* mosquitoes tend to be more arboreal and lay their eggs in treeholes 3 – 9 meters above the ground (Scholl & DeFoliart, 1977). Larval collections revealed that *Ae. triseriatus* mosquitoes have longer periods of maximum oviposition during the summer months than *Ae. hendersoni* mosquitoes (10 and 4 weeks, respectively) (Scholl & DeFoliart, 1977). The most important difference between these two sibling species is their vectorial capacity for LACV. Both species are susceptible to disseminated LACV infections following an infectious bloodmeal, however only a small percentage (0 – 10%) of *Ae. hendersoni* mosquitoes are able to transmit the virus to a susceptible host (Paulson

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et al., 1989, Watts et al., 1975a). Studies also showed that *Ae. hendersoni* can actually achieve greater LACV titers in their salivary glands than *Ae. triseriatus* mosquitoes, but fail to effectively transmit the virus. A salivary gland escape barrier is likely responsible for this observation (Paulson & Grimstad, 1989). This observation further indicates that *Ae. triseriatus* is the primary vector of LACV. Genetic comparisons of *Ae. triseriatus* and *Ae. hendersoni* could play an important role in identifying genetic determinants of LACV transmission.

5. Inhibitor of Apoptosis Genes

LACV induces a robust apoptotic response in mammalian cells, but shows no obvious pathology in mosquito cell culture (Blitvich et al., 2002). The LACV NSs protein has the ability to induce the apoptotic trigger, mitochondrial cytochrome C release, in cell-free *Xenopus* egg extracts and promote neuronal apoptosis in a mouse model. This protein is at least partially responsible for the apoptotic effects seen in mammals and bears similarity to the *Drosophila* pro-apoptotic protein, Reaper (Rpr) (Colon-Ramos et al., 2003). It seems that *Ae. triseriatus* must have evolved a system to counteract the pro-apoptotic effects of the NSs protein and survive LACV infection. Indeed controlling apoptosis to prevent oocyte death may be critical for a female *Ae. triseriatus* mosquito to transovarially transmit LACV. Perhaps a logical place to start investigating how *Ae. triseriatus* mosquitoes control LACV infection lies within inhibitor of apoptosis genes.

A. Apoptosis

Apoptosis, or programmed cell death, is an evolutionarily conserved process that regulates the normal development and homeostasis of multicellular organisms by

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removing unwanted, damaged, mutated, or infected cells (Reed, 2000, Twomey & McCarthy, 2005, Zimmermann et al., 2001). Inhibitor of apoptosis proteins (IAPs) play a key role in this process (Deveraux & Reed, 1999, Salvesen & Duckett, 2002). IAPs were first identified in baculoviruses and have since been identified in other viruses, invertebrates and vertebrates (Crook et al., 1993, Deveraux & Reed, 1999, Salvesen & Duckett, 2002). IAPs bind to and inhibit the activity of caspases, a family of cysteine proteases that orchestrate rapid cellular destruction by cleaving various target proteins (Shi, 2002, Shi, 2004). The anti-apoptotic activities of IAPs are antagonized by IAP binding motif (IBM) proteins, a family of pro-apoptotic proteins that share an IAP-binding tetrapeptide motif (Bergmann et al., 2003, Vaux & Silke, 2003, Verhagen et al., 2007). Members of this family include Reaper (Rpr), Head involution defective (HID), Grim, Sickle, and Jafrac2 in *Drosophila*, michelob_x in *Anopheles gambiae*, the michelob_x homolog in *Aedes aegypti*, and Smac/DIABLO, Omi/HtrA2 and GSPT1/eRF3 in mammals.

The defining characteristic of an IAP is the presence of at least one baculovirus IAP repeat (BIR) domain, with many IAPs also containing a C-terminal RING finger motif (Miller, 1999, Salvesen & Duckett, 2002). At least one BIR is required for anti-apoptotic activity, whereas the requirement for the RING motif depends upon the IAP and/or death-inducing stimuli. BIRs contain a highly conserved arrangement of cysteine and histidine residues that belong to a putative zinc-binding fold and function to mediate interactions between IAPs and various other proteins, including caspases and IBM proteins (Roy et al., 1997, Salvesen & Duckett, 2002, Vucic et al., 1997). BIRs also mediate the formation of IAP homodimers, which are the apparent functional form of

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these proteins (Hozak et al., 2000). RING motifs can function as E3 ligases by binding to E2 ubiquitin-conjugating enzymes and recruiting E2s to target proteins (Huang et al., 2000, Olson et al., 2003, Yang et al., 2000). IAPs have been shown to catalyze the ubiquitination of caspases and various IBM proteins, targeting them for proteasomal degradation. The ring motifs of IAPs can also promote auto-ubiquitination and subsequent degradation, which presumably promotes apoptosis.

Only a few apoptosis-related genes from mosquitoes have been characterized, despite the central role that these vectors play in disease transmission (Blitvich et al., 2002, Li et al., 2007, Zhou et al., 2005). Studies on apoptosis regulation in insects have mostly focused on *Drosophila* (Kornbluth & White, 2005). However, the recent completion of the *Anopheles gambiae* genome project has provided considerable insight into the genetic basis of apoptotic regulation in mosquitoes (Christophides et al., 2002). A larger number of apoptosis-related genes are encoded by the genome of *An. gambiae* than *Drosophila*, possibly due to the role that these mosquitoes play in pathogen transmission. For example, 7 IAPs were identified in *An. gambiae* whereas only 4 IAPs (designated *Drosophila* inhibitor of apoptosis 1 (*DIAP1*), *Drosophila* inhibitor of apoptosis 2 (*DIAP2*), *Deterin*, and *Bruce*) exist in *Drosophila* (Christophides et al., 2002, Hay et al., 1995, Jones et al., 2000, Vernooy et al., 2002). The recent availability of the sequence of the *Ae. aegypti* genome, the principal epidemic vector of both yellow fever and dengue viruses, should further increase our knowledge of apoptotic regulation in mosquitoes.

B. *Drosophila* Inhibitor of Apoptosis

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The *Drosophila melanogaster* genome encodes four inhibitor of apoptosis proteins involved in preventing cell death: DIAP1 (also known as thread), DIAP2, Deterin, and Bruce (Hay et al., 1995, Jones et al., 2000, Vernooy et al., 2002). DIAP1 and DIAP2 are in the inhibitor of apoptosis family of proteins because they contain two and three baculovirus inhibitor of apoptosis repeat (BIR) domains, respectively. Both proteins also contain a zinc-ring finger motif at their 3' end. However, the ring-finger motif does not necessarily contribute to inhibition of apoptosis (Hay et al., 1995). In fact the BIR domains alone seem to have greater anti-apoptotic function than the full length proteins (Vucic et al., 1998).

The *D. melanogaster* genome encodes seven functional caspases, which play a role in apoptosis (Vernooy et al., 2000). It is relatively well accepted that Dronc is the initiator caspase for the cell death pathway in *Drosophila*. This caspase contains a long prodomain and a caspase recruiting domain (CARD), typical of apical caspases, and its presence is necessary for cell death in embryonic cells (Quinn et al., 2000). Dark (the *Drosophila* homolog of mammalian Apaf-1 and *C. elegans* CED-4 cell death proteins) forms a complex with Dronc in *Drosophila* cell culture, resulting in the active form of Dronc (Quinn et al., 2000). It was later shown that down-regulation of DIAP1 leads to increased cell death, which begins with the Dronc and Dark proteins (Igaki et al., 2002). One possible apoptosis pathway involves DIAP1, Dronc, Dark, and Drice (Muro et al., 2002). dsRNA knockdown of the caspase Dronc followed by knockdown of DIAP1 leaves cells relatively healthy with very little apoptosis. Dark knockdown followed by DIAP1 knockdown is also quite effective at inhibiting apoptosis. The Dronc protein may be continuously self-processed into an intermediate form that may require the help of

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Dark (Muro et al., 2002). DIAP1 may be involved in removing this intermediate form of Dronc with the ubiquitin ligase activity of the protein. In this model, once DIAP1 levels are decreased, the intermediate form of Dronc accumulates, causing the effector caspase Drice to cleave it into its active form. This final cleavage of Dronc leads to the apoptosis cascade and eventually to cell death (Muro et al., 2002).

Rpr, HID, GRIM, and Sickle proteins have been shown to bind to DIAP1 (Christich et al., 2002, Vucic et al., 1997, Vucic et al., 1998). The N-terminal domains of Rpr, HID, and GRIM are shared; the first 37 amino acids of HID and Grim and the first 14 amino acids of Rpr are sufficient to confer binding to DIAP1 through the BIR motif and therefore increase apoptosis (Vucic et al., 1998). The Sickle gene shares a similar N-terminal region with Rpr, HID, and GRIM, and can also bind to the BIR domain of DIAP1 and increase apoptosis. However, this region alone is not sufficient to upregulate apoptosis in *D. melanogaster* (Christich et al., 2002). It is possible that the Rpr, HID, GRIM, and Sickle proteins are the proteins responsible for binding DIAP1 and blocking its ability to bind and destroy the Dronc intermediate as proposed in the Muro model (stated in the previous paragraph).

C. *Aedes triseriatus* Inhibitor of Apoptosis

A homologue to the DIAP1 protein has been identified in *Ae. triseriatus*. This sequence was originally found during an investigation of host mRNA cap scavenging by LACV. To identify host mRNA species that prime LACV transcription, the 5' end of the LACV S segment mRNA was sequenced. The predominant host sequence targeted by LACV cap scavenging enzymes in infected mosquito cells (C6/36 cells) and post-

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diapausing mosquito eggs was used as a probe to identify *Ae. triseriatus* mRNA with sequence similarity to *DIAP1* (Borucki et al., 2002, Dobie et al., 1997).

The apparent targeting of LACV cap scavenging to an inhibitor of apoptosis mRNA was very provocative. Subsequently, the *Ae. triseriatus* inhibitor of apoptosis 1 (AtIAP1) protein was identified (Blitvich et al., 2002). This gene is located at a single locus on chromosome two. The gene has two BIR domains and a ring-finger motif very similar to that of *DIAP1*. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blots were used to determine the mRNA and protein expression profiles of this gene. mRNA was detected in all life stages of *Ae. triseriatus* mosquitoes and in all adult organs tested (midgut, ovary, and salivary glands). The AtIAP1 protein, however, was only detected by Western blot in first instar larvae, early pupae, and adults. More specifically, AtIAP1 protein was detected primarily in the ovaries and to a much lesser extent in the salivary glands of adult females. This protein may play an important role in preventing LACV from causing apoptosis in mosquito cells (Blitvich et al., 2002). This hypothesis is further complicated because AtIAP1 mRNA has a variable portion in the 5' untranslated region, which may act to control AtIAP1 protein expression in different tissues, life stages, or LACV infection status (Beck et al., In Press).

6. Alternative Splicing of Messenger RNA in Invertebrates

Preliminary studies of the *Ae. triseriatus* inhibitor of apoptosis 1 (*AtIAP1*) gene suggested that gene expression is very complex. The 5' untranslated region (5'UTR) of the AtIAP1 mRNA has a variable region at the proximal end. This variation does not change the protein that is produced, but may play a role in protein expression in different tissue types and life stages. As mentioned above, AtIAP1 protein is only found in the

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ovaries and salivary glands of adult female mosquitoes while the RNA is found in all tissue types (Blitvich et al., 2002). It is possible that the variable region of this mRNA conditions what tissues will express AtIAP1 protein. It is also interesting that AtIAP1 protein is only found in the tissues involved in transmission of LACV and therefore certain mRNA variants may be more important in LACV infection than others.

A. Alternative Splicing in *Drosophila*

Alternative splicing of pre-mRNA in eukaryotic cells occurs at defined locations based on specific sequences. In *Drosophila melanogaster* (as well as *Caenorhabditis elegans* and *Saccharomyces cervisiae*) there are short conserved sequences at the intron boundaries that allow the recognition machinery to find and properly splice out an intron. There is also a conserved branch signal found near the 3' end of the intron. Using computer programs that mimic the splice site recognition machinery to analyze the *D. melanogaster* genome, exact intron boundaries can be correctly predicted about 95% of the time. Genomic analysis shows that the 5' splice site sequence is most commonly AAG/GTAAGTTT (with the / representing the splice site), but there is some variability in the sequence. Similarly, the 3' splice site sequence is most commonly TTTTTTTTTTTTTTTTCAG/AT and again there can be some variability in this sequence. Finally, the intron branch site sequence is generally TACTAAT with some variability possible and is located near the 3' end of the intron (Lim & Burge, 2001). An earlier analysis of pre-mRNAs confirmed that this branch sequence is located near the 3' end of *Drosophila* introns (Keller & Noon, 1985).

B. Alternative Splicing in *Aedes aegypti*

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There are currently few published studies of alternative splicing in mosquitoes. The examples that exist demonstrate a wide variety of functions for alternatively spliced mRNAs. One of the earliest published examples occurs in *Aedes aegypti*. The clathrin heavy chain (CHC) protein plays a structural role in forming the clathrin coated vesicles that are used in receptor-mediated endocytosis. The gene covers roughly 45 kilobases in the *Ae. aegypti* genome and is spliced to form three different CHC mRNA isoforms. Two of the isoforms (*Aa*CHCa-O and *Aa*CHCb-O) are expressed only in female germ-line cells and differ from one another in the 5' untranslated region (UTR) and at the distal portion of the protein coding sequence. The third isoform (*Aa*CHCb-S) is found in somatic cells and is identical to one of the germ-line isoforms (*Aa*CHCb-O) with the exception of having a much longer 3' UTR because it utilizes a different polyadenylation signal. The sequence of the longer 3' UTR leads to quicker mRNA decay, while the shorter 3' UTR of the ovarian transcripts probably leads to an increased stability of the mRNA (Kokoza & Raikhel, 1997).

Another example of alternative splicing in *Ae. aegypti* involves the homolog of *D. melanogaster Relish* gene. In *Drosophila*, this protein is involved in stimulating immune peptides used to mount antibacterial responses (Dushay et al., 1996, Engstrom et al., 1993). In *Drosophila*, Rel/NF-κB proteins, such as Relish, consist of both a Rel homology domain and an IκB domain (Dushay et al., 1996). The Rel homology domain is involved in dimerization and DNA binding while the IκB domain is involved in the cytoplasmic localization of NF- κB (Ghosh et al., 1998). In *Ae. aegypti* there are three isoforms of the Relish mRNA. The most common isoform contains a "full-length" Rel-homology domain, as well as, an IκB-like domain. This transcript is similar to the one

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seen in *Drosophila* and mammals. The second mRNA isoform lacks the IκB domain, which has been replaced with a 3' UTR. The final Relish isoform contains a truncated Rel-homolgy domain and a complete IκB-like domain. All three of these isoforms are upregulated following injection of the gram-negative bacteria *Enterobacter cloacae* (Shin et al., 2002).

A final example of alternative splicing in *Ae. aegypti* is seen in the ferritin light-chain homologue (LCH). LCH mRNA is upregulated in response to iron, H_2O_2, and hemin treatment in cultured *Ae. aegypti* cells (Aag2 cells). This protein is thought to be involved in sequestering free iron following a blood meal to protect the mosquito from oxidative stress. Alternate isoforms of LCH mRNA occur via splicing in the 5' UTR transcript. These alternative isoforms may allow different stimuli to upregulate their expression or they may play a role in tissue specific expression of LCH mRNA (Geiser et al., 2003).

C. Alternative Splicing in *Anopheles gambiae*

An. gambiae is similar to *Ae. aegypti* in that there are not very many published examples of alternative splicing. However, several important functions can be attributed to the examples that do exist. The first example is the glutathione S-transferase family and resistance to dichlorodiphenyl-trichloroethane (DDT). *An. gambiae* glutathione S-transferases are diversified due to alternative splicing of the mRNA transcript at the 5' end of the message leading to several mRNA variants that have different 5' exons spliced to the same 3' exon. This process occurs in both resistant and susceptible mosquitoes. However, there is greater mRNA expression in DDT-resistant mosquitoes (Hemingway

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et al., 1998). It is possible that specific glutathione S-transferase isoforms are more likely to be upregulated in the presence of DDT.

A second important function for alternative splicing of mRNA is that different isoforms can be used in response to different stimuli. For example, the *REL2* (homologous to *Drosophila Relish*) gene is alternatively spliced in order to properly regulate infection with either gram-positive or gram-negative bacteria. In *Drosophila*, the Toll pathway is stimulated by Dif (Meng et al., 1999) and is used to counteract gram-positive bacteria (Michel et al., 2001). The immunodeficiency pathway (IMD), which responds mostly to gram-negative bacteria (Lemaitre et al., 1995), is controlled by Relish (Silverman et al., 2000). While *Drosophila* uses two separate genes to stimulate the Toll and IMD pathways, *An. gambiae* uses splice variants from the same gene, *REL2*, to stimulate both types of responses. The two different REL2 mRNA variants lead to two different sized proteins, REL2-F and REL2-S, which control the response to gram-positive and gram-negative bacterial infections, respectively (Meister et al., 2005).

A final example of alternative splicing in *An. gambiae* involves the Down syndrome cell adhesion molecule (Dscam). This protein is a member of the Ig-superfamily and has been found in the hemolymph of *Drosophila*. The protein plays a role in phagocytosis of bacteria by hemocytes (Watson et al., 2005). mRNAs for this gene are made by splicing several exons together. However, the diversity of the protein is increased because each exon has several different variants. In *An. gambiae*, 31,920 different protein isoforms of Dscam can be produced. This protein may also play an important role in the nervous system (Graveley et al., 2004). mRNA transcripts are alternatively spliced to make different protein isoforms in response to different types of

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infections (e.g. gram-positive bacteria, gram-negative bacteria, and parasites). Differential expression of this protein in response to various pathogens may indicate that insects have a much more complex immune system than originally believed (Dong et al., 2006).

7. Summary of Research Aims

La Crosse virus remains an important public health problem in the United States. Transovarial transmission serves to maintain LACV in nature, yet little is known concerning the molecular determinants of *Ae. triseriatus* mosquitoes that condition the ability to TOT LACV. Previous investigators have proposed that some *Ae. triseriatus* females develop stabilized infections (Tesh & Beaty, 1983), but again little is known about the genetics that would allow some mosquitoes to maintain a stabilized infection. If particular females develop stabilized infections, movement of these females or their genes could be epidemiologically significant for LACV transmission. The major aims of this research were to test earlier conclusions about gene flow in *Ae. triseriatus* and to investigate the role of the *Ae. triseriatus* inhibitor of apoptosis protein 1 in vertical transmission of LACV.

The first aim was a population genetics study of *Aedes triseriatus* mosquitoes to determine if there are any barriers to gene flow in the southwestern Wisconsin, southeastern Minnesota, and northeastern Iowa collection area. The results from this experiment verify that *Ae. triseriatus* mosquitoes exist as a panmictic population over a large area and make it possible to work with field mosquitoes from a known interbreeding population (Chapter 2).

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The second aim of this project was to determine if polymorphisms in the *Ae. triseriatus* inhibitor of apoptosis 1 (*AtIAP1*) gene condition mosquitoes to be susceptible or refractory to transovarial infection with LACV. Field collected mosquito eggs were reared to adults and sequences of the *AtIAP1* gene of LACV positive and negative mosquitoes were compared to determine if particular single nucleotide polymorphisms (SNPs) are more common in LACV positive or negative mosquitoes. In addition, the expression profiles of AtIAP1 mRNA splice variants were analyzed to determine whether differences exist between *Ae. triseriatus* TOT permissive and refractory mosquito strains. The expression profiles of these mRNA variants were also characterized in different life stages and tissue types of *Ae. triseriatus* (Chapters 3 and 4).

The final experiment involved quantification of LACV in field collected mosquitoes and laboratory mosquitoes, specifically in the ovarian and midgut tissues. Virus load in these tissues, particularly the ovaries, could directly contribute to TOT. Increased virus loads may increase LACV TOT efficiency to eggs or it could impair fecundity. To determine the relationship between virus load and TOT, viral genome equivalents from each mosquito were determined and compared between field collected mosquitoes and TOT permissive and refractory laboratory mosquitoes. This experiment also demonstrated variation in LACV titers of TOT infected mosquitoes in the field. It is possible that, while many mosquitoes become infected transovarially, only a few of these have high enough viral titers to transmit either horizontally or vertically (Chapter 5).

Transovarial transmission is a critical process that allows LACV to persist in the environment. A better understanding of the genes conditioning the ability of *Ae. triseriatus* to TOT LACV may allow us to better protect people from LACV infection.

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Once the genetic loci controlling this phenotype are identified, field collections can be tested to determine which collections possess genotypes contributing to high levels of TOT. This knowledge will allow control measures to be focused in areas that are most likely involved in LACV persistence. Furthermore, demonstrating significant differences in ovarian titers of LACV infected field samples may be the first step in proving that a small percentage of LACV infected mosquitoes play the most important role in LACV persistence via TOT. Differences between mosquitoes that can accommodate high titer stabilized infections and those that cannot may be the key in determining the genetic factors involved in TOT of LACV.

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Chapter 2:

An Analysis of Gene Flow Among Midwestern Populations of the Mosquito, *Aedes triseriatus*

The work from this chapter has been published in The American Journal of Tropical Medicine and Hygiene:

Beck, E. T., Bosio, C. F., Geske, D. A., Blair, C. D., Beaty, B. J., & Black IV, W. C. An analysis of gene flow among Midwestern populations of the mosquito *Ochlerotatus triseriatus*. *Am J Trop Med Hyg* **73**, 534-540.

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1. Introduction

The Eastern treehole mosquito, *Aedes* (*Ochlerotatus*) *triseriatus*, is the primary vector of La Crosse virus (LACV) in the Midwestern United States (Thompson et al., 1972). Sinsko and Craig suggested that the species may exist in "ecological islands" maintained by limited gene flow between forested tracts of land in otherwise agricultural landscapes (Sinsko & Craig, 1979). Matthews and Craig used variation at 14 allozyme loci to examine gene flow among collections from Michigan, Indiana and Illinois (Matthews & Craig, 1980). They found that collections shared > 99% of their genetic markers suggesting, contrary to Sinsko and Craig, that *Ae. triseriatus* exists as a large panmictic population (Matthews & Craig, 1980, Sinsko & Craig, 1979).

In this study mitochondrial DNA (mtDNA) markers were used to reassess the results of Matthews and Craig (Matthews & Craig, 1980). Mitochondrial DNA is maternally inherited and does not recombine; therefore it can be used to examine maternal lineages (Avise, 1994). Mitochondrial DNA has previously been used to study phylogenetic relationships in several *Anopheles* species, as well as *Aedes (Stegomyia) aegypti* (Besansky et al., 1997, Caccone et al., 1996, Conn et al., 1997, Conn et al., 1998, De Merida et al., 1999, Gorrochotegui-Escalante et al., 2002, Gorrochotegui-Escalante et al., 2000, Mitchell et al., 1992).

Aedes triseriatus were collected from 36 sites in southwestern Wisconsin, southeastern Minnesota, and northeastern Iowa covering an area of ~14,000 km^2. The potential for the Mississippi River and Interstate-90 to serve as barriers to gene flow in adjoining regions of the states of Iowa, Minnesota, and Wisconsin was addressed. Genetic diversity at each collection site was examined and the effective migration rates

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($N_e m$) (the number of reproductive migrants per generation) and population sizes (N_e) (the number of reproductive individuals) were determined.

2. Materials and Methods

A. Mosquito Collection and Extraction of DNA

Aedes triseriatus eggs were collected from 5 oviposition traps in each of the 36 sites listed in Table 2.1. Collections were made from mid-July through September of 2002 by the La Crosse County Health Department in areas where La Crosse encephalitis cases occurred or that contained clusters of people at risk (e.g. wooded areas adjacent to houses with children, schools or playgrounds) (Fig. 2.1). Each trap consisted of a can (6.5 x 11 cm) painted black, half filled with tap water and containing seed germination paper along the inside perimeter. Each trap was placed at or slightly above ground level. Egg papers were recovered from traps after ten days, sent to Colorado State University, hatched, and reared to adults. Mosquitoes were analyzed for LACV infection by immunofluoresence assay (Beaty & Thompson, 1975). DNA was extracted from the thorax of each mosquito using the salt extraction method (Black & DuTeau, 1997). DNA was dissolved in 200 µL of Tris-EDTA (10 mM Tris, 1 mM EDTA) pH 8.0 and stored at -70°C.

B. Polymerase Chain Reaction Amplification, Single Strand Conformation Polymorphism (SSCP) Analysis and DNA Sequencing

The mitochondrial NADH dehydrogenase subunit 4 gene (ND4) was amplified using the thermocycling parameters and the primers described previously (Gorrochotegui-Escalante et al., 2000). However, *Taq* DNA polymerase (Promega, Madison, WI) was added at the beginning of each reaction. SSCP analysis was also

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Table 2.1. ***Aedes triseriatus*** **collection sites for gene flow analysis.**

Site	Site Abbr.	Region	State	Collection Date	Latitude	Longitude
Stevenstown	STEV	NE	WI	22-Aug-02	N 44°02'15.18"	W 91°10'13.14"
Boyscout Camp Cafeteria	BOCC	NE	WI	5-Aug-02	N 44°03'29.4"	W 91°16'57.66"
West Salem Riverview Ball Park	WSRP	NE	WI	4-Sep-02	N 43°54'31.98"	W 91°05'30.36"
Innsbruck Lower	INNL	NE	WI	13-Aug-02	N 43°54'16.38"	W 91°10'30.18"
West Salem Dave's House	WSDH	NE	WI	24-Sep-02	N 43°53'59.64"	W 91°05'7.08"
Rolling Stone Ball Field	ROBF	NW	MN	23-Aug-02	N 44°06'7.5"	W 91°49'11.28"
Bass Camp Hacienda	BCHA	NW	MN	20-Sep-02	N 44°09'13.32"	W 91°48'24.78"
Winona Hidden Valley Road	WHVR	NW	MN	6-Sep-02	N 44°03'48.96"	W 91°43'26.04"
Dakota I-90 Exit	DAKI	NW	MN	29-Jul-02	N 43°54'36.84"	W 91°21'18.78"
Dakota Elementary	DAKE	SW	MN	15-Aug-02	N 43°54'29.16"	W 91°21'35.22"
Dresbach Cemetery	DRES	NW	MN	6-Sep-02	N 43°53'15.6"	W 91°20'18"
St. Joseph's	STJO	SE	WI	5-Aug-02	N 43°47'0.42"	W 91°02'40.98"
Yanzer Rd.	YANZ	SE	WI	1-Oct-02	N 43°48'18.24"	W 91°07'8.4"
Mormon Coullee Park Basketball Ct	MCPB	SE	WI	5-Aug-02	N 43°45'42.42"	W 91°09'15.12"
Highway O 2000 Case Site	HOCS	SE	WI	19-Jul-02	N 43°34'58.74"	W 91°03'0.54"
Victory	VICT	SE	WI	19-Jul-02	N 43°28'50.34"	W 91°12'33.18"
Worman Rd.	WORM	SE	WI	24-Sep-02	N 43°30'38.76"	W 91°08'31.86"
Kendall Bike Trail	KEBT	SE	WI	2-Aug-02	N 43°47'47.22"	W 90°22'29.28
Wilton Ball Field	WIBF	SE	WI	2-Aug-02	N 43°48'44.7"	W 90°31'23.4
Oakdale Doug's Trucks	OADT	SE	WI	21-Aug-02	N 43°57'40.56"	W 90°23'11.4"
Steuben	STEU	SE	WI	20-Aug-02	N 43°10'51.36"	W 90°52'33.3"
Wauzeka Park	WAZP	SE	WI	29-Aug-02	N 43°05'14.82"	W 90°52'49.14"
Prairie Du Chien East Brunson	PDCB	SE	WI	20-Aug-02	N 43°02'21.72"	W 91°07'15.06"
Stoddard Village Park	STVP	SE	WI	19-Aug-02	N 43°39'31.62"	W 91°13'1.44"
Pike's Peak State Park	PPSP	SW	IA	27-Sep-02	N 42°59'41.34"	W 91°09'55.62"
New Albin	NEAL	SW	IA	27-Sep-02	N 43°29'34.08"	W 91°17'6"
Marquette Scenic Overlook	MARQ	SW	IA	27-Sep-02	N 43°02'44.04"	W 91°10'40.5"
Lansing Sports Complex	LASC	SW	IA	27-Sep-02	N 43°21'34.5"	W 91°12'56.52"
Hokah Como Falls	HOCF	SW	MN	18-Sep-02	N 43°45'39.54"	W 91°20'25.2"
Hokah St. Peters	HOSP	SW	MN	28-Aug-02	N 43°45'39.9"	W 91°20'41.46"
Houston Chase St.	HOCH	SW	MN	18-Sep-02	N 43°45'56.34"	W 91°33'47.28"
Caledonia Danielson	CALD	SW	MN	19-Jul-02	N 43°38'9.42"	W 91°29'11.52"
Caledonia Bulman	CALB	SW	MN	30-Jul-02	N 43°39'35.22"	W 91°32'57.18"
Caledonia Green Acres Rd.	CALG	SW	MN	18-Sep-02	N 43°37'19.62"	W 91°29'55.08"
Brownsville Ramsey St.	BRNR	SW	MN	17-Sep-02	N 43°41'27.66"	W 91°17'6.24"
Brownsville Wildcat Landing	BRNW	SW	MN	28-Aug-02	N 43°41'16.5"	W 91°16'32.7"

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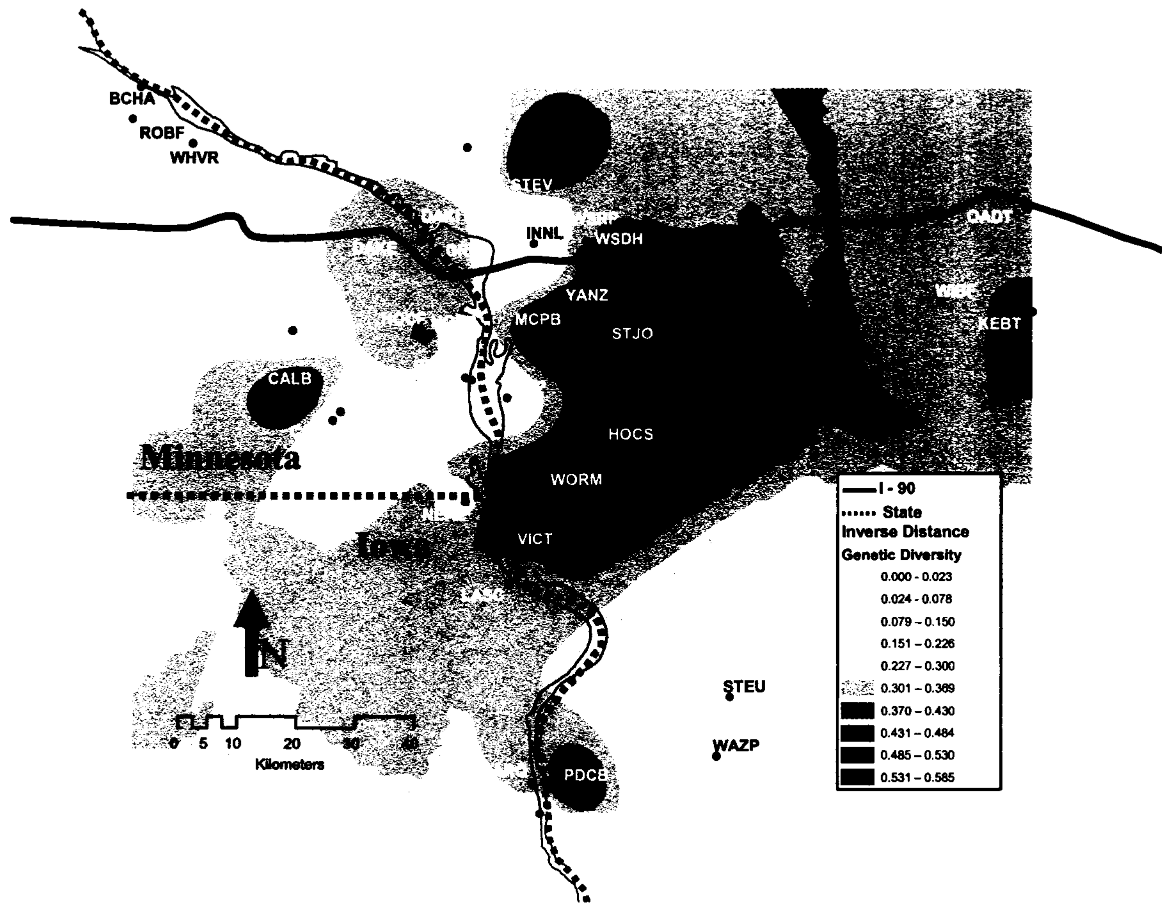

Figure 2.1. Map of collection sites. Shading indicates amount of genetic diversity at each location. Genetic diversity was transformed by taking the arcsine of the square root of the genetic diversity. Transformed values were used to create a weighted distance model using ArcGIS, to predict the amount of genetic diversity throughout the given study site. The equation of the model is $y = 0.3093x + 22.818$ (y = predicted genetic diversity; x = arcsine of the sq. root of genetic diversity). Genetic diversity at locations away from sampling sites was predicted using this equation and indicates the probability that two randomly chosen samples in a collection will have a different haplotype. The darker colors on the map indicate areas with greater haplotypic diversity.

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described previously by Black and DuTeau (1997). Prior to gel loading, 4 µL of PCR product was combined with 4 µL of denaturing loading buffer and heated to 95°C for 5 min and placed directly on ice for 5 min. Samples were loaded onto a 3% polyacrylamide gel containing 1X Tris-Borate EDTA (TBE) buffer (89 mM Tris, 89 mM Borate, 2 mM EDTA) and electrophoretically separated using a BioRad IPC gel apparatus (BioRad, Hercules, CA) at 15 milliamps for 10-12 hours. Gels were silver stained and examined for different banding patterns suggestive of differences in primary sequences (Black & DuTeau, 1997). DNA sequencing was performed by Davis Sequencing, Inc (Davis, CA). Fifteen samples were sequenced representing all four ND4 haplotypes. Samples from 27 of the 36 sites were chosen for SSCP screening. The chosen sites for SSCP sampling covered the entire region of the study.

C. Single Nucleotide Polymorphism (SNP) Analysis

The oligonucleotide ligation assay (OLA) is an inexpensive SNP assay that uses ligation between a biotinylated "allele specific detector" and a 3' fluorescein labeled "reporter" oligonucleotide. Heated OLA (HOLA) uses a thermal stable ligase and cycles of denaturing and reannealing on a thermal cycler for ligation and SNP detection (Black et al., 2006, Lynd et al., 2005) (Fig. 2.2). Allele specific detectors (bold and underlined sites indicate a polymorphic site in the ND4 gene) at SNP site 21 were ND4tris21A-dtc: 5' Biotin-CCT AAG GCY CAT GTT GAA GC**<u>T</u>** 3', ND4tris21G-dtc: 5' Biotin-CCT AAG GCY CAT GTT GAA GC**<u>C</u>** 3', and the reporter was ND4tris21-rpt 5' PO_4-CCT GTT TCA GGA TCA ATA A-Fluorescein 3'. Allele specific detectors at SNP site 234 were ND4tris234C-dtc: 5' Biotin-GCT TAT TCT TCT GTT GCT CAT AT**<u>G</u>** 3', ND4tris234T-dtc 5': 5' Biotin-GCT TAT TCT TCT GTT GCT CAT AT**<u>A</u>** 3',

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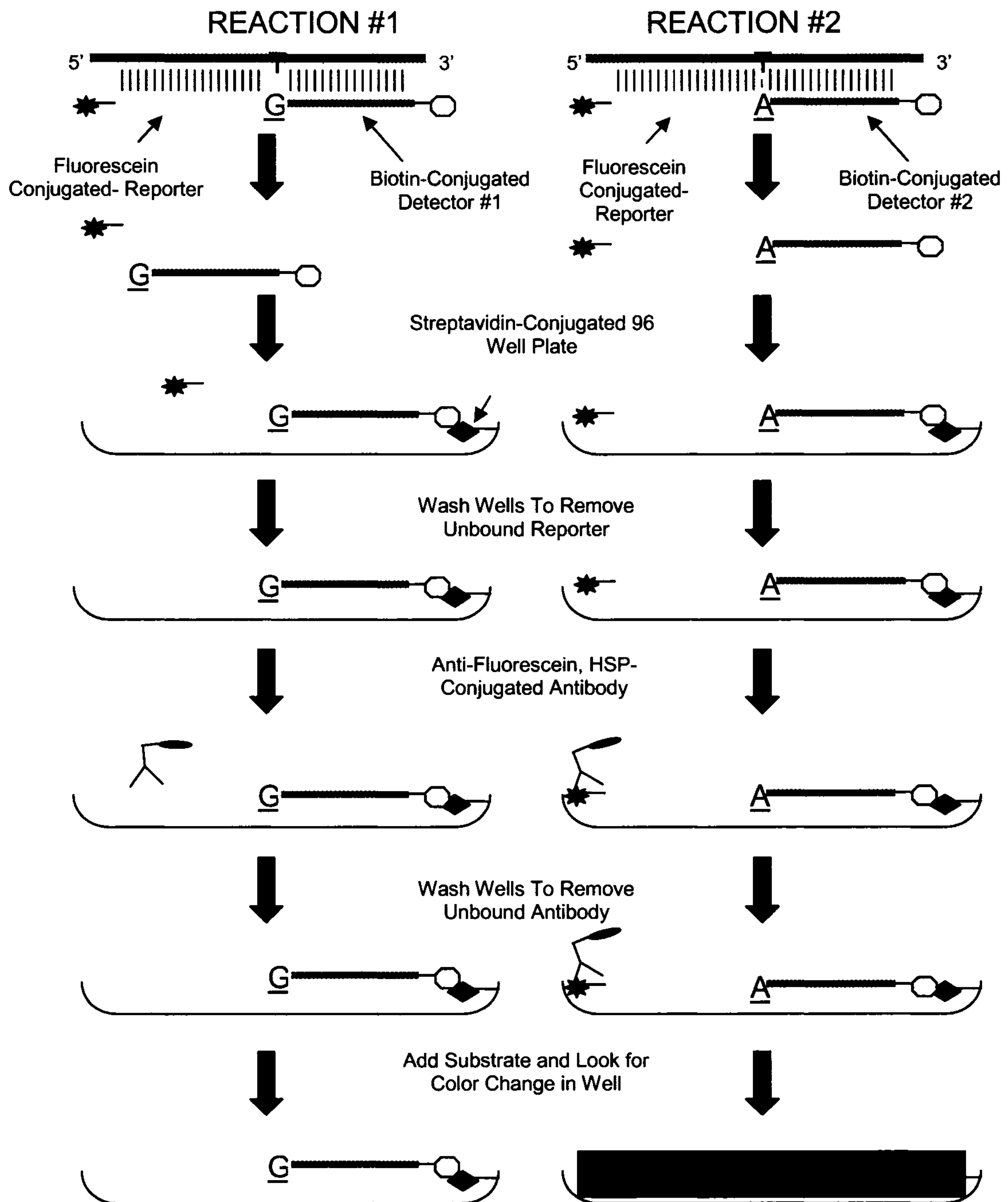

Figure 2.2. Schematic diagram of the heated oligonucleotide assay (HOLA). This diagram provides a general overview of how the HOLA assay discriminates between nucleotides of a SNP.

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and the reporter was ND4tris234-rpt: 5' PO_4-GGA ATT GTA TTA AGA GGG T-Fluorescein 3'. One 96 well plate was used for ND4 PCR, a second plate for HOLA, and a third for ligation detection. Successful ligation was detected using horseradish peroxidase (HRP) conjugated, anti-fluorescein antibody from Roche. HRP activity was detected by addition of 3,3',5,5' tetramethylbenzidine (TMB) (Sigma) (Fig. 2.3).

D. Population Genetics Analyses

ND4 frequencies were estimated in each collection using ARLEQUIN version 2.000 (University of Geneva, Geneva, Switzerland). Genetic diversity (the probability that two randomly chosen haplotypes in a collection are different) was estimated in each collection (equation 8.5 from (Nei, 1987). This genetic diversity is equivalent to heterozygosity in diploid data. Pairwise F_{ST} (a measure of non-random mating among subpopulations) and linearized $F_{ST} = (F_{ST}/(1 - F_{ST}))$ values were estimated among populations and analyses of molecular variance (AMOVA) were performed (Excoffier et al., 1992). The data were analyzed by grouping sites to the north and south of Interstate-90, sites to the east and west of the Mississippi River, and sites in the NW, NE, SW, and SE quadrants of the study area using the interstate and the river as the quadrant dividers.

A dendrogram was constructed among all of the collections using the NEIGHBOR program in PHYLIP3.61 using linearized F_{ST} values (Felsenstein, 1993). This value was also plotted against the natural logarithm of pairwise geographic distances among collections to test for isolation by distance (Slatkin, 1993). The Mantel test was used to determine if geographic distance acts as a barrier to gene flow in *Ae. triseriatus* (Mantel, 1967).

ArcGIS (Environmental Systems Research Institute (ESRI), Redlands, CA, USA)

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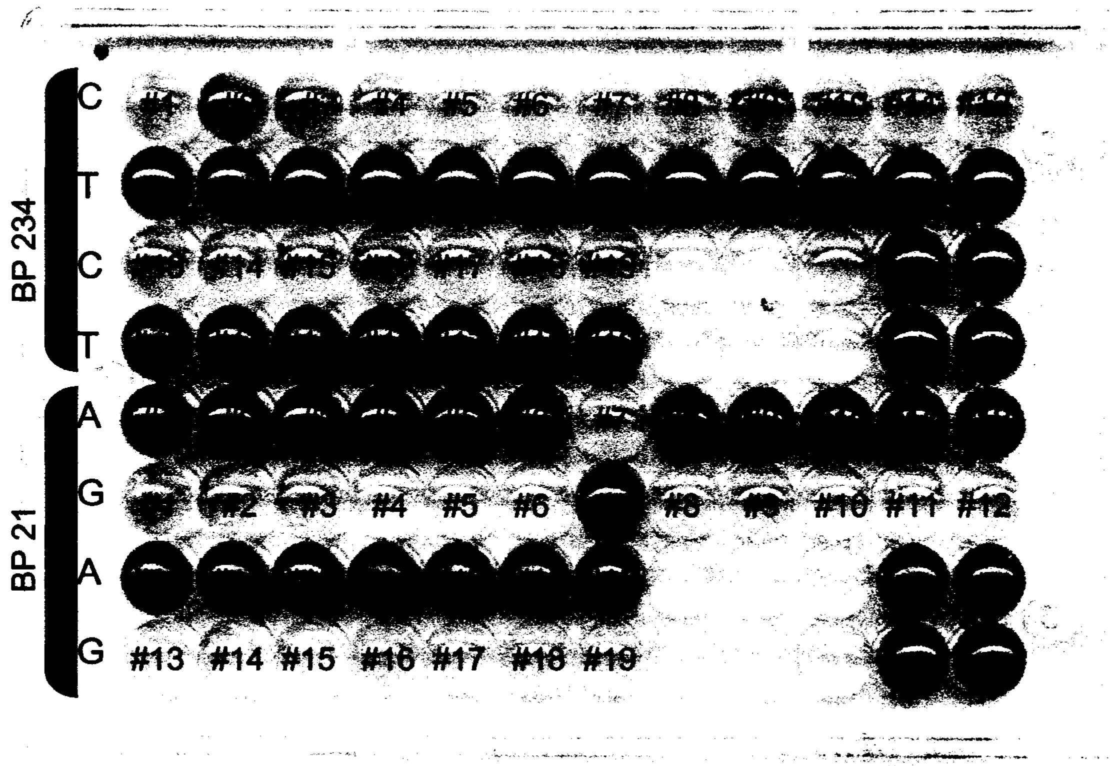

Figure 2.3. Results of ND4 HOLA analysis for *Ae. triseriatus*. This picture shows results from an HOLA assay at base pairs 21 and 234 of the ND4 PCR fragment amplified from *Ae. triseriatus*. The "+" represents positive controls made from plasmid DNA to show that both reactions worked. A positive reaction (dark blue in color) determines which nucleotide is present at both polymorphic sites for samples 1-19.

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was used to examine the spatial distribution of genetic diversity. Genetic diversity was transformed by taking the arcsine of the square root of genetic diversity to linearize the data (equation 13.5 from (Zar, 1999) because values near 0 or 1 (untransformed values) are nonlinear. Inverse distance weighted (IDW) interpolation was used to predict genetic diversity values outside of collection sites. IDW assumes that genetic diversity values will be more similar among nearby points. The interpolated diversities are therefore a weighted average of the scatter points where the weights are assigned based on the distance to each scatter point. The weight diminishes as the distance from the interpolation point to the scatter point increases. Similarly, population genetics assumes that genetic diversity should be spatially autocorrelated. Proximate collection sites are more likely to have similar genetic diversity than distant sites.

3. Results

A. Mitochondrial Haplotype Analysis

We used SSCP analysis on 564 of the 1,697 mosquitoes from different collection sites encompassing the entire study area. Only four different ND4 haplotypes were observed (Fig. 2.4). These contained 8 variable sites, seven of which were in complete linkage disequilibrium. Haplotypes 1 and 3, as well as haplotypes 2 and 4, differed by only one base pair and all haplotypes could be differentiated using SSCP analysis. All of the 8 substitutions among the four haplotypes occurred in a third codon position and seven involved transitions. Only substitution 234 encoded a nonsynonymous Met ←→ Ile mutation (Fig. 2.5).

Frequencies of haplotypes 1-4 among 564 mosquitoes were 0.812, 0.023, 0.158, and 0.007, respectively (Table 2.2). SSCP analysis required more work and haplotypes

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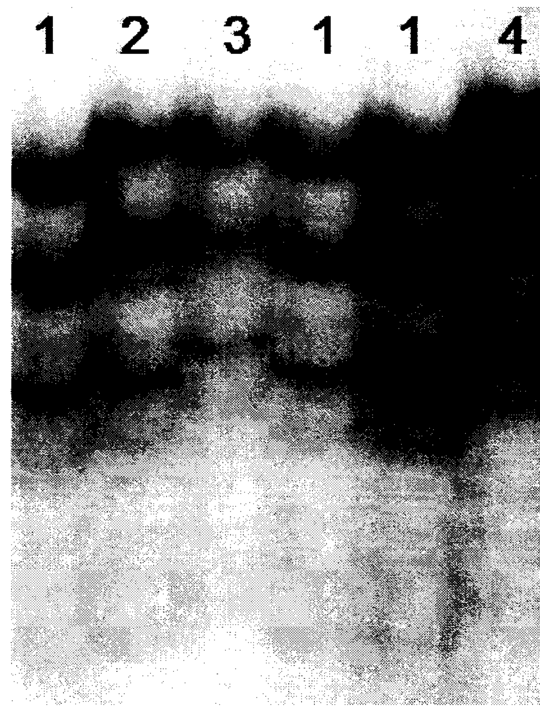

Figure 2.4. ***Ae. triseriatus*** **ND4 haplotypes visualized by SSCP.** Amplification of a portion of the ND4 gene showed only 4 different SSCP banding patterns when 564 individual mosquitoes were analyzed. These four patterns were distinguishable when looking at a silver stained gel. The number at the top of the lane corresponds to the haplotype number of each sample.

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```
                    9           21                         48    54
            P  K  A  H  V  E  A  P  V  S  G  S  I  I  L  A  G  V  L  L  K
Ae. tris 1  CCTAAGGCACATGTTGAAGCTCCTGTTTCAGGATCAATAATTTTAGCAGGAGTTTTATTAAAA
Ae. tris 2  ........G......................................G.....C.........
Ae. tris 3  ....................C..........................................
Ae. tris 4  ........G...........C..........................G.....C.........

                    72
            L  G  G  Y  G  L  L  R  V  F  S  I  L  Q  I  L  G  I  K  F  N
Ae. tris 1  TTAGGGGGGTATGGTTTACTTCGAGTTTTTTCAATTTTACAAATTTTAGGTATAAAATTTAAT
Ae. tris 2  ........T......................................................
Ae. tris 3  ...............................................................
Ae. tris 4  ........T......................................................

            F  I  W  I  R  I  R  L  I  G  G  I  L  V  R  L  I  C  L  W  Q
Ae. tris 1  TTTATATGAATTAGAATTAGATTAATTGGAGGAATTTTAGTAAGATTAATTTGTTTATGACAA
Ae. tris 2  ...............................................................
Ae. tris 3  ...............................................................
Ae. tris 4  ...............................................................

                       201                              234
            I  D  L  K  A  L  I  A  Y  S  S  V  A  H  I  G  I  V  L  R  G
Ae. tris 1  ATAGATTTAAAAGCTTTAATTGCTTATTCTTCTGTTGCTCATATAGGAATTGTATTAAGAGGG
Ae. tris 2  ...........G................................G..................
Ae. tris 3  ...............................................................
Ae. tris 4  ...........G................................G..................

                                            285
            L  L  T  I  T  Y  W  G  L  N  G  S  Y  T  L  I  I  A  H  G  L
Ae. tris 1  TTATTAACTATAACTTATTGAGGATTAAATGGATCTTATACTTTAATAATTGCTCATGGATTA
Ae. tris 2  ................................G..............................
Ae. tris 3  ...............................................................
Ae. tris 4  ................................G..............................

            C  S  S  G  L  F  C  L  A  N  I  S  Y  E  R
Ae. tris 1  TGTTCATCCGGGTTATTTTGTTTAGCTAATATTTCTTATGAACG
Ae. tris 2  ............................................
Ae. tris 3  ............................................
Ae. tris 4  ............................................
```

Figure 2.5. Sequence alignment of the four ND4 haplotypes detected by SSCP analysis of 564 mosquitoes. Bases 21 and 234 were used for SNP analysis with the HOLA assay to determine the ND4 haplotype of all 1,697 mosquitoes.

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Table 2.2. SSCP sample numbers and SNP haplotype frequencies.

Site Abbrev.	n for SSCP	n for SNP	Haplotype #1 Frequency	Haplotype #2 Frequency	Haplotype #3 Frequency	Haplotype #4 Frequency
STEV	0	49	0.57	0.02	0.41	0.00
BOCC	19	49	0.92	0.00	0.08	0.00
WSRP	21	50	0.84	0.10	0.06	0.00
INNL	19	48	1.00	0.00	0.00	0.00
WSDH	0	53	0.49	0.00	0.51	0.00
ROBF	0	50	0.94	0.00	0.06	0.00
BCHA	0	50	0.92	0.08	0.00	0.00
WHVR	21	49	1.00	0.00	0.00	0.00
DAKI	41	50	0.76	0.02	0.22	0.00
DAKE	10	50	0.64	0.12	0.24	0.00
DRES	50	50	.74	0.02	0.24	0.00
STJO	20	48	0.67	0.06	0.27	0.00
YANZ	22	50	0.72	0.00	0.28	0.00
MCPB	16	48	0.46	0.00	0.54	0.00
HOCS	0	49	0.67	0.02	0.31	0.00
VICT	19	47	0.49	0.09	0.43	0.00
WORM	15	50	0.66	0.02	0.32	0.00
KEBT	21	50	0.76	0.02	0.22	0.00
WIBF	21	48	0.79	0.10	0.10	0.00
OADT	0	64	0.81	0.00	0.19	0.00
STEU	21	47	1.00	0.00	0.00	0.00
WAZP	0	48	1.00	0.00	0.00	0.00
PDCB	21	50	0.62	0.00	0.38	0.00
STVP	21	48	0.88	0.00	0.13	0.00
PPSP	21	50	0.92	0.00	0.06	0.02
NEAL	21	42	0.83	0.00	0.17	0.00
MARQ	27	27	0.81	0.15	0.04	0.00
LASC	0	50	0.82	0.00	0.18	0.00
HOCF	21	49	0.92	0.00	0.08	0.00
HOSP	16	50	0.62	0.26	0.12	0.00
HOCH	21	50	0.88	0.00	0.12	0.00
CALD	10	30	0.83	0.00	0.17	0.00
CALB	7	24	0.46	0.00	0.54	0.00
CALG	0	50	0.92	0.02	0.06	0.00
BRNR	21	50	0.92	0.00	0.08	0.00
BRNW	21	30	0.83	0.03	0.00	0.13

N for SNP Analysis = 1697. Haplotype frequency data is calculated from SNP analysis.

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were more difficult to discern than with the HOLA SNP detection assay. The haplotypes determined by SSCP and HOLA were identical in all 564 mosquitoes. Haplotypes were therefore determined using HOLA. Frequencies of haplotypes 1-4 among all 1,697 mosquitoes by HOLA were 0.784, 0.031, 0.183, and 0.003, respectively (Table 2.2).

B. Nested Analysis of Haplotype Frequencies

The ND4 haplotype frequencies were partitioned using AMOVA within collections, between collections in a quadrant, and between quadrants. Most of the variation (86.3%) arose within collections, 13.2% arose among collections within a quadrant, and very little arose between quadrants (0.6%) (Table 2.3). The effective migration rates were determined using the equation $N_em = (1-F_{ST})/4F_{ST}$ (Slatkin, 1993). The N_em among mosquitoes in all four quadrants was 45 reproductive migrants/generation, an extremely high rate of gene flow.

Haplotype frequencies were next partitioned within collections, among collections, and between collections north or south of Interstate-90. Again, most of the variation (86.9%) arose within collections, while the remainder arose among collections either north or south of the interstate. A negative variance (-0.15%) arose between collections on either side of the interstate. This value indicates that there is more variance within collections on one side than there is between collections on opposite sides of the interstate (Table 2.4). Interstate-90 does not serve as a barrier to gene flow. Results of the AMOVA comparing collections east and west of the Mississippi River were similar (Table 2.5). Most variance (86.2%) arose within collections, 12.4% arose among collections located either east or west of the river, and only 1.4% occurred between collections on either side of the river. N_em between groups on either side of the

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Table 2.3. AMOVA analysis of groups of *Aedes triseriatus* collections from all four quadrants.

Source of Variation	d.f.	Sum of Squares	Variance Components	% Variance
Between collections in the 4 quadrants	3	5.014	0.00098	0.55
Among collections in each quadrant	31	38.702	0.02337	13.16
Among mosquitoes in a collection	1612	247.043	0.15325	86.29
Total	1646	290.758	0.17760	

Fixation Indices:

$F_{\text{among quadrants}}$ 0.006 — 45 Reproductive Migrants/Generation

$F_{\text{among collections in quadrants}}$ 0.132***

$F_{\text{mosquitoes in a collection}}$ 0.137***

***$p \leq 0.0001$

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Table 2.4. AMOVA analysis of *Aedes triseriatus* collections from north and south of Interstate 90.

Source of Variation	d.f.	Sum of Squares	Variance Components	% Variance
Between collections north or south of I-90	1	1.172	-0.00025	-0.15
Among collections north or south of I-90	33	42.544	0.02419	13.28
Among mosquitoes in a collection	1612	247.043	0.15325	86.87
Total	1646	290.758	0.17721	

Fixation Indices:

$F_{\text{between north and south of I90}}$	-0.002	no barrier to gene flow
$F_{\text{among collections in region}}$	0.133***	
$F_{\text{mosquitoes in a collection}}$	0.131***	

***$p \leq 0.0001$

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Table 2.5. AMOVA analysis of *Aedes triseriatus* collections from east and west of the Mississippi River.

Source of Variation	d.f.	Sum of Squares	Variance Components	% Variance
Between collections east or west of Mississippi River	1	3.347	0.00262	1.40
Among collections east or west of Mississippi River	33	40.369	0.02277	12.40
Among mosquitoes in a collection	1612	247.043	0.15325	86.20
Total	1646	290.758	0.17864	

Fixation Indices:

$F_{\text{between east and west of river}}$ 0.014 $\simeq$18 Reproductive migrants/generation
$F_{\text{among collections in regions}}$ 0.126***
$F_{\text{mosquitoes in a collection}}$ 0.138***
***$p \leq 0.0001$

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Mississippi River was 18 reproductive migrants/generation.

C. Cluster Analysis and Genetic Diversity

Cluster analysis placed the collections into two groups. One group contained collections with high genetic diversity and the other contained collections with low diversity. Clusters did not correspond to the geographic locations of collections. The variation in genetic diversity within each collection was enormous, varying between 0.0000 for collections WHVR, INNL, STEU, and WAZP to 0.5846 in the VICT collection (Fig. 2.6). In many cases, populations that were located within a few km of one another had large differences in genetic diversity.

Transformed genetic diversity values were used to predict genetic diversity throughout the study area (Fig. 2.1). The equation of the model is $y = 0.3093x + 22.818$, $r^2 = 0.195$ where y is the predicted genetic diversity and x is the arcsine of the square root of genetic diversity. The index of dispersion (ID = variance/mean, equation 4.1 from (Rosenberg, 2000) was estimated using the mean and variance of the transformed values to test the hypothesis that genetic variability was randomly distributed over the study area. The ID was significantly greater than 1 ($p = 1.77 \times 10^{-8}$), indicating a "contagious" or clustered distribution of variability. This is evident from the map in that there is no smooth pattern of increasing or decreasing genetic diversity. Also, the map shows that sites of high variability are often located very close to sites with low variability (e.g. CALB and CALG, VICT and NEAL, and PDCB and PPSP).

D. Genetic Isolation by Distance

The linearized pairwise F_{ST} values were regressed on the natural logarithm of the pairwise geographic distance to test whether geographic distance acts as a barrier to gene

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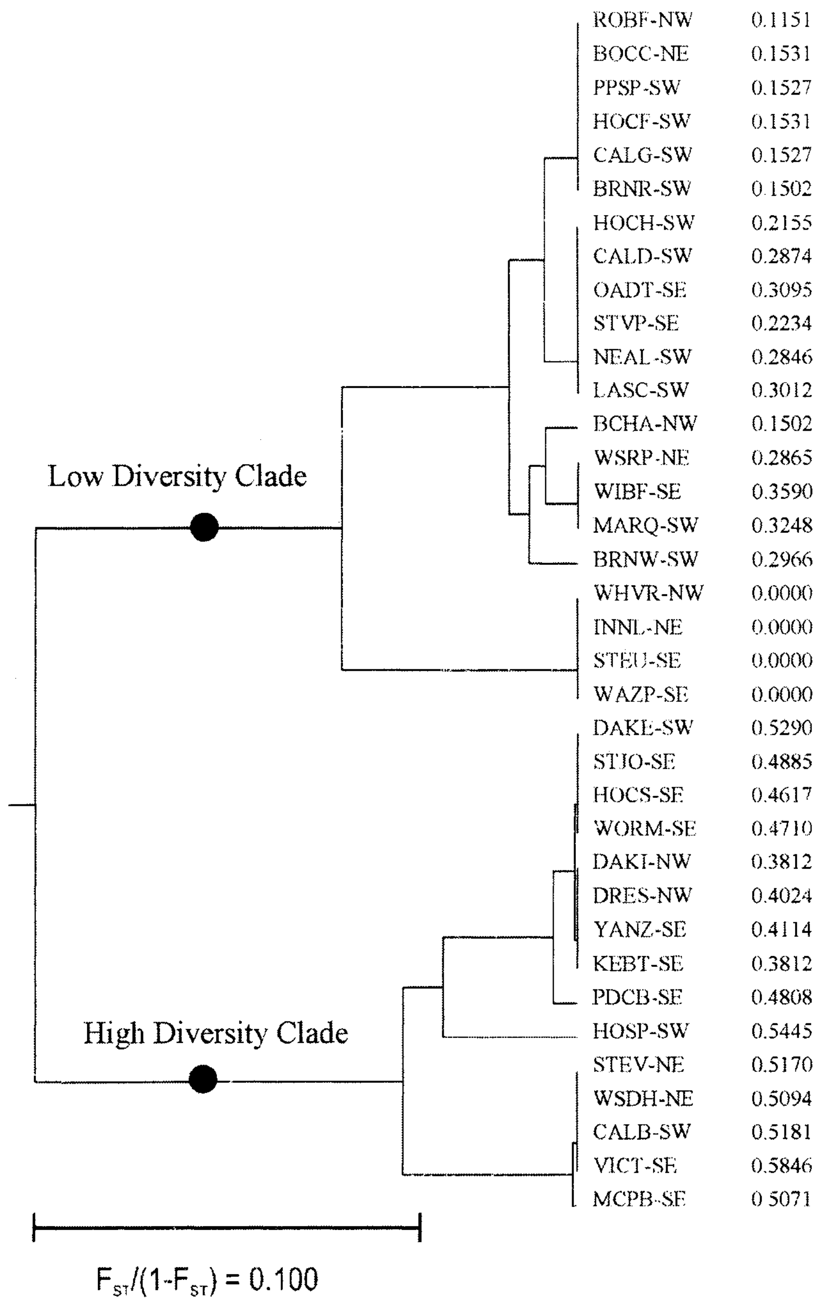

Figure 2.6. Unweighted pair-group method with arithmetic averaging cluster analysis of linearized pairwise F_{ST} values ($F_{ST}/(1-F_{ST})$) in conjunction with the Neighbor program in PHYLIP. There are two clusters: one contained collections with low genetic diversity and the other contained collections with high genetic diversity. The quadrant abbreviation (north/south refers to location relative to Interstate-90; east/west refers to location relative to the Mississippi River) and genetic diversity values are listed next to the site abbreviation.

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flow in *Ae. triseriatus* in this study area (Fig. 2.7). The estimated slope was not significantly greater than zero suggesting that there is no isolation by distance. It also shows that there were large genetic distances among proximate collections and small differences among distant collections.

4. Discussion

The amount of diversity in the mtDNA ND4 gene in *Ae. triseriatus* is low when compared to the same gene in *Ae. aegypti*. A similar sample of *Ae. aegypti* from throughout Mexico detected 25 different ND4 haplotypes (Gorrochotegui-Escalante et al., 2000; 2002). Only a survey from throughout the geographic range of *Ae. triseriatus* in the eastern half of North America including Mexico will determine if low mtDNA diversity is general to the species. Such a survey should also involve nuclear diploid markers. A continued pattern of low mtDNA diversity would suggest a historical bottleneck in *Ae. triseriatus*. Alternatively, high mtDNA diversity in other collections would suggest that a local historical bottleneck occurred during the establishment of northern midwestern *Ae. triseriatus* populations.

F_{ST} estimates were calculated from among *Ae. triseriatus* collections from northwestern Indiana and southwestern Michigan using the allozyme frequency data at 14 enzyme loci reported by Matthews and Craig (Table 2.6) (Matthews & Craig, 1980). The $N_e m$ of 22 reproductive migrants/generation estimated from their study falls within the range of 18-45 reproductive migrants/generation found in the present study. These results therefore largely agree with the results of Matthews and Craig and do not support the suggestion by Sinsko and Craig that the species exists in "ecological islands" in agricultural landscapes (Matthews & Craig, 1980, Sinsko & Craig, 1979). A major

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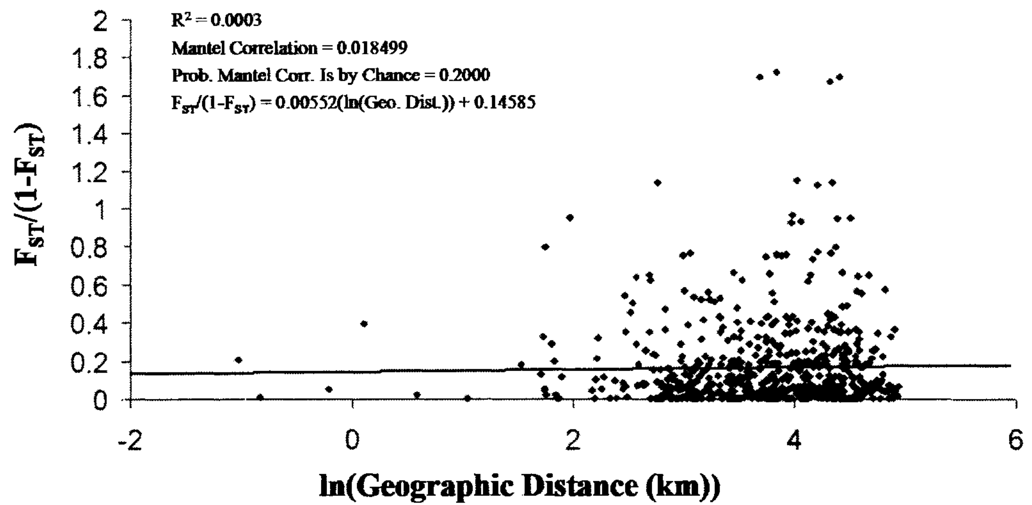

Figure 2.7. Regression analysis of linearized pairwise F_{ST} values ($F_{ST}/(1-F_{ST})$) and natural log of the pairwise geographic distances using the Mantel program.

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Table 2.6. F_{ST} values from allele frequencies in Matthews and Craig (1980). $N_e m \simeq$ 22 reproductive migrants/generation.

Locus	$F_{among\ collections}$
ODH	0.026
PGM	0.008
HBD	0.009
IDH-2	0.008
MDH-2	0.008
ME	0.013
GPD	0.003
PGI	0.013
SOD-5	0.001
IDH-1	0.015
FUM	0.004
AK	0.004
HK-4	0.000
6-PGD	0.003
Average	0.011

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difference between the current results and those of Matthews and Craig concerns the genetic variability encountered at individual collection sites (Matthews & Craig, 1980). In this study, variability ranged from 0.000 - 0.585 while it only ranged from 0.202 – 0.257 in Matthews and Craig (Matthews & Craig, 1980). This 10-fold disparity could arise from the types of genetic markers used. Variation in isozyme loci may be constrained by purifying selection, while nucleotide variation in the mitochondrial ND4 gene was probably largely neutral. Seven of the 8 substitutions were synonymous and involved transitions (Fig. 2.5).

However, substitution rates alone fail to explain why many collections exhibited no variation, while others were highly variable (Fig. 2.6) and why there were geographical clusters of collections with high variability. For example, BOCC and STEV were close to one another as were CALB and CALG, but in both cases collections differed greatly in genetic variability (Fig. 2.1). Genetic variability was therefore discontinuous or grainy at a local level. Several collection sites showed no genetic diversity (WHVR, INNL, STEU, and WAZP), while others showed a large amount (DAKE, HOSP, CALB, and VICT). Differences, in genetic diversity, between sites may be attributed to differences in oviposition behavior. Some egg collections may have been from one or two females, while others may be from many females. Active mosquito source reduction programs could be responsible for differences in number of females ovipositing at a given site and therefore could have generated the observed clustering of genetic diversity.

It is also possible that the collection methods are responsible for the clustering effect. In some of our collections, eggs only occurred in 1-2 ovitraps. However, the

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number of ovitraps at each site containing eggs was not recorded. Instead a qualitative observation that not all ovitraps contained samples was recorded, making it possible that one female could have laid all of the eggs at one site. This is highly unlikely but this possibility cannot be assessed because eggs from the 5 ovitraps at a location were combined prior to rearing them to adults. For future studies it would be wise to record this information so that an accurate comparison can be made between genetic diversity and the number of oviposition cans containing eggs.

The present study utilized different field collecting techniques from those of Matthews and Craig (Matthews & Craig, 1980). In these studies 5 ovitrap papers were collected at each site while Matthews and Craig removed first and second instar larvae from a minimum of 5 treeholes at each collection site (Matthews & Craig, 1980). In both studies, adults on which the genetic analyses were completed were reared in the laboratory. Conducting a formal ecological or genetic analysis (Apostol et al., 1993; 1994) of eggs found in individual traps would make it possible to assess the frequency of egg dumping (laying all eggs in one container) or skip oviposition (laying eggs in several containers) by *Ae. triseriatus*. If egg dumping (semelparity) is common in *Ae. triseriatus,* this would explain why a large proportion (~13%) of variation arose among collections (Tables 2.3-2.5) within a quadrant or region even though mosquitoes across the study area were panmictic.

This survey of variation in mtDNA was completed rapidly because of the switch from an SSCP to a SNP format for haplotype detection. ND4 haplotypes were initially determined using SSCP, which identified four unique ND4 haplotypes among 564 initial mosquitoes. A subset of 27 of the 36 collection sites were screened for polymorphisms

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using SSCP analysis to obtain a broad overview of diversity in the study area. It is possible that SSCP analysis of all samples would have revealed additional low frequency haplotypes, however, addition of these haplotypes would not have greatly affected the diversity analysis. After sequence analysis of all four SSCP haplotypes, only eight polymorphic sites were detected. Seven of the eight SNP sites were in linkage disequilibrium suggesting two maternal lineages of *Ae. triseriatus* in the study area. This disequilibrium also simplified detection of the four ND4 haplotypes by simply identifying the base present at two SNP sites (bases 21 and 234). Use of HOLA allowed for rapid determination of genotypes in all 1,697 mosquitoes (Black et al., 2006, Lynd et al., 2005).

These results suggest a general model of gene flow in *Ae. triseriatus* throughout our study area in the upper Midwest. The species exists as a single, panmictic adult population. However, within this panmictic population, local genetic drift arises, caused by one or a few females ovipositing in larval breeding containers (e.g. tires, cans, and treeholes). From generation to generation, there is sufficient gene flow to mix families arising from individual breeding sites and eliminate founders effects due to drift. With this knowledge, *Ae. triseriatus* mosquitoes from this portion of country can be treated as if they were from one large collection site. More importantly, neutral mutations conditioning transovarial transmission of La Crosse virus should be evenly distributed throughout this study area.

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Chapter 3:

Association Mapping of the Inhibitor of Apoptosis 1 gene with Transovarial Transmission of La Crosse Virus in *Aedes triseriatus*

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1. Introduction

The Eastern treehole mosquito, *Aedes (Ochlerotatus) triseriatus* (Say), is the primary vector of La Crosse virus (LACV), a leading cause of pediatric encephalitis in the Unites States (Watts et al., 1972). An important part of the LACV transmission cycle in the field involves the infection of ovaries in an infected mosquito and subsequent transovarial and transtadial transmission of the virus to her adult offspring, which are then infected and capable of transmission. Transovarial transmission (TOT) is also an important part of LACV overwintering in temperate climates (Beaty & Thompson, 1975, McGaw et al., 1998, Watts et al., 1973; 1974; 1975b). TOT refractory and permissive strains of *Ae. triseriatus* have been selected (Graham et al., 1999) and three quantitative trait loci were mapped and shown to contribute additively to a female's ability to TOT LACV (Graham et al., 2003).

In order for LACV to be transmitted transovarially, the virus must infect but not disrupt ovarian tissues. The NSs protein of LACV is similar to the *Drosophila* pro-apoptotic protein, Reaper (Colon-Ramos et al., 2003), and in mammalian cells and tissues, NSs expression or LACV infection may promote apoptosis. In contrast, LACV induced apoptosis has not been detected in LACV infected mosquito tissues. A candidate protein that may suppress apoptosis in infected tissues is the *Aedes triseriatus* inhibitor of apoptosis protein 1 (AtIAP1) (Blitvich et al., 2002), which is an ortholog of the well characterized *Drosophila* inhibitor of apoptosis 1 (DIAP1). DIAP1 ubiquitinates the apical caspase Dronc to stop activation of downstream caspases that would eventually lead to apoptosis (Palaga & Osborne, 2002). For apoptosis to occur, Reaper, Hid, Grim, and Sickle proteins must bind at their IAP binding motifs (IBMs) to the Baculovirus

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inhibitor of apoptosis (BIR) domains of DIAP1 (Bergmann et al., 2003). This binding blocks the ability of DIAP1 to inactivate Dronc and the apoptotic cascade begins (Chai et al., 2000, Liu et al., 2000, Wang et al., 1999, Wu et al., 2000). AtIAP1 may act in a similar fashion to DIAP1 to counter the potential apoptotic effect of LACV in mosquitoes.

Previous observations concerning *AtIAP1* have also led us to consider it as a candidate gene affecting LACV TOT. LACV is known to scavenge the 5' methylated guanine cap plus the adjacent oligonucleotide from host mRNAs to prime transcription of viral mRNAs (Beaty et al., 2000). Dobie et al. (1997) found that LACV predominantly scavenged the cap from an mRNA similar to AtIAP1 in a persistently infected *Ae. albopictus* larval cell line and in *Ae. triseriatus* eggs emerging from diapause (Borucki et al., 2002, Dobie et al., 1997).

The biology of the LACV TOT system provides a unique opportunity to exploit association mapping to determine if specific *AtIAP1* genotypes condition efficient TOT and overwintering. *Ae. triseriatus* eggs were collected from oviposition sites throughout southwestern Wisconsin, southeastern Minnesota, and northeastern Iowa. These were hatched, reared to adults, tested for LACV infection and then separated into TOT+ (infected) and TOT- (uninfected) groups. The *AtIAP1* gene of individual mosquitoes from both groups was amplified by polymerase chain reaction (PCR) and sequenced. The results from this analysis were used to compare genotype frequencies of each segregating site between TOT+ and TOT- mosquitoes to test the hypothesis that the *AtIAP1* genotype conditions TOT of LACV. The heated oligonucleotide ligation assay (HOLA) was used to determine genotypes at these five loci in a prospective case-control

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study. In this study uninfected mosquitoes were chosen from those collections that also contained infected samples in order to maximize the possibility that mosquitoes were exposed to LACV, but remained uninfected.

2. Materials and Methods

A. Mosquito Collection and DNA Extraction

Aedes triseriatus eggs were collected by the La Crosse County Health Department from LACV endemic areas in southwestern Wisconsin, southeastern Minnesota, and northeastern Iowa where La Crosse encephalitis cases were reported. The eggs were collected from June through August of 2004 in cans that were painted black, half filled with tap water, and lined with seed germination paper as an oviposition substrate. Five traps were used at each site and placed at or slightly above ground level. The egg liners were collected after 10 days and sent to Colorado State University where the eggs were hatched and reared to adults. Adults were sacrificed and assayed for LACV using an immunofluorescence assay (Beaty & Thompson, 1975). DNA was extracted from the thorax of each mosquito, using the salt extraction method (Black & DuTeau, 1997), dissolved in 200 μL Tris-EDTA buffer (10 mM Tris, 1 mM EDTA), pH 8.0 and stored at -70° C.

B. PCR and DNA Sequencing

The *AtIAP1* gene was amplified from each sample using three overlapping primer sets (Table 3.1 and Fig. 3.1). IAP1F and IAP2R amplified the region from beginning of the 5' UTR to the middle of the first BIR domain. IAP3F and IAP4R were designed to overlap the region amplified by IAP1F and IAP2R beginning at the first BIR domain to a region close to the end of the second BIR domain. IAP5F and IAP6R amplified the

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Table 3.1. Primer sequences used for PCR amplification of the *AtIAP1* gene.

Primer Name	Sequence (5' to 3')	Optimal Annealing Temp
IAP0F	ACCATAAATGCATCTTCCAC	51
IAP1F	GGACCAAGAGTAGACGAAGAG	51
IAP2R	GCCCGACATAGTAAAAGC	51
IAP3F	GGACGGTTTTGTTCATCA	51
IAP4R	TACCACATGGCATGCTGT	51
IAP5F	CCTCAAGGATTGGGAAGC	51
IAP6R	CCAAAAACGATCACCTTTATTTTA	51

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```
-416 agatcaatgttttcccatctggaccaagagtagacgaacagcatcagcatctttccgtgcatcatcgccatcatcaccat -337
                     IAP1F---------------->

-336 tgaagaagagagcgccaacc--gagagagagcagtgcaattgcaacttgccatcacgacggagctgttgcaccactcacc -257

-256 actaccagatcacaacaaaaacatttccgagctgaactgct-ggaaaaaccccggagaacaagattgaatcagtctctg -177

-176 agcaacagccgcggactcaagatcgctgtcttcgc---cgtcgtcgtcg------t---agtggtagtcgatccttgact -97

 -96 gcacttttccatcttctgtacctacttctccttcctcgacgatctgtcatccctatcggaatcgtacgcgagagcagag -17

                     M  T  A  Q  V  L  S  I  P  R  D  F  T  D  N  K  G  K  D  E  S  P
 -16 cagcatccgtaagaatATGACGGCGCAAGTACTGTCAATACCGCGGATTTCACAGATAACAAAGGAAAAGATGAATCCC 64

      F  V  G  V  T  G  A  S  S  V  S  V  S  Y  H  T  E  V  A [R  L  D  S  F  R  N]
  65 CCTTTGTAGGAGTAACTGGGGCGTCGTCGGTGTCAGTGTCGTACCATACGGAGGTCGCCCGGTTGGACTCGTTCCGGAAC 144

    [W  T  V  L  F  I  T  K  A  E  L  A  R  Y  G  F  Y  Y  V  G  P  N  D  M  V  K  C]
 145 TGGACGGTTTTGTTCATCACCAAGGCTGAGCTGGCCCGGTACGGCTTTTACTATGTCGGGCCAAACGATATGGTTAAATG 224
     IAP3F------------->                         <------------IAP2R

    [Y  F  C  R  V  E  I  G  L  W  E  P  N  D  N  V  L  S  E  H  L  R  W  S  P  Y  C]
 225 TTACTTCTGTCGAGTGGAAATCGGACTATGGGAGCCAAACGACAACGTTCTTTCGGAACACCTAAGATGGTCCCCGTACT 304

    [P  L  L  R] K  R  Q  T  N  N  V  P  I  D  A  S  F  L  D  Q  L  P  E  P  S  Y
 305 GTCCACTGCTGCGTAAACGGCAAACCAACAATGTTCCAATCGATGCCAGCTTTCTGGATCAGCTGCCGGAACCGAGCTAC 384

      D  T  C  G  I  R  I  R  E  N  S  V  A  E  N  A  Y [S  S  S  D  R  S  S  S  G  S]
 385 GATACCTGTGGCATTAGGATACGGGAAAATTCCGTGGCAGAAAATGCATACTCTTCGTCGGACCGAAGCAGTAGCGGATC 464

    [L  S  S  P  P  S  S  L  T  S  E  S  S  M  L  S] N  G  P  I  P  Q  Q  L  P  A  P
 465 CCTATCGTCTCCACCCAGCTCCCTAACGTCCGAATCCAGTATGTTATCTAATGGCCCGATCCCCCAACAACTTCCTGCGC 544

      A  L  P  Q  Q  K  R  P  E  Y  P  N  Y  A  I  E  A  K [R  L  E  S  Y  E  D  W]
 545 CCGCGCTGCCACAGCAGAAACGGCCGGAATATCCCAACTATGCCATCGAGGCGAAGCGACTGGAAAGCTACGAAGATTGG 624

    [P  K  F  M  K  Q  K  P  K  E  L  S  D  A  G  F  F  Y  T  G  K  G  D  R  V  K  C]
 625 CCAAAGTTCATGAAACAGAAGCCCAAGGAACTAAGCGATGCCGGATTTTTCTACACTGGCAAAGGCGATCGTGTCAAATG 704

    [F  S  C  G  G  G  L  K  D  W  E  A  E  D  E  P  W  E  Q  H  A  M  W  Y  S  N  C]
 705 CTTCAGCTGCGGTGGCGGCCTCAAGGATTGGGAAGCGGAGGACGAACCTTGGGAACAGCATGCCATGTGGTATAGTAATT 784
                        IAP5F------------->                <-------------IAP4R

    [E  Y  L  K] L  M  K  G  E  E  Y  I  A  Q  C  L  A  K  K  D  N  P  S  D  Q  K
 785 GTGAGTACCTGAAACTAATGAAAGGCGAAGAATACATCGCCCAATGTCTGGCCAAGAAGGATAATCCTTCTGATCAGAAA 864

      G  S  D  V  G  P [S  N  S  P  Q  P  S  T  S  G  V  T  S  A  A  T  T  S  L  P  S]
 865 GGCTCCGACGTTGGGCCGTCGAATTCGCCGCAACCGTCAACGTCCGGAGTAACATCCGCTGCAACAACCTCGCTCCCATC 944

    [S  Q  S  S  S  L  S  A  S  A  E  E  V  N  S  P  L  G  G  S  S  S] G  M  E  E  D
 945 GTCCCAATCGTCCAGTCTGAGTGCATCGGCCGAAGAAGTCAATTCTCCGCTCGGCGGCAGTAGTAGTGGCATGGAGGAAG 1024

      E  D  E  P  N  R  K  L  D  T  S  R  I  C  K  I  C  Y  V  N  E  Y  N  T  A  F
1025 ATGAAGATGAACCAAATCGTAAATTAGACACGAGTCGGATCTGCAAGATTTGCTACGTCAACGAGTACAATACGGCTTTC 1104

      S  P  C  G  H  V  V  A  C  A  K  C  A  S  S  V  T  K  C  P  L  C  R  K  P  F  T
1105 TCACCCTGTGGCATGTAGTGGCGTGTGCCAAATGCGCCTCGTCGGTCACCAAGTGTCCGCTCTGTCGGAAACCGTTTAC 1184

      N  V  M  R  I  Y  L  M  *
1185 CAATGTGATGAGAATTTACTTGATGTGAaagttcagaaataaaataaaggtgatcgttttggaaaaaaaaaaaaa    1261
                                           <-------------------IAP6
```

Figure 3.1. ***Aedes triseriatus*** **inhibitor of apoptosis 1 gene.** Nucleotides in the 5' UTR are labeled with negative numbers. The 3' UTR begins at nucleotide 1,213. BIR domain amino acids appear in the gray boxes, amino acids in the serine-rich domain appear in clear boxes, and amino acids in the zinc-ring finger motif are in bold italics (Blitvich et al., 2002). Primer sequences are italicized and underlined. Potential QTNs, as determined by complete sequence analysis with PGtheta, are highlighted in gray (nucleotides -361, -268, 542, 555, and 570).

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region from the middle of the second BIR domain to the 3' polyadenylation site of the gene and thus overlapped the domain amplified by IAP3F and IAP4R (Fig. 3.1). PCR was completed with the following thermocycling parameters 1 min at 95° C, a 1 min at 51° C, and 2 min at 72° C; this was repeated 35 times. Products were separated on a 1% agarose gel containing tris-acetate-EDTA buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.3). DNA bands were excised and purified using the Qiaquick gel extraction kit (Qiagen, Valencia, CA). Gel-extracted PCR products were sequenced using the PCR primers at Colorado State University's Macromolecular Resources (Fort Collins, CO). Several samples had an insertion/deletion polymorphism preventing direct sequencing. These samples were instead cloned into the pCR2.1-Topo vector (Invitrogen, Carlsbad, CA) and the inserts of several clones were sequenced. The complete AtIAP1 gene, sequenced in three overlapping pieces, was assembled. Sequence trace files were carefully analyzed and corrected where necessary. They were then aligned using Seqman II version 5.01 (DNAstar Inc., Madison, WI). Complete sequences were determined for 45 LACV+ and 46 LACV- mosquitoes. Genotypes were recorded using the coding scheme in PGenome (Gorrochotegui-Escalante et al., 2005).

C. Analysis of Sequence Variability

The computer program DnaSP 4.10 (Rozas et al., 2003) estimated nucleotide diversity (π) (Nei, 1987), the standard deviation of π (Nei, 1987) and the F^* test for neutrality (Fu & Li, 1993). These analyses were performed on the 91 genotypes that were completely sequenced.

D. Analysis of Genotype Frequencies

Wright's F_{IS} summarizes the relationship between observed and expected

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heterozygotes at each segregating site (Wright, 1965).

$$F_{IS} = 1 - \left(\frac{H_{o(i)}}{H_{e(i)}} \right)$$

where Ho(i) and He(i) are respectively the observed and expected frequencies of heterozygotes containing nucleotide i at a segregating site. Weir and Cockerham's f is an estimator of F_{IS} that is unbiased by small or unequal sample sizes (Weir & Cockerham, 1984) and is calculated as:

$$f = \frac{b}{b + c}$$

where

$$b = \frac{1}{2(\bar{n} - 1)} \left(\sum_y n_y H_{e(iy)} - \frac{(2\bar{n} - 1)}{2\bar{n}} \sum_y n_y H_{o(iy)} \right)$$

$$c = \frac{\sum_y n_y H_{o(iy)}}{2\bar{n}}$$

$\bar{n} = \sum_y n_y$ / number of collections, $H_{e(iy)} = 1 - \sum_i p_i^2$ and p_i is the frequency of nucleotide i at a segregating site and n_y is the size of collection y.

E. Linkage Disequilibrium Analysis

Linkage disequilibrium among all pairs of segregating sites was analyzed with the program PGLD (Gorrochotegui-Escalante et al., 2005) to calculate Ohta's five D-statistics (Ohta, 1982a, Ohta, 1982b). The disequilibrium between two segregating sites (D^2_{ST}) was estimated and a χ^2 analysis of this result was performed. After applying Bonferroni's correction, a half matrix of the results of pairwise comparisons was plotted. In addition, D^2_{ST} was regressed on the number of nucleotides between segregating sites. *A priori* sites that are closer together are expected to be in greater disequilibrium than

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those sites that are farther apart. The significance of this regression using Mantel's test was assessed (Mantel, 1967).

F. Association Mapping Based on Allele and Genotype Frequencies

PGTheta (Gorrochotegui-Escalante et al., 2005) is a program that compares nucleotide frequencies at segregating sites among, in this case, TOT+ and TOT- mosquitoes. At each segregating site, θ (Weir & Cockerham, 1984) was estimated and its consistency was assessed using the permutation procedure of Doerge and Churchill (1996). Potential quantitative trait nucleotides (QTNs) were assigned when the original estimate of θ exceeded 95% of the lowest θ values obtained by permutation. PGCon (Gorrochotegui-Escalante et al., 2005) performed contingency χ^2 tests at the putative QTNs assigned by PGTheta to determine if TOT rates were significantly different among genotypes.

G. AtIAP1 Heated Oligonucleotide Ligation Assay

The heated oligonucleotide ligation assay (Black et al., 2006, Lynd et al., 2005) was used to determine the genotypes of 300 additional mosquitoes (150 TOT+ and 150 TOT-) at the 5 putative QTNs identified by PGTheta in the analysis of full sequences. These mosquitoes were collected from the same regions as the initial samples. PCR on each sample used primers IAP0F (located 199 bp upstream of the sequence in Fig. 3.1) and IAP4R to amplify a portion of the *AtIAP1* gene that contained all five putative QTNs. HOLA reactions were conducted as described by Black et al. (2006) with the oligonucleotides in Table 3.2.

Genotypic results from HOLA analysis were combined with the genotypic results from the previous sequence analysis to give a total 195 TOT+ and 196 TOT- mosquitoes.

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Table 3.2. Reporter and detector oligonucleotides used in genotyping *AtIAP1* segregating sites -361, -208, 542, 555 and 570.

Oligonucleotide Name	Sequence (5' to 3')	Optimal Ligation Temp (°C)
IAP-361dtcA	Biotin-TGATGATGGCRAYGATGCACT	60
IAP-361dtcC	Biotin-TGATGATGGCRAYGATGCACG	
IAP-361rpt	PO4-GAAAGATGCTGATGCTGT-Fluorescein	
IAP-208dtcA	Biotin-TCAATCTTGTTCTCCGGGGGT	51
IAP-208dtcC	Biotin-TCAATCTTGTTCTCCGGGGGG	
IAP-208rpt	PO4-TTTTCCGCRGTTCAGCTC-Fluorescein	
IAP542dtcC	Biotin-TTTCTGCTGWGGCAGCGCGGGCG	58
IAP542dtcT	Biotin-TTTCTGCTGWGGCAGCGCGGGCA	
IAP542rpt	PO4-CAGGAAGTTGTTGGGGGA-Fluorescein	
IAP555dtcA	Biotin-ATTCYGGCCGTTTCTGCTGT	58
IAP555dtcT	Biotin-ATTCYGGCCGTTTCTGCTGA	
IAP555rpt	PO4-GGCAGCGCGGGCRCAGGA-Fluorescein	
IAP570dtcA	Biotin-ATGGCATAGTTGGGATATTCT	58
IAP570dtcG	Biotin-ATGGCATAGTTGGGATATTCC	
IAP570rpt	PO4-GGCCGTTTCTGCTGWGGC-Fluorescein	

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Contingency χ^2 analyses were again performed to determine if the five polymorphic sites had significant genotypic variation.

H. Association Mapping Based on *ND4* Haplotype Frequencies

False genotype-phenotype associations can arise if phenotypes are compared among genetically disparate collections. These false associations arise because of admixtures of different populations arising from gene flow barriers rather than because they condition the phenotype under study. One means to test for potential confounding admixtures is to assess genetic differentiation among phenotype groups using a neutral marker; a marker that wouldn't be expected *a priori* to condition the phenotype of interest. Two sites in the mitochondrial *ND4* gene used in a previous study (Beck et al., 2005) were analyzed by HOLA to estimate haplotype frequencies in each sample. Allelic frequencies were compared between all 195 TOT+ and 196 TOT- mosquitoes, using the PGTheta program, to determine if there are any associations between segregating sites and LACV TOT. A contingency χ^2 test was performed to determine if *ND4* haplotypes were associated with TOT.

3. Results

A. Analysis of Sequence Variability

The amplified *AtIAP1* sequence was 1,665 nucleotides in length with 416 bases in the 5' UTR, 1,212 bases in the coding region, and 37 bases in the 3'UTR (Fig. 3.1). In total, 113 segregating sites were found in the 5' UTR, 144 sites occurred in the coding region, and a single site was found in the 3' UTR for a total of 258 segregating sites (Table 3.3). The overall nucleotide diversity (π) was 0.01133 and θ/site was estimated at 0.026. The average number of nucleotide differences (k) among pairs of mosquitoes was

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Table 3.3. Nucleotide frequency at Segregating Sites in the *Aedes triseriatus* Inhibitor of Apoptosis 1 gene.

Nuc.	Freq. of the Most Common Nucleotide	Hexp	Hobs	Substitution	Codon	Amino Acid
-390	0.7692	0.3551		a > g		
-389	0.6538	0.4527		g > t		
-370	0.9630	0.0713		g > a		
-363	0.9556	0.0849	0.0000	t > g		
-361	0.7167	0.4061	0.0112	c > a		
-353	0.9780	0.0430	0.0000	a > g		
-351	0.9451	0.1038	0.0000	c > t		
-344	0.9890	0.0218	0.0000	a > t		
-341	0.9670, 0.0220	0.0643	0.0000	a > c > t		
-340	0.9615	0.0740	0.0112	c > t		
-335	0.9176, 0.0549	0.1542	0.0112	g > a > -		
-334	0.9725	0.0535	0.0112	a > -		
-333	0.9615, 0.0275	0.0746	0.0112	a > - > g		
-332	0.9451	0.1038	0.0000	g > c		
-326	0.9945	0.0109		a > g		
-324	0.9945	0.0109	0.0112	c > a		
-321	0.9780, 0.0110	0.0433	0.0225	c > g = t		
-320	0.9670	0.0638	0.0000	a > g		
-319	0.9890	0.0218	0.0000	a > g		
-318	0.9121, 0.0549, 0.0275	0.1643	0.0449	c > a > g > t		
-316	0.9615	0.0740	0.0562	- > g		
-315	0.9615	0.0740	0.0562	- > a		
-314	0.9451	0.1038	0.0000	g > -		
-313	0.9451	0.1038	0.0000	a > -		
-310	0.9890	0.0218	0.0000	g > c		
-298	0.9945	0.0109	0.0112	a > g		
-286	0.9835	0.0325	0.0337	c > t		
-282	0.9341, 0.0440	0.1250	0.0000	a > - > t		
-281	0.9560	0.0841	0.0000	c > -		
-280	0.9560	0.0841	0.0000	g > -		
-279	0.9890	0.0218	0.0000	a > -		
-278	0.9890	0.0218	0.0000	c > -		
-277	0.9780, 0.0110	0.0433	0.0000	g > c = -		
-276	0.9945	0.0109	0.0112	g > -		
-274	0.9945	0.0109	0.0112	g > -		
-272	0.9890	0.0218	0.0000	t > c		
-271	0.9890	0.0218	0.0000	g > a		
-270	0.9945	0.0109	0.0112	t > -		
-269	0.9945	0.0109	0.0112	t > -		

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-266	0.9890	0.0218	0.0000	a > g
-265	0.9890	0.0218	0.0000	c > t
-263	0.9890	0.0218	0.0000	a > g
-259	0.9945	0.0109		a > g
-257	0.9560	0.0841	0.0225	c > t
-254	0.9670	0.0638	0.0000	t > c
-244	0.9780	0.0430	0.0000	c > t
-235	0.9945	0.0109	0.0112	c > t
-228	0.9286	0.1326	0.0337	g > a
-227	0.9890	0.0218	0.0000	a > g
-225	0.9945	0.0109	0.0112	c > t
-224	0.9890	0.0218	0.0000	t > c
-223	0.9451	0.1038	0.0000	g > c
-222	0.9505	0.0941	0.0112	a > g
-220	0.9396	0.1135	0.0112	c > t
-219	0.7363	0.3883	0.2247	t > c
-217	0.9835	0.0325	0.0337	c > t
-212	0.9725, 0.0220	0.0537	0.0562	a > g > c
-208	0.9396	0.1135	0.0562	a > c
-202	0.9945	0.0109	0.0112	g > t
-195	0.9890	0.0218	0.0000	a > c
-170	0.9890	0.0218	0.0000	a > g
-169	0.9890	0.0218	0.0000	g > a
-167	0.9890	0.0218	0.0225	c > t
-160	0.9945	0.0109	0.0112	t > a
-150	0.9945	0.0109		t > c
-149	0.9945	0.0109	0.0112	g > t
-142	0.9396, 0.0495	0.1146	0.0337	c > t > -
-141	0.9670	0.0638	0.0225	- > c
-140	0.9670	0.0638	0.0225	- > g
-139	0.9670	0.0638	0.0225	- > t
-138	0.9945	0.0109	0.0112	c > -
-137	0.9945	0.0109	0.0112	g > -
-136	0.9780, 0.0165	0.0432	0.0112	t > c > -
-135	0.9835, 0.0110	0.0326	0.0337	c > t > -
-134	0.9945	0.0109	0.0112	g > -
-128	0.9945	0.0109	0.0112	g > a
-126	0.9890	0.0218	0.0000	c > -
-125	0.9780, 0.0110	0.0433	0.0000	g > c = -
-124	0.9890	0.0218	0.0000	- > t
-123	0.9890	0.0218	0.0000	- > c
-122	0.9890, 0.0055	0.0218	0.0112	- > g = c
-121	0.9890	0.0218	0.0000	t > -
-120	0.9615	0.0740	0.0112	- > c
-119	0.9615	0.0740	0.0112	- > g
-118	0.9615	0.0740	0.0112	- > t

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-114	0.9725	0.0535	0.0112	g > c		
-101	0.9890	0.0218	0.0000	t > g		
-94	0.9890	0.0218	0.0000	a > g		
-77	0.9560	0.0841	0.0000	t > c		
-76	0.9286	0.1326	0.0112	a > t		
-75	0.9286	0.1326	0.0112	c > t		
-74	0.9890	0.0218	0.0225	c > t		
-68	0.9890	0.0218	0.0000	c > t		
-58	0.9945	0.0109	0.0112	g > a		
-57	0.9890	0.0218	0.0000	a > g		
-50	0.9835	0.0325	0.0112	g > a		
-48	0.9890	0.0218	0.0000	c > t		
-47	0.9231	0.1420	0.0000	a > g		
-46	0.9505	0.0941	0.0562	t > c		
-38	0.989, 0.0055	0.0218	0.0225	g > a = t		
-31	0.6154	0.4734	0.0674	a > t		
-30	0.9835	0.0325	0.0112	a > t		
-29	0.9780	0.0430	0.0000	c > t		
-26	0.9890	0.0218	0.0000	g > a		
-22	0.9890	0.0218	0.0225	g > a		
-19	0.9890	0.0218	0.0000	g > t		
-18	0.9505	0.0941	0.0337	a > g		
-17	0.9890	0.0218	0.0000	g > t		
-10	0.9890	0.0218	0.0000	c > t		
-7	0.9670	0.0638	0.0674	t > c		
-6	0.9890	0.0218	0.0000	a > g		
-5	0.9890	0.0218	0.0000	a > g		
-4	0.9615	0.0740	0.0112	g > a		
6	0.9945	0.0109		G > A	ACG > ACA	T
18	0.9890	0.0218	0.0000	G > C	CTG > CTC	L
24	0.9890	0.0218	0.0000	A > T	ATA > ATT	I
36	0.8901	0.1956	0.1124	C > T	TTC > TTT	F
61	0.9780	0.0430	0.0000	T > C	TCC > CCC	S > P
64	0.9890	0.0218	0.0000	C > T	CCC > TCC	P > S
71	0.9451	0.1038	0.0000	T > -	GTA > G-A =	V >
72	0.9451	0.1038	0.0000	A > -	GT- > G--	Fr.shift
73	0.9725, 0.0165	0.0539	0.0112	G > A > C	GGA > GCA > GGG > GGC > AGA > GG- > CGA = GCG > GCC > GC- = ACA = CCA > AGG = AGC*	G > A > G > G > R > Fr.shift > R = A > A > Fr.shift = T = P > R = S*
74	0.8352	0.2753	0.0449	G > C		
75	0.8956, 0.0495, 0.0440	0.1934	0.0112	A > G > C > -		

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76	0.9451, 0.0440	0.1047	0.0000	G > - > C	GTA > -TA > CTA	V > Fr.shift > L
81	0.9451	0.1038	0.0000	T > C	ACT > ACC	T
83	0.9890	0.0218	0.0000	G > A	GGG > GGA > GAG > GAA	G > G > E > E
84	0.9121	0.1603	0.0225	G > A		
87	0.9890	0.0218	0.0225	G > A	GCG > GCA	A
96	0.9890	0.0218	0.0000	G > A	GTG > GTA	V
120	0.9945	0.0109	0.0112	C > T	GTC > GTT	V
132	0.9890	0.0218		C > T	GAC > GAT	D
133	0.9890	0.0218	0.0000	T > A	TCG > TCA = ACG > ACA	S > S = T > T
135	0.9835	0.0325	0.0112	G > A		
138	0.9890	0.0218	0.0000	C > T	TTC > TTT	F
152	0.9945	0.0109	0.0112	T > C	GTT > GCT	V > A
154	0.9780	0.0430	0.0225	T > C	TCG > TTG > CCG > CTG > TCA > TTA > CCA > CTA	S > L > P > L > S > L > P > L
155	0.5659	0.4913	0.2472	C > T		
156	0.9890	0.0218	0.0000	G > A		
162	0.9890	0.0218	0.0000	C > T	ATC > ATT	I
169	0.9945	0.0109	0.0112	G > A	GCT > GAT > ACT > AAT	A > D > T > N
170	0.9890	0.0218	0.0000	C > A		
174	0.9890	0.0218	0.0000	G > A	GAG > GAA	E
180	0.9835	0.0325	0.0000	C > T	GCC > GCT	A
189	0.8626, 0.0824	0.2461	0.0112	C > G > A	GGC > GGG > GGA	G
191	0.9945	0.0109		T > A	TTT > TAT	F > Y
194	0.9945	0.0109	0.0112	A > C	TAC > TAA = TCC > TCA	Y > stop > S > S
195	0.9945	0.0109	0.0112	C > A		
201	0.9945	0.0109	0.0112	C > T	GTC > GTT	V
205	0.9231	0.1420	0.0225	C > G	CCA > GCA > CCC > GCC	P > A > P > A
207	0.9890	0.0218	0.0000	A > C		
213	0.9945	0.0109	0.0112	T > C	GAT > GAC	D
227	0.9945	0.0109	0.0112	A > T	TAC > TTC	Y > F
237	0.9835	0.0325	0.0337	A > C	CGA > CGC	R
246	0.9945	0.0109	0.0112	C > T	ATC > ATT	I
249	0.9945	0.0109	0.0112	A > G	GGA > GGG	G
252	0.9396	0.1135	0.0112	G > A	CTG > CTA	L
258	0.9945	0.0109	0.0112	G > A	GAG > GAA	E
267	0.9945	0.0109	0.0112	C > T	GAC > GAT	D
276	0.9835	0.0325	0.0112	T > C	CTT > CTC	L

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297	0.7198, 0.2747	0.4064	0.2809	C > A > G	TCC > TCA > TCG	S
303	0.9945	0.0109	0.0112	C > T	TAC > TAT	Y
313	0.9945	0.0109	0.0112	C > T	CTG > TTG	L
318	0.9835	0.0325	0.0112	T > A	CGT > CGA	R
327	0.9670	0.0638	0.0000	A > G	CAA > CAG	Q
330	0.9670	0.0638	0.0000	C > A	ACC > ACA	T
333	0.9945	0.0109	0.0112	C > T	ACC > ACT	T
354	0.9945	0.0109	0.0112	C > T	AGC > AGT	S
366	0.9231	0.1420	0.0899	G > A	CAG > CAA	Q
369	0.9615	0.0740	0.0112	G > T	CTG > CTT	L
372	0.9725	0.0535	0.0337	G > A	CCG > CCA	P
384	0.9835	0.0325	0.0337	C > T	TAC > TAT	Y
390	0.9945	0.0109	0.0112	C > A	ACC > ACA	S
402	0.9725	0.0535	0.0562	G > A	AGG > AGA	R
417	0.9945	0.0109	0.0112	C > T	TCC > TCT	S
420	0.9835	0.0325	0.0225	G > A	GTG > GTA	V
423	0.9725	0.0535	0.0562	A > T	GCA > GCT	A
429	0.9890	0.0218	0.0000	T > C	AAT > AAC	N
438	0.9890	0.0218	0.0225	T > A	TCT > TCA	S
441	0.8956	0.1870	0.2135	G > A	TCG > TCA	S
444	0.9835	0.0325	0.0337	G > C	TCG > TCC	S
448	0.9615	0.0740	0.0787	C > A	CGA > AGA	A
456	0.9670	0.0638	0.0000	T > C	AGT > AGC	S
462	0.7637, 0.1538	0.3863	0.1011	T > A > C	GGT > GGA > GGC	G
468	0.9945	0.0109	0.0112	A > G	CTA > CTG	L
471	0.9890	0.0218	0.0225	G > A	TCG > TCA	S
480	0.9890	0.0218	0.0225	C > T	CCC > CCT	P
483	0.9451	0.1038	0.0000	C > T	AGC > AGT	S
490	0.9945	0.0109	0.0112	A > C	ACG > CCG	T > P
508	0.9451	0.1038	0.0000	T > C	TTA > CTA	L
523	0.9890	0.0218	0.0112	A > G	ATC > GTC	I > V
526	0.7802	0.3430	0.2247	C > G	CCC > GCC > CCT > GCT	P > A > P > A
528	0.9890	0.0218	0.0000	C > T		
540	0.9945	0.0109	0.0112	T > A	CCT > CCA	P
541	0.9451	0.1038	0.0000	G > C	GCG > GTG > GCA > CTG > GTA > CCG > CCA > CTA	A > V > A > L > V > P > P > L
542	0.7637	0.3609	0.3258	C > T		
543	0.9780	0.0430	0.0000	G > A		
546	0.9945	0.0109	0.0112	C > T	CCC > CCT	P
549	0.9396	0.1135	0.0112	G > A	GCG > GCA	A
555	0.6703	0.4420	0.0225	A > T	CCA > CCT	P
561	0.9670	0.0638	0.0449	G > A	CAG > CAA	Q
565	0.9780	0.0430	0.0449	C > A	CGG > AGG	R

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570	0.9176	0.1512	0.1573	G > A	CCG > CCA	P
594	0.9835	0.0325	0.0337	G > A	GAG > GAA	E
597	0.9615	0.0740	0.0112	G > A	GCG > GCA	A
600	0.8516	0.2528	0.2135	G > A	AAG > AAA	K
603	0.5549, 0.2473	0.5918	0.4831	A > C > G	CGA > CGC > CGG	R
604	0.5989	0.4804	0.1910	C > T	CTG > TTG >	L
606	0.7418	0.3831	0.2809	G > A	CTA > TTA	
615	0.9945	0.0109	0.0112	C > T	TAC > TAT	Y
639	0.5879	0.4845	0.3483	G > A	AAG > AAA	K
645	0.9945	0.0109	0.0112	G > A	AAG > AAA	K
657	0.9945	0.0109	0.0112	A > T	CTA > CTT	L
666	0.8956	0.1870	0.0787	C > T	GCC > GCT	A
675	0.9945	0.0109	0.0112	C > T	TTC > TTT	F
681	0.9396, 0.0440	0.1149	0.1011	T > C > A	ACT > ACC > ACA	T
693	0.9780, 0.0165	0.0432	0.0225	T > G > C	GAT > GAG > GAC	D > E > D
694	0.9890	0.0218	0.0000	C > G	CGA > CGT > GGA > GGT	R > R > G > G
696	0.8242	0.2898	0.0225	A > T		
699	0.9725, 0.0220	0.0537	0.0337	C > G > A	GTC > GTG > GTA	V
710	0.9890	0.0218	0.0000	G > T	AGC > ATC	S > I
723	0.9890, 0.0055	0.0218	0.0225	C > G = A	GGC > GGG = GGA	G
727	0.9890	0.0218	0.0000	A > C	AAG > AAA > CAG > CAA	K > K > Q > Q
729	0.9835	0.0325	0.0112	G > A		
744	0.9111	0.1620	0.0225	G > A	GAG > GAA	E
775	0.9945	0.0109	0.0112	T > C	TAT > CAT	Y > H
783	0.9451	0.1038	0.0000	T > C	AAT > AAC	N
806	0.9945	0.0109	0.0112	A > C	AAA > ACA	K > T
816	0.9341	0.1231	0.0000	A > G	GAA > GAG	E
822	0.9945	0.0109	0.0112	C > T	ATC > ATT	I
825	0.9670	0.0638	0.0000	C > T	GCC > GCT	A
861	0.9945	0.0109	0.0112	G > C	CAG > CAC	Q > H
879	0.9451, 0.0495	0.1043	0.0112	G > T > C	GGG > GGT > GGC	G
918	0.9780	0.0430	0.0000	A > T	ACA > ACT	T
921	0.9890	0.0218	0.0000	C > T	TCC > TCT	S
954	0.9725	0.0535	0.0225	G > A	TCG > TCA	S
966	0.9560	0.0841	0.0000	T > C	AGT > AGC	S
969	0.9560	0.0841	0.0000	A > T	GCA > GCT	A
972	0.9505	0.0941	0.0225	G > A	TCG > TCA	S
984	0.9945	0.0109	0.0112	C > T	GTC > GTT	V

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994	0.6758	0.4382	0.0112	C > A	CTC > ATC	L > I
998	0.9945	0.0109	0.0112	G > C	GGC > GCC	G > A
1010	0.9945	0.0109	0.0112	G > T	AGT > ATT	S > I
1041	0.9890	0.0218	0.0000	T > C	AAT > AAC	N
1059	0.9945	0.0109	0.0112	T > C	AGT > AGC	S
1064	0.9451	0.1038	0.0000	T > C	ATC > ACC	I > T > I >
1065	0.9560	0.0841	0.0000	C > T	> ATT > ACT	T
1089	0.6044	0.4782	0.0225	A > G	GAA > GAG	E
1104	0.9890	0.0218	0.0000	C > T	TTC > TTT	F
1107	0.8791	0.2126	0.0674	G > A	TCG > TCA	S
1110	0.9670, 0.0110	0.0645	0.0000	C > A = G = T	CCC > CCA = CCG = CCT	P
1116	0.7033	0.4173	0.0674	G > A	GGG > GGA	G
1122	0.9945	0.0109	0.0112	A > G	GTA > GTG	V
1137	0.9505	0.0941	0.0112	A > G	AAA > AAG	K
1155	0.9890	0.0218	0.0000	C > T	ACC > ACT	T
1164	0.7527	0.3723	0.0787	A > G	CCA > CCG	P
1167	0.9670	0.0638	0.0000	C > T	CTC > CTT	L
1221	0.9888	0.0221	0.0000	a > t	A	

* All remaining codon possibilities have a frequency of less than 0.0005
Polymorphic sites that are boxed occur within the same amino acid codon
Highlighted sites are the ones shown to be significant using PGtheta analysis of *AtIAP1* sequences.

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17.432. π varied between 0.000 and 0.595 across the gene (Fig. 3.2) with π being ~13.5 times greater in synonymous vs. nonsynonymous substitutions. DNAsp 4.10 does not estimate nucleotide diversity with missing data or indels, so the first 53 and the last 25 nucleotides were not included in this analysis.

F_{IS} varies between -1 and 1 with a positive value indicating an excess of homozygotes and a negative value indicating an excess of heterozygotes. Segregating sites in the *AtIAP1* gene had a consistent excess of homozygotes. Of the 258 segregating sites only 25 had a negative F_{is}. The majority of these sites were found within the coding sequence and more specifically near the 1st serine rich domain. This is the region with the greatest amount of nucleotide diversity.

Fu and Li's F^* is a normalized comparison of all mutations (η) relative to the number of those appearing once ("singletons"- η_s). The underlying assumption of this test is that $F^* > 0$ ($\eta > \eta_s$) under balancing selection, that $F^* = 0$ ($\eta = \eta_s$) under neutral selection, and that $F^* < 0$ ($\eta < \eta_s$) under purifying selection. Our analysis (Fig. 3.3) shows that the majority of the gene has a negative F^* value suggesting a general pattern of purifying selection. Furthermore, the negative F^* value is significant in the BIR domains suggesting that mutations in these regions may compromise fitness and are rapidly eliminated through purifying selection. These observations are consistent with the F_{IS} analysis because an excess of homozygotes is also indicative of purifying selection. Furthermore, the region of the gene with an F^* value around zero, indicating neutral selection, is the same as where there is a small excess of heterozygotes.

B. Linkage Disequilibrium Analysis

Polymorphisms occurring between the first BIR and serine-rich domains were

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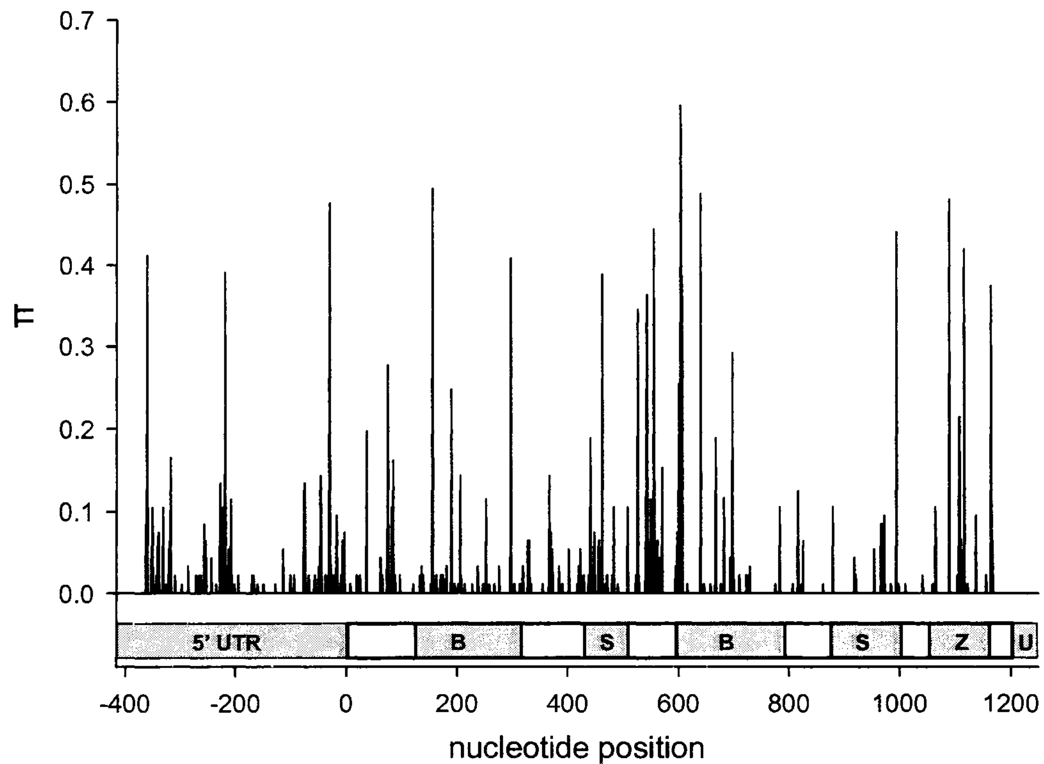

Figure 3.2. Plot of nucleotide diversity (π) across *AtIAP1*. 5' UTR = 5' untranslated region, B = BIR, S = serine-rich domain, Z = zinc-finger motif, and U = 3' untranslated region. Coding region begins at nucleotide 0 and ends at nucleotide 1,212.

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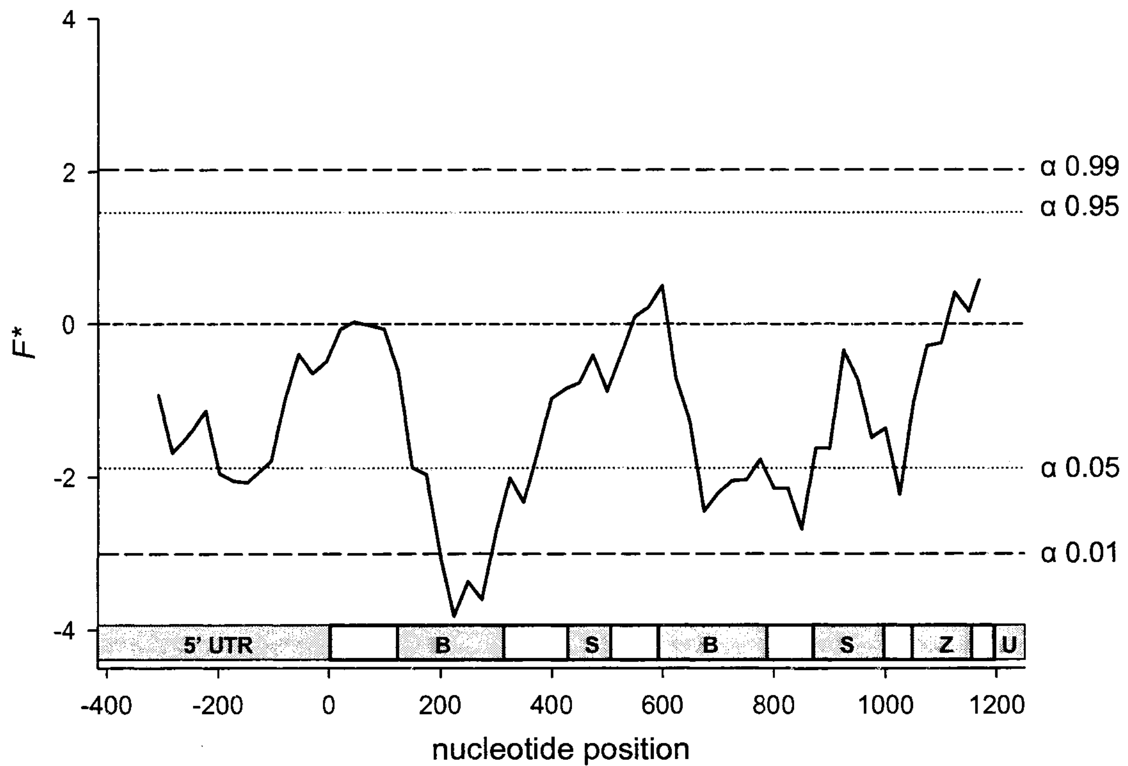

Figure 3.3. Plot of Fu and Li's F^* across *AtIAP1*. A significant negative value indicates a substitution pattern consistent with purifying selection. A significant positive value indicates patterns consistent with balancing selection. 5' UTR = 5' untranslated region, B = BIR, S = serine-rich domain, Z = zinc-finger motif, and U = 3' untranslated region. Coding region begins at nucleotide 0 and ends at nucleotide 1,212.

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more likely to be in linkage disequilibrium amongst themselves and with other segregating sites (Fig. 3.4). There was little linkage disequilibrium among segregating sites in the 5' UTR. Regression analysis of $D^{2'}_{ST}$ on the number of nucleotides between segregating sites estimated $D^{2'}_{ST} = 1.79 \times 10^{-6} x$ nucleotides + 5.87×10^{-3} with $r^2 = 1.58 \times 10^{-3}$. Neither the y- intercept nor the slopes were significantly greater than 0. Linkage disequilibrium does not appear to decrease with physical distance between segregating sites, i.e. the recombination frequency between mutations occurring at nearby sites is similar to the recombination frequency between those mutations that occur farther apart.

C. Association Mapping Based on Allele and Genotype Frequencies

PGTheta identified five sites (at nucleotides -361, -208, 542, 555, and 570) in which θ estimated from the original dataset was greater than 95% of permuted θs (Fig. 3.5). Two of these putative QTNs were found in the 5' UTR, the other three were found between the first serine-rich domain and the second BIR domain. QTNs 555 and 570 encode synonymous substitutions. QTN 542 encodes a transition in the second codon (GCG ⇔ GTG) causing an A ⇔ V amino acid substitution . Because the two other positions in this codon are also polymorphic (albeit with fairly low frequency), leucine and proline are also possible amino acid substitutions. QTNs 542 and 555 were in linkage disequilibrium. The LACV infection rate was significantly different ($p < 0.05$) among genotypes at QTNs -361, 555, and 570, but not at sites -208 and 542 (Fig. 3.6).

D. Assessing the Risk for False Positives Using the Mitochondrial *ND4* Gene

ND4 mitochondrial haplotypes were determined in each mosquito and then compared among TOT+ and TOT- mosquitoes. None of the segregating sites have a θ

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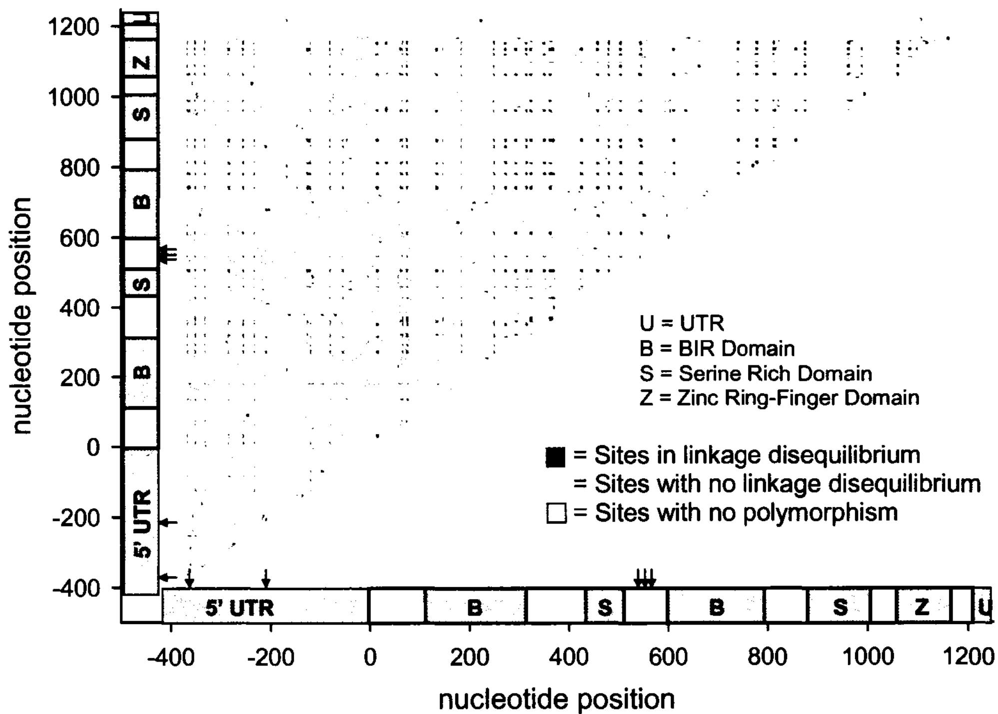

Figure 3.4. A half-matrix showing linkage disequilibrium coefficient D^2_{ST} between *AtIAP1* segregating sites. 5' UTR = 5' untranslated region, B = BIR, S = serine-rich domain, Z = zinc-finger motif, and U = 3' untranslated region. Arrows indicate location of five potential QTNs. Coding region begins at nucleotide 0 and ends at nucleotide 1,212.

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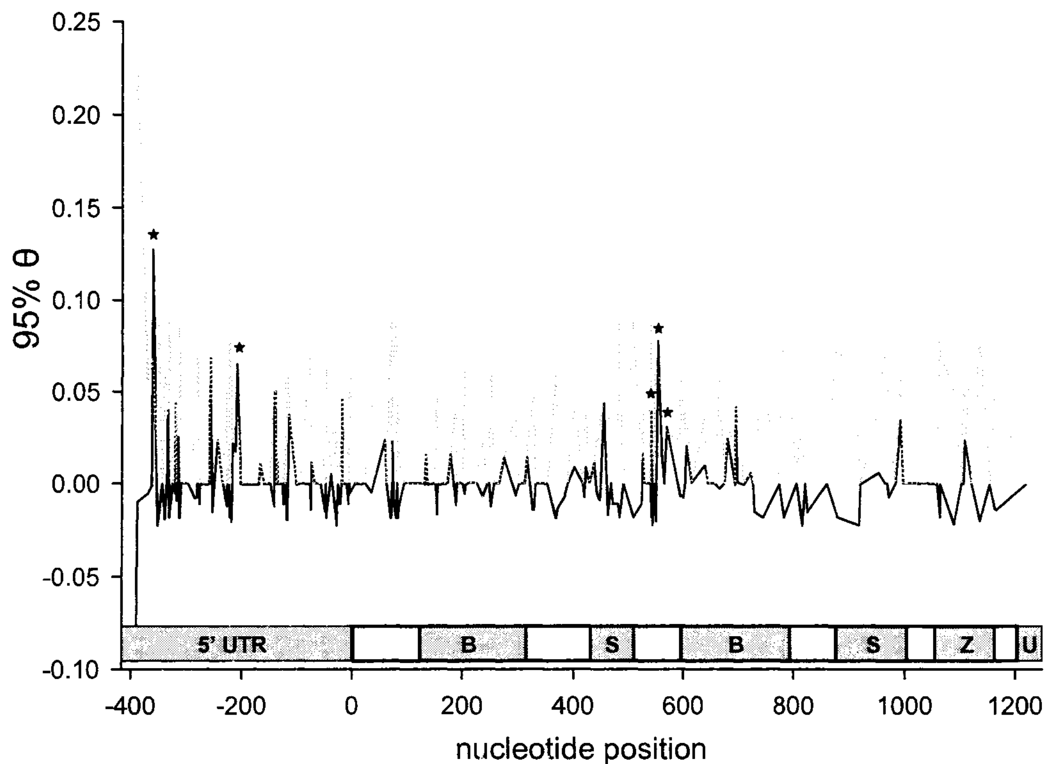

Figure 3.5. Association mapping of *AtIAP1* comparing polymorphic nucleotide frequencies between TOT+ and TOT- mosquitoes using PGtheta. An asterisk indicates a position where the estimated θ exceeds the lowest 95% permuted θs. 5' UTR = 5' untranslated region, B = BIR, S = serine-rich domain, Z = zinc-finger motif, and U = 3' untranslated region. Coding region begins at nucleotide 0 and ends at nucleotide 1212.

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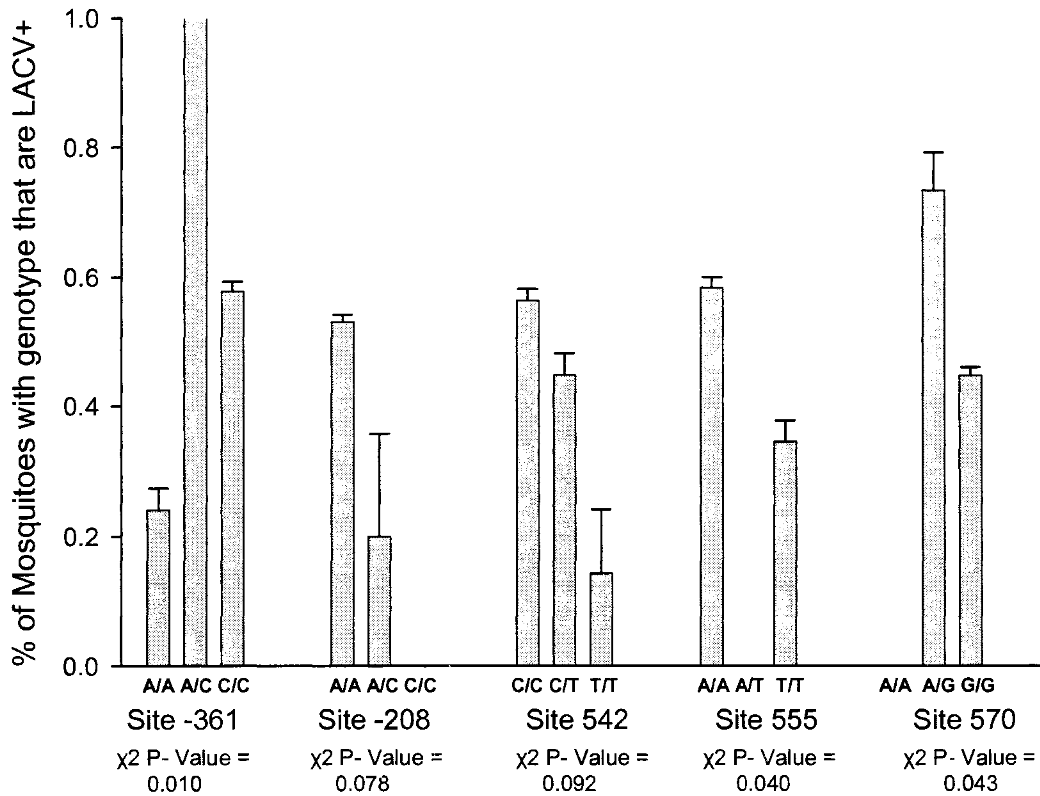

Figure 3.6. Association mapping of genotype frequency based on *AtIAP1* sequencing analysis. Sequence analysis of 45 LACV+ and 46 LACV- *Ae. triseriatus* mosquitoes shows significant differences in allele frequency at sites -361, -208, 542, 555, and 570. χ^2 analysis of these five nucleotides reveals significant genotypic differences among LACV+ and LACV- samples at sites -361, 555, and 570.

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value greater than the 95% lowest permuted θs suggesting that the mosquitoes used in the association mapping are not an admixture (Fig. 3.7).

E. Association Mapping Based on Single Nucleotide Polymorphisms

To evaluate further the 5 putative QTN's, further analyses were performed on an additional 150 LACV+ and 150 LACV- mosquitoes using HOLA. These results were analyzed separately and then combined with the results from the fully sequenced mosquitoes. In both analyses, the TOT infection rate was not statistically different among genotypes at all five putative QTN sites (Figs. 3.8 and 3.9).

4. Discussion

Five significant QTNs associated with TOT were detected in a sequence analysis of 91 mosquitoes. However, the subsequent prospective case control study failed to validate any of these 5 QTNs. False positive QTNs were detected in the initial sequence analysis, probably because of small sample sizes or possibly due to sequencing errors. Irrespective of the reasons, this study did not find an association between *AtIAP1* polymorphisms and TOT in field collected *Ae. triseriatus*.

Implicit assumptions in the study design reduce its power to detect valid QTNs. The design assumes that the *Ae. triseriatus* mothers of the offspring in this study were uniformly susceptible to infection with LACV and that all mothers were exposed to LACV. If true, then the only differences among reared TOT+ and TOT- adults would arise from genetic differences among mothers in the genes that condition TOT. The first assumption is probably valid; typically *Ae. triseriatus* are uniformly susceptible to oral or vertical infection with LACV (Woodring et al., 1998). The second assumption is obviously valid with TOT+ offspring but is probably false among TOT- offspring. The

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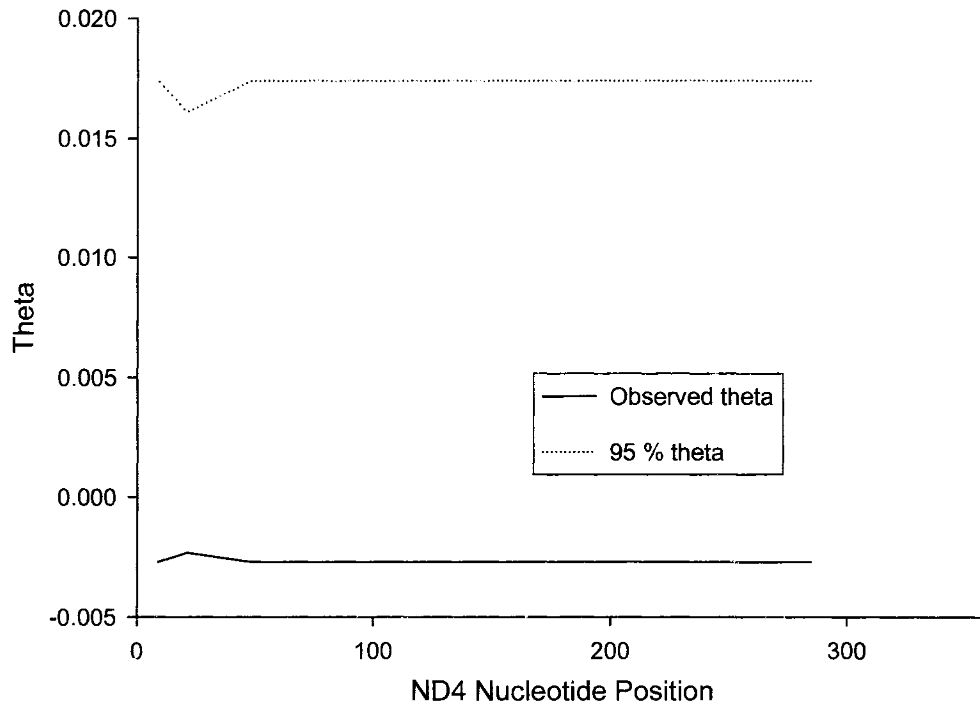

Figure 3.7. Association mapping of NADH subunit 4 mtDNA comparing nucleotide frequencies between TOT+ and TOT- mosquitoes using PGtheta. Single nucleotide polymorphisms in the NAD dehydrogenase subunit 4 were compared between LACV infected and uninfected mosquito samples. A potential quantitative trait nucleotide (QTN) would be indicated if the observed theta value was greater than the 95% theta value. In a neutral gene such as this one, we would not expect to find any QTNs.

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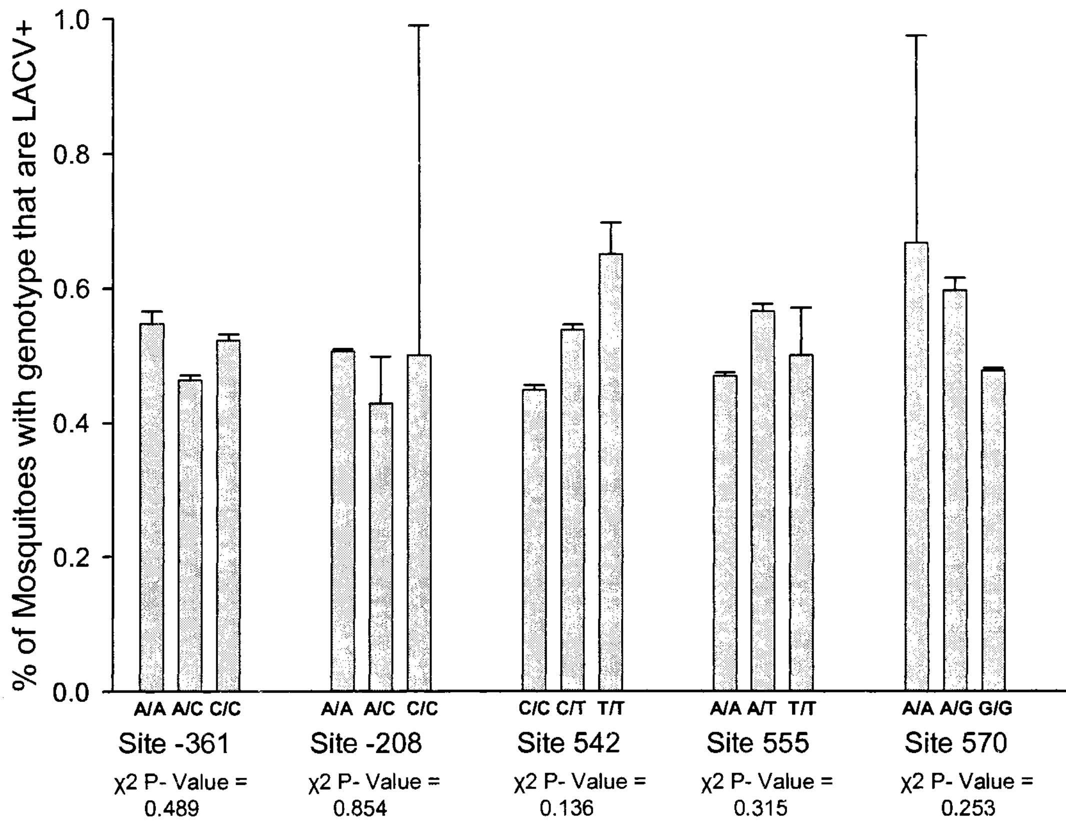

Figure 3.8. Association mapping of genotype frequency based on *AtIAP1* HOLA analysis. 300 previously ungenotyped individuals were genotyped at the five potential QTNs found in the *AtIAP1* gene by sequence analysis. After further analysis with a larger sample, the associations detected by sequencing did not hold up.

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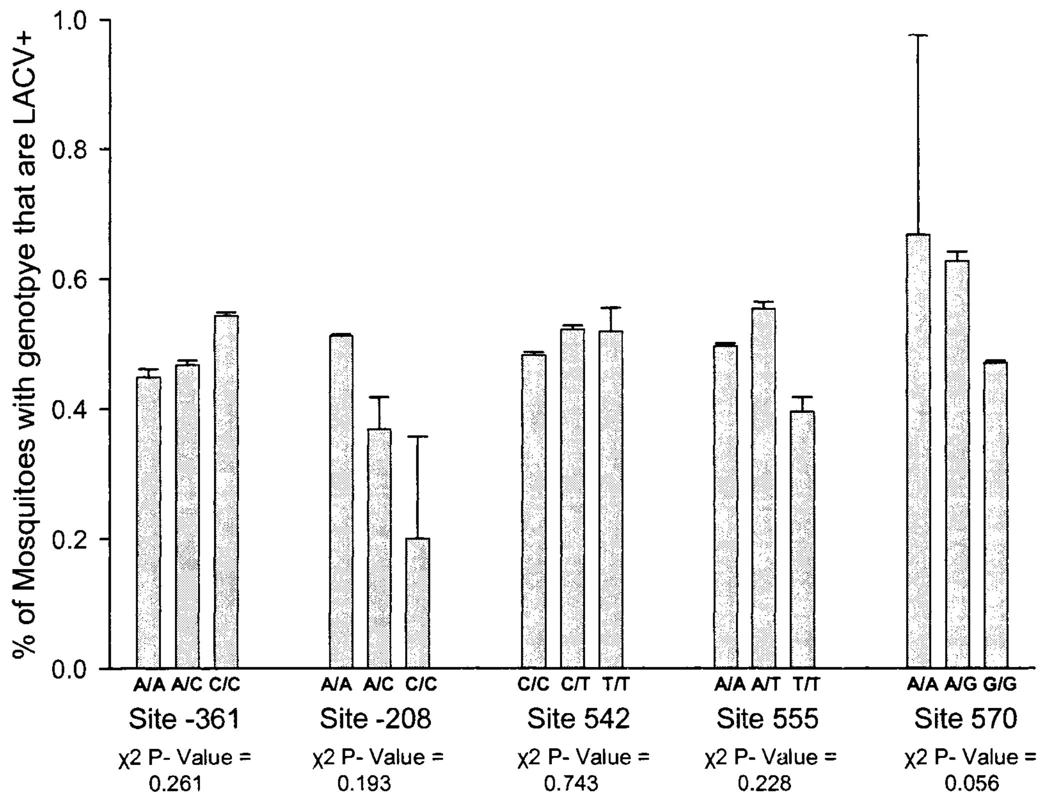

Figure 3.9. Association mapping of genotype frequency based on *AtIAP1* sequencing and HOLA analysis. This figure combines the results found in figures 3.6 and 3.8 and compares the % of LACV positive mosquitoes with a given genotype using all samples (those genotyped by sequencing and HOLA). This analysis has the largest sample size (and thus the most power) and again fails to validate any associations between single nucleotide polymorphisms and TOT potential.

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Minimal Field Infection Rate of *Ae. triseriatus* with LACV is 3.4 - 12.7/1000; making it difficult to verify whether offspring are uninfected because their mothers were never exposed to LACV or because the mothers were genetically incapable of TOT (Clark et al., 1983). This assumption could have been eliminated by returning mosquitoes to the laboratory, uniformly exposing them to LACV with either oral or intrathoracic inoculation, and then collecting eggs and analyzing the resulting offspring. This was the procedure followed by Gorrochotegui-Escalante et al. (2005) in association mapping of the *Early Trypsin* gene with susceptibility to Dengue 2 virus. However, this strategy is problematic with *Ae. triseriatus* because they are difficult to colonize from the field.

Problems arising from non-uniform exposure of a study group do not preclude association mapping studies. Human genetic epidemiologists have to deal with non-uniform exposure in identifying genetic factors that condition genetic susceptibility to heritable or infectious diseases. Nevertheless, non-uniform exposure does lower the power of association mapping to detect valid QTN. Only QTNs with large effects on phenotype are likely to be detected.

One way of evaluating the 5 QTNs detected in the present study would be to repeat the study in the same geographic region but in additional years or in other geographic regions in which *Ae. triseriatus* occurs. If the QTNs are valid then they should be detected in other populations. There are 258 segregating sites in *AtIAP1* in the upper Midwest. If we assume that the same number of segregating sites will be found in other locations then with $\alpha = 0.05$ we would expect ~13 QTN (0.05 x 258) to be detected in another panmictic unit by random chance. However, the likelihood of the same segregating site being detected as a QTN in two panmictic units is $(1/258)^2 = 1.5 \times 10^{-5}$ or

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5.8×10^{-8} in three panmictic units. Thus replication of QTN analyses across panmictic units is a potential method for validating a QTN with a low probability of detecting a false QTN.

Replication of the study can also be used to qualitatively assess the validity and repeatability of the phenotype-genotype associations as shown in Figure 3.6. If a segregating site has two alternate nucleotides N_1 and N_2 then there are 3 possible phenotype-genotype associations: TOT rate (N_1/N_1), TOT rate (N_1/N_2), and TOT rate (N_2/N_2). If any of the QTNs detected in this study are valid then the way that specific genotypes both qualitatively and quantitatively condition a phenotype should be repeatable across panmictic sampling units.

This population genetic analysis of *AtIAP1* revealed 258 segregating sites. Of the 144 sites in the coding region, 37 were nonsynonymous. The overall nucleotide diversity $\pi = 0.011$ and θ/site = 0.026 and Figure 3.2 indicated that the variation was fairly uniformly distributed across the genome. In contrast with the *Ae. aegypti Early trypsin* gene, π was nearly equivalent at 0.012 but θ/site was 0.010, only one third of that found in *AtIAP1*. In the *Ae. aegypti* a*bundant trypsin* gene, π was only slightly lower at 0.009 and the θ/per site (0.009) was again one third of that found in AtIAP1. Thus the average diversity per site is one third as large in *AtIAP1*.

This greater diversity could arise due to relaxation of selection, or through balancing or diversifying selection. The high proportion of synonymous substitutions is not consistent with diversifying or balancing selection. Furthermore, the overall negative values of F^* (Fig. 3.3) indicate that the numbers of singletons exceeded the overall numbers of shared mutations which is strong evidence of purifying selection. Significant

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negative F^* estimates occurred in the BIR domains. In *Drosophila*, BIR domains are targeted by pro-apoptotic caspases to neutralize DIAP1. Amino acid substitutions in this portion of the gene would compromise this function in AtIAP1 and probably the fitness of *Ae. triseriatus*.

Previous studies of gene flow in mosquitoes from the same geographic region as examined here showed that *Ae. triseriatus* exists as a large panmictic population (Beck et al., 2005) in the upper Midwest with little evidence of genetic drift. Neutral theory predicts that with large effective population size, neutral mutations can only be maintained by weak positive selection (Kimura, 1983). Otherwise the large numbers of singletons and the greater θ/site seen in AtIAP1 are difficult to explain.

While mutations in the coding sequence of *AtIAP1* lack associations with ability of *Ae. triseriatus* to transovarially transmit LACV, this study does not necessarily rule out the involvement of *AtIAP1* in TOT of LACV. It is possible that this gene or other apoptosis-related genes may yet play a role in this phenotype. Unique splicing found in the distal portion of the 5' UTR of AtIAP1 mRNA may contribute to conditioning TOT. These splicing events could lead to differences in AtIAP1 protein expression or mRNA stability and are explored in greater detail in chapter four of this dissertation. It is possible that protein levels rather than single nucleotide polymorphisms are involved in conditioning TOT. Other candidate genes that have either pro- or anti-apoptotic properties in mosquitoes include: Michelob_x (Zhou et al., 2005) and Ancaspase-7 (Abraham et al., 2004). While both of these genes were described from the *Anopheles gambiae* genome, it is likely that they have a homolog in *Ae. triseriatus*. Determining which genes (and

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which QTNs within those genes) control TOT could lead to better LACV risk assessment in a given area.

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Chapter 4:

Alternative Splicing Generates Multiple mRNA Variants of the *Aedes triseriatus* Inhibitor of Apoptosis 1 Gene

A portion of the work from this chapter has been accepted for publication in Insect Biochemistry and Molecular Biology:

Beck, E. T., Blair, C. D., Black IV, W. C., Beaty, B. J., & Blitvich, B. J. Alternative splicing generates multiple transcripts of the inhibitor of apoptosis 1 gene in *Aedes* and *Culex* spp. *Insect Biochem Mol Biol* **In Press**.

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1. Introduction

A homologue of the *Drosophila* inhibitor of apoptosis 1 (*DIAP1*) gene was recently characterized from the La Crosse virus vector, *Aedes triseriatus* (Say) and designated *AtIAP1* (Blitvich et al., 2002). The *AtIAP1* translation product is a 403 amino acid protein that contains 2 BIRs and a RING finger motif. AtIAP1 mRNA was detectable by RT-PCR using primers that amplify a 1,461 nt region (nt -252 to +1209) in all mosquito life-stages (embryos, first–fourth instar larvae, early and late pupae, and adults) and adult tissues (midguts, ovaries, and salivary glands) examined. In contrast, the AtIAP1 protein was detectable by western blot only in certain developmental stages (first instar larvae, early pupae, and adults) and tissues (salivary glands and ovaries). Taken together, these data suggest that expression of AtIAP1 is post-transcriptionally regulated (Blitvich et al., 2002). More recent studies characterized an IAP1 from *Ae. albopictus* (designated *AaIAP1*). This is the only other mosquito gene with significant similarity to a known apoptotic suppressor to be studied in detail (Li et al., 2007). Expression of AaIAP1 protected lepidopteran cells from Hid-induced apoptosis, and vertebrate cells from bluetongue virus-induced apoptosis. Homology to both *DIAP1* and *AaIAP1*, two known inhibitor of apoptosis genes, further indicates that AtIAP1 functions as an inhibitor of apoptosis.

The 5' untranslated region (5'UTR) of the AtIAP1 mRNA is unusually long, and possesses significant secondary structure when analyzed using the MFOLD RNA folding server (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html), suggesting that it has an important role in post-transcriptional regulation (Blitvich et al., 2002). Unusually long 5'UTRs are also present in the mRNAs of other IAPs, such as X-linked inhibitor of

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apoptosis (XIAP) and human inhibitor of apoptosis 2 (HIAP2) (1.6 kb and 1.2 kb, respectively), suggesting that this feature is common among IAPs. The 5'UTR of XIAP1 mRNA contains an internal ribosomal entry site (IRES) that regulates translation in response to serum starvation and low dose gamma irradiation in human cell lines (Holcik & Korneluk, 2000, Holcik et al., 1999, Nevins et al., 2003). HIAP2 translation is regulated by an inducible IRES in response to endoplasmic reticulum stress (Warnakulasuriyarachchi et al., 2004).

Previous studies identified heterogeneous sequences at the distal end of the AtIAP1 mRNA 5'UTR (Blitvich et al., 2002). Four cDNA clones were analyzed by 5' rapid amplification of cDNA ends (5' RACE) and automated sequencing. The 5'UTRs encoded by these cDNAs varied in length. The 401 nt immediately upstream of the open reading frame (ORF) were identical for each clone, but the distal nucleotide sequences possessed no significant similarity. Since that time, studies have shown that there are at least five AtIAP1 mRNA variants that only differ in the distal end of the 5'UTR (Beck et al., In Press). Similar variable sequences are found in the 5'UTR of other mosquito IAP1 genes including those from *Ae. albopictus* (Skuse), *Ae. aegypti* (Linnaeus), *Culex pipiens* Linnaeus and *Cx. tarsalis* Coquillett (Beck et al., In Press).

Alternative splicing has been reported frequently in other organisms, including in an estimated 35-60% of all human genes (Mironov et al., 1999, Stamm et al., 2005). Many other apoptosis-regulatory genes are expressed as distinct mRNA variants or protein isoforms resulting from alternative splicing, which demonstrates that this mode of gene regulation plays a major role in the control of apoptosis (Schwerk & Schulze-Osthoff, 2005, Wu et al., 2003). Three DIAP1 mRNA variants were predicted during the

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computational analysis of the *Drosophila melanogaster* genome. Each transcript shares the same ORF and a 78 nt region of the 5'UTR, but the distal ends of their 5'UTRs contain heterogeneous sequences of 352, 197 or 158 nt (GenBank accession nos. NM_079377, NM_168644, NM_168645; isoforms A, B and C, respectively). Similar predictions were made for the *DIAP2* gene, indicating that alternative splicing is responsible for generating two mRNA variants differing only at the distal ends of their 5'UTRs (GenBank accession nos. NM_057779 and NM_176182; isoforms A and B, respectively) (Adams et al., 2000). The biological significance of these DIAP1 and DIAP2 mRNA variants has not been studied.

While there is no information available on the developmental or tissue-specific distribution of invertebrate IAPs, the expression profiles of several mammalian IAPs that exist as alternative splice variants have been determined. The murine survivin protein exists as three different protein isoforms and is involved in inhibition of caspase-3. The tissue-specific expression of the 3 survivin mRNA variants was assessed by RT-PCR in adult mice, with $survivin_{121}$ detectable in all 10 tissues examined, $survivin_{140}$ detectable only in the thymus and testis, and $survivin_{40}$ not detectable in any of the tissues examined (Conway et al., 2000). Differential expression patterns were also reported for the two Livin splice variants. Livin α mRNA was not detectable by RT-PCR in any of the 8 human fetal tissues examined, whereas Livin β mRNA was detectable only in fetal kidney, heart and spleen (Ashhab et al., 2001). These results indicate that mRNA variants may be produced to function in tissue specific circumstances.

While studies in the previous chapter failed to identify any single nucleotide polymorphisms conditioning TOT of LACV, it is possible that differences in AtIAP1

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mRNA variant expression functions in this capacity. Previous studies reveal that the 5' end of the LACV S-segment mRNAs contain 9-15 nucleotides of host-derived sequence (Dobie et al., 1997). In diapausing *Ae. triseriatus* embryos that were infected via TOT the most common host sequence is CCACTCGCCACT (Dobie et al., 1997). This sequence can be found in the homogeneous region of the AtIAP1 5' UTR with the caveat that the underlined "G" in the sequence is more commonly an "A" (Blitvich et al., 2002). The predicted secondary structure of each AtIAP1 mRNA variant reveals differences in accessibility of this sequence indicating that predominant expression of a particular AtIAP1 mRNA variant may create increased opportunities for LACV to effectively scavenge the cap.

The following experiments were performed to determine whether AtIAP1 mRNA variants are formed through alternative splicing or whether they are products of separate genes. Sequence alignments of AtIAP1 mRNA variants with other Culicine IAP1 mRNA variants were also analyzed to determine whether heterogeneous sequences are conserved across mosquito species. The expression profiles of each mRNA variant were characterized to determine which variants are most abundant in particular life stages and tissue types. Finally, to determine if particular AtIAP1 mRNA variants play a role in conditioning TOT of LACV, expression profiles of each AtIAP1 mRNA variant were obtained for the midguts and ovaries of bloodfed TOT permissive and TOT refractory strains of *Ae. triseriatus*.

2. Materials and Methods

A. Genomic Sequencing of *AtIAP1*

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Because the genome of *Ae. triseriatus* has not been fully sequenced, the genomic sequence of *AtIAP1* was determined using the Universal GenomeWalker Kit (Clontech, Palo Alto, CA). *Ae. triseriatus* DNA was extracted from colony mosquitoes using the salt extraction method (Black & DuTeau, 1997) and digested according to GenomeWalker manufacturer's specifications. Adaptors were then ligated to the digested DNA and PCR amplified using a gene specific primer in conjunction with an adaptor specific primer. Several walks were done to obtain as much sequence upstream of the *AtIAP1* gene as possible.

B. Genomic Sequence Analysis of *AeIAP1*

In order to determine whether the genomic schematic of the *AtIAP1* gene is conserved among other Culicine mosquitoes, the genomic locations of the *Ae. aegypti* inhibitor of apoptosis 1 (*AeIAP1*) 5'UTR heterogeneous sequences were determined by BLAST search of the *Ae. aegypti* genome (http://msc.tigr.org/aedes/aedes.shtml). This search revealed that the *AeIAP1* gene is located on supercontig 1.368 of the genome (GenBank accession no. CH477553). Using this sequence, the location of the heterogeneous 5'UTR sequences in reference to the coding region of the *AeIAP1* gene were determined.

C. 5'UTR Heterogeneous Sequence Alignments

19 Culicine IAP1 heterogeneous 5'UTR sequences were aligned using Seqman (DNAStar, Madison, WI). Sequences from three IAP1 mRNA variants from *Cx. pipiens* and *Ae. aegypti*, four IAP1 mRNA variants from *Cx. tarsalis* and *Ae. albopictus*, and five IAP1 mRNA variants from *Ae. triseriatus* were assembled to determine whether these sequences are conserved across Culicine species (Beck et al., In Press). The assembling

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parameters required a 50% minimum match percentage in order for sequences to fall into the same group.

D. AtIAP1 RNA Folding Predictions

The secondary structure of the entire 5'UTR of each AtIAP1 mRNA variant was analyzed using the DINAMelt server found on the Rensselaer and Wadsworth bioinformatics server (Markham & Zuker, 2005). The predictions were made using RNA (2.3) energy rules at a temperature of 28° C with a 300 base pair maximum limit between paired bases.

E. *Aedes triseriatus* Life Stage and Tissue RNA Isolation

Ae. triseriatus (Say) mosquitoes were reared at 24°C and 75% relative humidity with a photocycle of 16:8 (L:D). Total RNA was extracted from whole *Ae. triseriatus* mosquitoes at various developmental stages (embryos, larvae, pupae, and adults) and from adult female organs (ovaries, midguts, and salivary glands) using TRIzol Reagent (Invitrogen, Carlsbad, CA). Briefly, whole mosquitoes were homogenized in 500 µL of TRIzol Reagent. Numbers of whole mosquitoes used in each reaction were as follows: embryos (100), first instar larvae (50), second instar larvae (25), third instar larvae (10), fourth instar larvae (5), early pupae (5), adult males (5), non bloodfed females (5), and 24 hour-post bloodfed females (5). Larvae and pupae were collected within 24 hours of molting. Organs were dissected from groups of five adult non-bloodfed female mosquitoes or 24 hour-post bloodfed females and homogenized in 50 µl of TRIzol Reagent. Following homogenization, total RNA was extracted following the manufacturer's instructions.

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F. *Aedes triseriatus* TOT Permissive and Refractory Ovarian and Midgut Tissue RNA Extraction

Ae. triseriatus strains selected for high and low TOT rates following a LACV infectious bloodmeal were also used in this experiment (and will be referred to as TOT permissive and TOT refractory, respectively) (Graham et al., 1999). The TOT permissive mosquitoes were hatched and reared to adults. The eggs from this strain of mosquito are already infected because the selection process involves collecting eggs from mosquitoes that are verified as having a high TOT rate. The TOT refractory mosquitoes are selected in the opposite manner and uninfected eggs are collected from mosquitoes with a low TOT rate. This strain was given a LACV infectious bloodmeal consisting of defibrinated sheep blood and supernatant plus cellular debris from LACV-infected BHK-21 cells (1:1) with a titer of 10^6 $TCID_{50}$/0.01 mL. After the bloodmeal, females were placed in a colony cage with male TOT refractory mosquitoes and allowed to mate and lay eggs. The females were given a second bloodmeal 14 days post LACV-infectious meal and the resulting eggs were collected and held in the insectary for two weeks (to allow the eggs to mature). The eggs from both TOT permissive and refractory strains were then reared to adults. Potentially infected TOT permissive and TOT refractory adult mosquitoes were tested for the presence of LACV antigen by immunofluorescence assay on tissue from individual legs (Beaty & Thompson, 1975). Mosquitoes were sorted into groups containing LACV positive and negative samples and were offered a non-infectious bloodmeal. Midguts and ovaries were then dissected from individual mosquitoes three days post bloodfeed in four groups: bloodfed LACV+ and LACV- TOT permissive mosquitoes and bloodfed LACV+ and LACV- TOT refractory mosquitoes, and

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homogenized in 50 μL of L-15 medium. RNA was isolated from individual tissue samples by adding 300 μL of TRIzol Reagent and following the manufacturer's protocol.

G. Quantitative Reverse Transcriptase PCR of AtIAP1 mRNA Variants

Following RNA extraction, each sample was analyzed using the Qiagen Quantitect SYBR Green RT-PCR kit (Qiagen, Chatsworth, CA) to determine the relative quantities of each AtIAP1 mRNA splice variant and actin (primer sequences can be found in Table 4.1) on an Opticon 2 Real-Time Thermocycler (Bio-Rad, Hercules, CA). Reactions were run in duplicate and amplified according to the manufacturer's protocols (using 20 μL reactions instead of 50 μL reactions). The fluorescence threshold was set at 0.014 using the logarithmic scale and the CT values for each sample were recorded and averaged among duplicates. The ΔCT was then calculated by subtracting the averaged actin CT value from the averaged CT value of each variant. The ΔCT was then averaged for each AtIAP1 mRNA variant from similar samples (particular life stage, tissue type, or strain). Finally, the corrected CT was calculated by multiplying ΔCT by -1 and adding 15. Multiplication by -1 was done to make the graph more intuitive with larger bars representing larger amounts of transcript. 15 is an arbitrary number that was added to ensure that all values were positive and neither of these calculations change the ratios between the samples. An example calculation for the quantification of mRNA variant 1 of the pupal life stage follows:

Average Sample AtIAP variant CT = (CT for pupae sample 1 replicate 1 AtIAP variant 1 + CT for pupae sample 1 replicate 2 AtIAP variant 1)/2

Δ CT Sample = (Average pupae sample 1 AtIAP variant 1 CT – Average pupae sample 1 Actin CT)

Life stage average AtIAP variant ΔCT = (pupae sample 1 AtIAP variant 1 ΔCT + pupae sample 2 AtIAP variant 1 ΔCT + pupae sample 3 AtIAP variant 1 ΔCT)/3

Life Stage Corrected ΔCT = -1(Life stage average AtIAP1 variant 1 ΔCT) + 15

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Table 4.1. Primers used for Q-RT-PCR of *Ae. triseriatus* AtIAP1 mRNA splice variants.

Primer Name	Primer Sequence (5' – 3')	mRNA Amplified
ActinF	GAA CAC CCA GTG CTG TTG AC	*Ae. triseriatus* Actin
ActinR	GTA CGA CCG GAA GCG TAC AG	*Ae. triseriatus* Actin
IAP1F	TCC ATT CGT CTT TGC TTG TC	*Ae. triseriatus* AtIAP1 variant #1
IAP1R	GCA ATT GCA CTG CTC TCT CTC	*Ae. triseriatus* AtIAP1 variant #1
IAP2F	CGA AAA AGG ATT TGC TGC TG	*Ae. triseriatus* AtIAP1 variant #2
IAP2R	CTC TTG GTC CAG ATG GGA AA	*Ae. triseriatus* AtIAP1 variant #2
IAP3F	CAT TGT GTC TCG CAT CGT CT	*Ae. triseriatus* AtIAP1 variant #3
IAP3R	GCG CTC TCT TCT TCA ATG GT	*Ae. triseriatus* AtIAP1 variant #3
IAP4F	CCA TTG TAA CTG GTC CTG GTC	*Ae. triseriatus* AtIAP1 variant #4
IAP4R	CTC TTG GTC CAG ATG GGA AA	*Ae. triseriatus* AtIAP1 variant #4
IAP5F	TCG ATT TGT GAT GTT TGG TG	*Ae. triseriatus* AtIAP1 variant #5
IAP5R	GCA ATT GCA CTG CTC TCT CTC	*Ae. triseriatus* AtIAP1 variant #5

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3. Results

A. Partial Genomic Sequencing of the *Ae. triseriatus IAP1* Gene

Using genome walking, ~6 kb of new sequence data was generated and three of the five heterogeneous 5'UTR sequences were identified in the *Ae. triseriatus* genome (GenBank accession no. EF059537, Fig. 4.1). The heterogeneous sequence from AtIAP1 mRNA variant 1 was located immediately upstream of the homogeneous 5'UTR sequence. The heterogeneous sequence from AtIAP1 mRNA variant 4 was located 2,110 base pairs upstream of the homogeneous 5'UTR sequence. Finally, the heterogeneous 5'UTR sequence from AtIAP1 mRNA variant 3 was also found, but the reverse primer bound incorrectly, prohibiting determination of the exact genomic position of the sequence. The heterogeneous sequences from mRNA variants 2 and 5 were not amplified from the *Ae. triseriatus* genome. Because at least two of the five heterogeneous 5'UTR sequences, variants 1 and 4, were found upstream of the *AtIAP1* ORF alternative splicing is a more likely explanation for the mRNA variants than multiple gene copies.

B. Genomic Location of *Ae. aegypti* Heterogeneous 5'UTR Sequences

Two of the three heterogeneous 5'UTR sequences of AeIAP1 were located in the known genome sequence of *Ae. aegypti* (Fig. 4.1). The heterogeneous sequence of AeIAP1 mRNA variant 2 begins 470 base pairs upstream of the 5' homogeneous UTR sequence. The heterogeneous sequence of AeIAP1 mRNA variant 1 begins 119.78 kb upstream from the homogeneous 5'UTR sequence. Both of these variable sequences are nearly identical in the AIDL laboratory strain (Rexville D) and the *Ae. aegypti* Liverpool strain found in Genbank. The heterogeneous sequence of AeIAP1 mRNA variant 3 was

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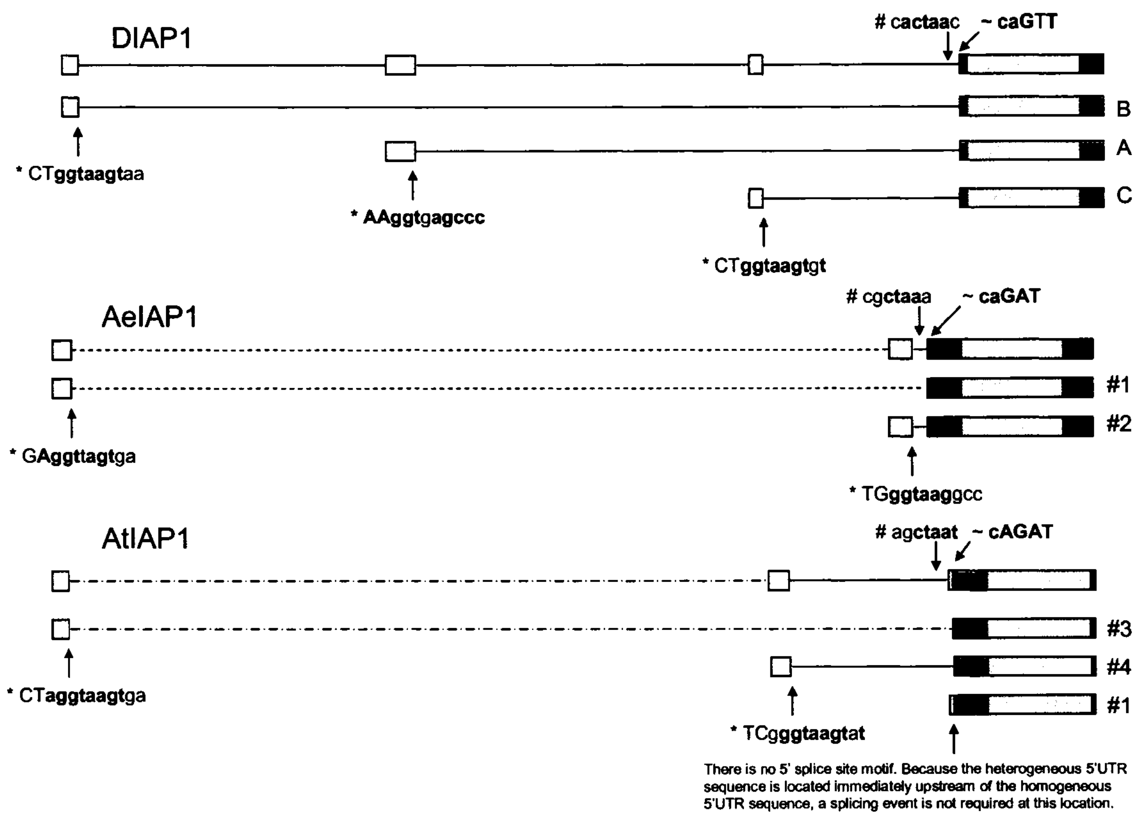

Figure 4.1. Genomic schematics of the *DIAP1*, *AeIAP1*, and *AtIAP1* genes. The schematics are drawn to scale (the full length *DIAP1* gene represents roughly 13 kb) with the exception of the dotted line in the *AeIAP1* figure (this line represents roughly 115 kb of DNA) and dotted and dashed line in the *AtIAP1* figure (this line represents an unknown genomic distance). Red boxes indicate untranslated regions, grey indicates the protein coding sequence, and blue indicates 5'UTR variable regions. Uppercase letters represent nucleotides located in the exon while lowercase letters represent nucleotides found in the intron. Bold letters indicate nucleotides matching the consensus sequences of *D. melanogaster* 5' splice site (sequences indicated by an *), 3' branch site (sequences indicated by a #), and 3' splice site (sequences indicated by a ~) intron motifs.

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not found in the *Ae. aegypti* genome when a Blast search was performed. The *AeIAP1* gene (and the *AtIAP1* gene) contain sequence motifs that are similar to splice site motifs found in *D. melanogaster* introns (Fig. 4.1) (Lim & Burge, 2001). These sequences are found directly following the heterogeneous 5'UTR sequences (the 5' intron splice site motif), preceding the 5' UTR homogeneous sequences (the 3' intron splice site motif), and 70 – 135 base pairs before the beginning of the homogeneous 5'UTR sequences (the 3' intron branch site motif). A similar pattern is seen in the genomic arrangement of *DIAP1* (Fig. 4.1), which is predicted to generate multiple mRNA variants through alternative splicing (Adams et al., 2000).

C. Alignment of IAP1 Heterogeneous 5'UTR Sequences of 5 Culicine Mosquitoes

Alignments of 19 variable IAP1 5'UTR sequences from five different Culicine mosquitoes shows that the 5'UTR variable sequences appear to be conserved across species, but not across genera. The aligned sequences formed five groups made up of four, three, three, three, and two sequences each. Four of the sequences failed to align with any other sequences. Each group predominantly consisted of sequences from either the *Aedes* or *Culex* genera, but not both (with the exception of group 1) (Fig. 4.2).

D. Secondary Structure Predictions of the 5'UTRs of AtIAP1 mRNA Variants

The secondary structures of all five complete AtIAP1 5'UTR sequences were predicted using the DINAmelt server (Markham & Zuker, 2005). The results showed that the homogeneous portion of the 5'UTR generally folds in a similar manner for most of the mRNA variants. The variation in secondary structure was generally seen at the 5' end of the sequences, which corresponds to the location of the heterogeneous portion of the UTR (Fig. 4.3).

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Group 1

```
                      1 .........+.........+.........+.........+.........+.........+.........+  70
Cx. pipiens 3           AGTTTTGCT.CTGGCC.TCGGAGCT....AGAAAA.G.CGT..ACGT.TTC....GGG.A.AA..ACTCG
Ae. triseriatus 5       AGTT.CGGT.CTGTCT.TCGGACCTCTTGAGAGTGCG.CGCGTACCC.TAC....GAA.AAAGTT.TT.G
Ae. albopictus 3        AGTTTCGGTT.TACCC.TCGGAGCTCTT..GAGTGCG.CGCGAACCCAA.CT..A.CCC.T.CT.ACT.G
Ae. aegypti 3           ..TTTCCGTTCTACCCCTTGGRAGCTYTTYGAGAGCGGSGCGCGGCGWTTCCCCAGCCCATACTTATTAG
                        ****    *  *  *  * **         **    *  *          *                * *

                     71 .........+.........+.........+.........+.........+.........+.........+ 140
Cx. pipiens 3           C.A.AACCTTTT...TTTGTTGTTGT.TGTTTTCGCTCCCTTTCAA.AACAGTTTTGCAACTTGAGAGGG
Ae. triseriatus 5       T.ATAATTTCAGC.A....A.GA.G..T..TTTT..TTTTGT..GT.ATGATTTGCAAAGAGTACAAGGG
Ae. albopictus 3        TGTGAAAAGTTTCCCTTTGA..ATGAGTGA...T..TTTTTTTCGGCAAGAGT.G.CCATT.TGCAAGGG
Ae. aegypti 3           GGAGKATTTTTTCCATTTGG.GATGKGKGATTTT..TTTTTTTCGGCAAGAGT.GCCCATT.TGCAAGGG
                             *                  *           *    *     *  * *     *   *   ****

                    141 .........+.........+.........+.........+.........+.........+.........+ 210
Cx. pipiens 3           GGAA.AAA.CTAGTTT.AATTACGTCACA.CAATAAT.A..A..ACG..AA.GGGATCTCG.CGC.GT.T
Ae. triseriatus 5       GGAAGAAA.CTAGTTT.AATTACGTCATAATAATAATTACTATAAGGGGAAYGGCAT....ACT..GTGT
Ae. albopictus 3        GGAAGAAA.CTAGTTT.AATTACGTCATAACAGTAATTACTATTAGGGGAACGGCAT.TGGACT..GTGT
Ae. aegypti 3           GGAAGAAAACTAGTTTAAATTACGTCATAACAGTAATTACTATTAGGGGAACGGCAT.TGGACTATGTGT
                        **** *** ******* ********** *  * **** *  *  * *  ** ** **         ** *

                    211 .........+.........+.........+.........+.........+.........+.........+ 280
Cx. pipiens 3           TGTGAGTGAGAAAAAAAGAA.ACGGGGGTTG.TAATTTCTGTGAAACGAACGAAA.C.G.G.GGT.G.AA
Ae. triseriatus 5       TTT...T..G..W..CG....A.A.TAGAAGAGA.T.AC.GGCGA..GGCA.AAAACTGCGCGCTTGGAT
Ae. albopictus 3        TGTGC.TACGC.TT.CGCTTCACAATAGAAG.TAA.CAC.A.CGA.CGTGCGAGAACTACGTTTTTGCAA
Ae. aegypti 3           TTTGC.TACGCGTTTYGCTTCACAATAGAAGATAGTCAC.GTCGAACAGGCGAAAACTGCGCGTTTGCRA
                        * *   *  *           *        *  *    *     *       * * *   *   * *

                    281 .........+.........+.........+.........+.........+.........+.........+ 350
Cx. pipiens 3           G.TT...TTGTGGGA.AAATAGCAC.G.GATGG.T..G.CAGGGA.AATTGTGAA.ATTGAATG.CGTTC
Ae. triseriatus 5       GCTGGAATAGTAGGCGAAATCGGA.TGC.TT...TTTG..AAAAGCGAGCG.GAA.ATTTTC.GAC.AGT
Ae. albopictus 3        TCTA.AGTTGAAG.A.ATATTGTA.WA.GT.GGATTTG.CAAAACCAAGTGAGTTTATTTGCTGACGAAG
Ae. aegypti 3           GCTGAATTGAAGGA.AAATT..TACTGTGTTGGCTTTGGCGAAACCAAGCCAATTTATTTACTGACGGAG
                          *    *    *   *  *   *          *  *         *        ***    * *

                    351 .........+.........+.........+.........+...... 396
Cx. pipiens 3           TTCTCTCT.GGCTGGC.TGGCTG.GCGCTAT.GAGTGCAGAG....
Ae. triseriatus 5       TTTGAYAGTGGAA.A..TCGATTTGTGATGTTTGGTGTTTACCTCC
Ae. albopictus 3        .T.TA.ATTGGAAGAAATCGATCTACGATGTTGGTTGYCTGCCTCC
Ae. aegypti 3           ATATCTATTTGAARAAATC...........................
                         *        *      *** *    *  * *    **     ****
```

Group 2

```
                      1 .........+.........+.........+.........+.........+.........+.........+  70
Cx. tarsalis 3          CTACACAGTAYTKTTRCRAARTTCGARCGAATCGTCTAGCGTGAAGG..TTGTTTT.TC...AAAATGWT
Cx. tarsalis 2          .TG.ACAGTACTGTTGCAAAGTTCGAACGAATCGTCTAGCGTGAAG........................
Cx. pipiens 1           ....ACAGTACAGTTACAAAGTTCGAACGAATCGTGTAGAGTGAGGGCATTTTTTTCTCTTCATAATGAT
                            ******   ** * ** ***** ******** *** **** *   ** **** **   * **** *

                     71 .........+...  83
Cx. tarsalis 3          TTACTGCATTGAR
Cx. tarsalis 2          .............
Cx. pipiens 1           TTACCACATTGAG
                        ****  ******
```


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Group 3

```
                      1 .........+.........+.........+.........+.........+.........+.........+  70
Ae. aegypti 1             AGTTTCTTGCCCCGATCATCGCGAGGTCGTTCACGTTCACAGAGTTGCGAAAAGTGGATTTTGTGTGCGA
Ae. albopictus 2          ....................TCGAG TCG...A.GTT.A..GTCTTGTGTC..GTGGACGTCGTGT.CGA
Ae. triseriatus 3         ..................................ACAGTC..GAGAAAAA.G.AAGT.GTACGCCGA
                                              **** ***   * *** *  *    * *       * *  * **   ***

                     71 .........+.........+.........+.........+.........+.........+.........+ 140
Ae. aegypti 1             GAGTGCTGTTGTGTCG.TGTGGTTGTC.GATCGAGTGTTTTTTCC.GTCGT.................T
Ae. albopictus 2          ACGTTGTGT.G.GTCGGTCTAGTTTTGTGAAAG.GTGTTTTTCCCCGTCGT.GAAA......AAACA.GT
Ae. triseriatus 3         GAG..CTGTTGTGT.GATCGTGA.GTTTGAGTGT.TTTTTTTTCTCGTTGTCGAAAGTGTGGAAAAGTGT
                            *   *** * ** * *   *   *  **  *  * ***** *  ** **                  *

                    141 .........+.........+.........+.........+.........+.........+.........+ 210
Ae. aegypti 1             ..TTTCGCGAGTTTTC.AGTGCATTTCCCATTGTCGAA.CAGTTGGAAAATGGATTCGCAAGCTCCTGTT
Ae. albopictus 2          ..TTTCGTGAGTTTTCCATCGTATTTCCGATCGTCAAA.TAGTTGGAAAATGGATTCGCAAGCCCCCGTC
Ae. triseriatus 3         CATTTTCCGAAATTTCCTCCTAAATTTC.AACGTTGCTGCAGTTGGAAAATGGATTY.CCA..TTGTGTC
                            ***   **  ****      * ** * *   *      * *************  *  *       **

                    211 .........+.........+.........+.........+.........+.... 264
Ae. aegypti 1             TGCCATCAGTGAT.ATTTGG.CTCGAGC.AAGAAAG.GCCTC..CGA.......
Ae. albopictus 2          CATCATCGTTAACGACCAGG.CGCGCGCTACGAAGGCGTTTCT.CAAAGGCTCA
Ae. triseriatus 3         TCGCATCGTCTAC.ATTAGGACCAGAACGAGGAAAGCTCCCCTGCT........
                             ****    *  *   ** *  *  * * *** *     *  *
```

Group 4

```
                      1 .........+.........+.........+.........+.........+.........+.........+  70
Ae. albopictus 4          CTGGTTCGTCTCTCGTATTTGTATTGTATTCATTAGATTTAACGTAAGCCTGGCAGTTATAAATGATTGT
Ae. aegypti 2             ......................................................................
Ae. triseriatus 1         ......................................................................

                     71 .........+.........+.........+.........+.........+.........+.........+ 140
Ae. albopictus 4          TGAAATCAAGGATTATTGTGATAATCCTTTCACCATTGTTGTAGCATGTGAATGATAACAAGTTTTCATT
Ae. aegypti 2             ........................................ATAG.ACGC.AAT..TA.CTTATTTGAAGA
Ae. triseriatus 1         ......................................................................
                                                                  *** * *  ***  ** *   ***  *

                    141 .........+.........+.........+.........+.........+.........+.........+ 210
Ae. albopictus 4          ACTGATCGTATAA.TCTCA.CAGCTG.TCATAGCCCGCAAATTGAGCGACTTTCTTGAAAAATTGTTCAA
Ae. aegypti 2             ACCAGTTGTGTAACTCTGTTCCTCTGATTAT.GGTAGCAGATTGAGCGACTTTCTTTATAAA......AA
Ae. triseriatus 1         ......................................................................
                          **   * ** *** ***   *  *** * ** *   *** *************** * ***      **

                    211 .........+.........+.........+.........+.........+.........+.........+ 280
Ae. albopictus 4          AACCAGCTCGAGAGTGCAACTTTCTACCGACAATCGATGGACCCTGTACCAGGCTACCAAACACAACTAT
Ae. aegypti 2             A.CTCGCTCAACTGCACAACTTTCTACCAATAATCGACGGA....G.A...GGGT.CCAAACCTAACTAT
Ae. triseriatus 1         ......................................................................
                          * *  **** *  *  ************ * ****** ***    * *   ** * ******  ******

                    281 .........+.........+.........+.........+.........+.........+.........+ 350
Ae. albopictus 4          CCAAAGTGAAACGATC..CGGTGG..CACAAGTCGTCAACCAGCTGCTCTCC.CACAACCACCCTTTTC.
Ae. aegypti 2             CCAAAGTGAAACGATCTGCGGTGGCACACAAGTCGTCAACCTGCTACCCGCTTCACCGCCATCGTTTCCT
Ae. triseriatus 1         ......................................................................
                          ****************  ******  *************** *** * * *  ***  *** * *** *

                    351 .........+.........+.........+.........+.........+.........+.........+ 420
Ae. albopictus 4          CCATACCCACAAAATGCAGTTTTCCACCGGATTTCCACTCTACCTAAATGCCATTTTGGGAAGGCTCCCC
Ae. aegypti 2             CCA.AAG.A.AAAATGCA.TTTTCCACCGGATTTCCACTCTG..T....GCCATTTTG............
Ae. triseriatus 1         ......................................................................
                          *** *   * ******** ********************** *    *********

                    421 .........+.........+.........+.........+.........+.........+.........+ 490
Ae. albopictus 4          AGCTTGTAGCCTCCCGCATATCAACCCACCCAGGCAGGATAAACTTTGCTAAATAAGTCTTTTCT.TTTC
Ae. aegypti 2             ......................................................................
Ae. triseriatus 1         ..........................................................TTTCTTCTCTTTC
                                                                                    * * **** ****

                    491 .........+.........+.........+.........+........ 538
Ae. albopictus 4          TCTTTCTCTTTCCAT.C.TTTTTTCTCTCACAAACACTGCCCGCACAC
Ae. aegypti 2             ................................................
Ae. triseriatus 1         TCTTTCTCTTTCCATTCGTCTTTGCT.TGTCGCACAC...........
                          *************** * * *** ** *  *  ****
```


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Group 5

```
                     1 .........+.........+.........+.........+.........-.........+.........+  70
Cx. pipiens 2          ACATCAGCACTCAGCACTGGCTGCAACGTAACCAAAAGGCT.TG.....TGTGAAGTGTGTGGTGAGCTG
Cx. tarsalis 4         ..........TC.GCACTCT.TGCAACGTAACCAAAAGGCAGTGCCCCCTGTGAAGTGCGTGGTGAGCGG
                                 ** *****   *******************  **     ********* ********* *

                    71 .........+.........+.........+.........+.........+.........+.........+ 140
Cx. pipiens 2          ATTTTGTTGGATTTTAAGCAGTTTTCGTCGCGTTTCGTCCATCAAAAGTGTACAAAATGGATTGCGAGTC
Cx. tarsalis 4         ATTTTGCTGGATTTTTAGCAGTTTTTATCGCTTTACGTCCATCAAAACTTTACAAAATGGATCGTTGGTC
                       ****** ******** *********  **** ** ************ * ************ *   ***

                   141 .........+.........+.........+.........+.........+. 191
Cx. pipiens 2          GTTTGTGCATCACGGCCACTAGGATTCCCGTCCTGTTTGGCTGGATTTCAG
Cx. tarsalis 4         GTRCGTGCACCAGGGCCACTAGGATTTTTGTCCCGGTTGGCTGGATTTCAG
                       **  ***** ** *************   **** * ***************
```

Figure 4.2. Alignment of the IAP1 5'UTR variable regions of Culicine mosquitoes. This figure shows alignments of the IAP1 5'UTR variable regions from *Ae. aegypti*, *Ae. albopictus*, *Ae. triseriatus*, *Cx. pipiens*, and *Cx. tarsalis*. These alignments do not include the conserved portion of each 5'UTR. In general, *Aedes* sequences align best with those from other *Aedes* species and *Culex* sequences align best with those from other *Culex* species. The variable sequences from *Ae. albopictus* mRNA variant 1, *Ae. triseriatus* mRNA variants 2 and 4, and *Cx. tarsalis* mRNA variant 1 did not show significant homology to any other mRNA variable sequences. An asterisk indicates the nucleotide is conserved among all sequences in the alignment.

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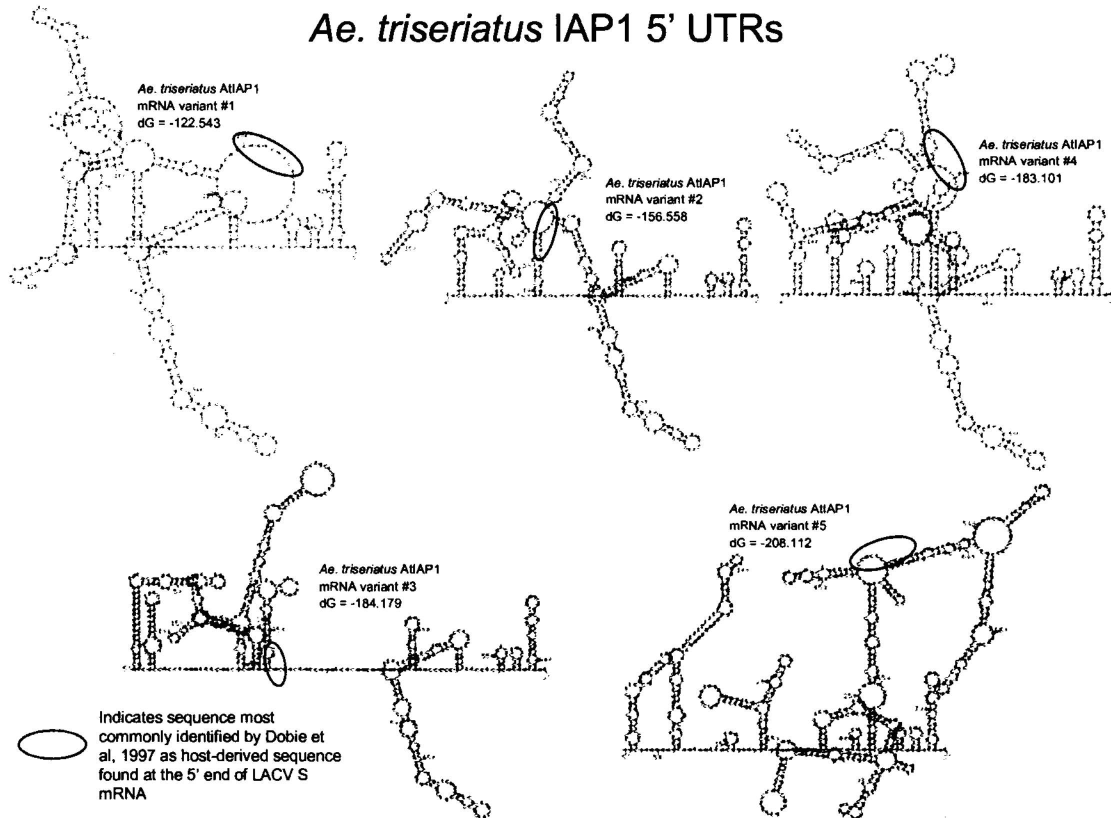

Figure 4.3. Secondary structure predictions of the 5'UTRs of AtIAP1 mRNA variants. Secondary structure predictions were made using the 5'UTR sequence of each AtIAP1 mRNA variant. Each sequence consisted of the variable portion of the 5'UTR plus the conserved region. In general the predicted structures were similar at the 3' end of the UTR where the conserved region is located. The 5' portion of each sequence, the variable portion, showed obvious differences in secondary structure that may act to control translation in different life stages and tissue types. The red circle indicates the location of the sequence most commonly identified by Dobie et al., 1997 as host derived sequence found at the 5' end of LACV S mRNA in diapausing TOT infected *Ae. triseriatus* eggs.

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E. Relative Frequency of AtIAP1 mRNA Variants in *Ae. triseriatus* Life Stages

The life-stage expression of each AtIAP1 mRNA variant was characterized using quantitative reverse-transcriptase PCR (Table 4.2 and Fig. 4.4). Total RNA was extracted from 9 *Ae. triseriatus* life-stages; embryos (8 days old), larvae (first, second, third, and fourth instars within 24 hours after molting), pupae (within 24 hours of pupation) and adults (pre-bloodfed females, 24 hour post-bloodfed females, and males). All five mRNA variants could be detected in each *Ae. triseriatus* life stage. In all life stages besides 2nd and 3rd instar larvae mRNA variant three was the most abundant. mRNA variant four was the most abundant in 2nd and 3rd instar larvae. mRNA variants one and two made up a small percentage of all AtIAP1 mRNA transcripts and were the least abundant in each life stage. The only detectable pattern was that mRNA variants three and five were the first and second most abundant AtIAP1 transcripts, respectively, in stages requiring the greatest developmental changes (i.e. eggs, fourth instar larvae, and pupae). It should also be noted that each CT represents a twofold difference of initial RNA transcript. Thus the fold difference between two samples is calculated as: $2^{\text{difference in corrected CT}}$. An analysis of variance (ANOVA) showed that all AtIAP1 mRNA variant expression profiles were significantly affected by life stage (Table 4.3).

F. Relative Frequency of AtIAP1 mRNA Variants in *Ae. triseriatus* Tissues

All five AtIAP1 mRNA variants were detected in all tissue types that were examined: salivary glands, ovaries, and midguts from pre-bloodfed (4 days post-eclosion) and 24 hours post blood-fed (fed at 4 days post-eclosion) females (Table 4.4 and Fig 4.5). Unlike the different *Ae. triseriatus* life stages, there were obvious differences in AtIAP1 mRNA variant expression patterns in *Ae. triseriatus* tissues. Variant four was the most

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Table 4.2. Relative frequency of AtIAP1 mRNA variants in *Ae. triseriatus* life stages.

Ae. triseriatus Life Stage	AtIAP1 mRNA Variant	ΔCt Sample 1 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 2 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 3 (Ave. variant duplicates - Ave. Actin duplicates)	Average ΔCt (Ave ΔCt for each variant of each life stage)	Corrected ΔCt ((-1 * Average ΔCt)+15)	Standard Deviation of Corrected ΔCt
Eggs	Variant #1	9.71	6.16	7.57	7.81	7.19	1.792
	Variant #2	13.37	13.31	13.19	13.29	1.71	0.092
	Variant #3	2.92	2.90	2.98	2.93	12.07	0.042
	Variant #4	5.61	5.44	5.56	5.54	9.47	0.083
	Variant #5	2.80	3.07	3.25	3.04	11.97	0.231
1st Instar larvae	Variant #1	7.93	7.40	7.41	7.58	7.42	0.306
	Variant #2	9.71	10.33	10.02	10.02	4.98	0.310
	Variant #3	4.22	4.63	4.53	4.46	10.54	0.210
	Variant #4	4.99	5.24	5.68	5.30	9.7	0.344
	Variant #5	5.50	5.29	5.83	5.54	9.46	0.277
2nd Instar Larvae	Variant #1	11.22	9.70	9.71	10.21	4.79	0.878
	Variant #2	12.87	13.14	13.20	13.07	1.93	0.174
	Variant #3	6.79	6.83	6.38	6.66	8.34	0.246
	Variant #4	5.82	6.25	6.00	6.02	8.98	0.216
	Variant #5	7.03	6.98	8.19	7.40	7.6	0.688
3rd Instar Larvae	Variant #1	10.40	9.57	9.11	9.69	5.31	0.653
	Variant #2	14.13	15.30	14.46	14.63	0.37	0.598
	Variant #3	6.16	4.21	4.62	5.00	10.01	1.030
	Variant #4	5.15	4.77	4.54	4.82	10.19	0.308
	Variant #5	6.59	5.20	5.14	5.64	9.36	0.823
4th Instar Larvae	Variant #1	12.38	10.36	11.03	11.26	3.74	1.029
	Variant #2	12.96	13.23	13.89	13.36	1.64	0.473
	Variant #3	7.26	6.46	7.61	7.11	7.9	0.591
	Variant #4	7.65	6.77	10.48	8.30	6.7	1.940
	Variant #5	6.90	6.43	8.13	7.15	7.85	0.874
Pupae	Variant #1	8.77	8.87	8.56	8.73	6.27	0.157
	Variant #2	10.61	10.06	10.17	10.28	4.73	0.287
	Variant #3	4.55	4.58	4.40	4.51	10.5	0.100
	Variant #4	9.87	10.86	10.51	10.41	4.59	0.507
	Variant #5	4.50	4.53	4.57	4.53	10.47	0.035
Pre-BF Females	Variant #1	9.35	10.18	9.95	9.83	5.17	0.429
	Variant #2	11.51	12.68	13.04	12.41	2.59	0.806
	Variant #3	4.14	5.31	5.70	5.05	9.96	0.816
	Variant #4	5.59	6.80	7.47	6.62	8.38	0.952
	Variant #5	5.14	7.35	7.42	6.63	8.37	1.294
Post-BF Females	Variant #1	8.30	7.98	8.53	8.27	6.73	0.272
	Variant #2	11.49	11.11	10.10	10.90	4.10	0.718
	Variant #3	2.90	3.00	3.41	3.10	11.90	0.270
	Variant #4	6.78	5.30	3.41	5.16	9.84	1.689
	Variant #5	5.30	5.38	4 51	5.06	9.94	0.481
Male Adults	Variant #1	10.01	9.72	8.98	9.57	5.43	0.531
	Variant #2	12.25	12.64	12.52	12.47	2.53	0.205
	Variant #3	4.44	4.73	4.23	4.46	10.53	0.251
	Variant #4	7.47	7.39	7.26	7.37	7.63	0.101
	Variant #5	6.64	6.84	6.70	6.73	8.27	0.103

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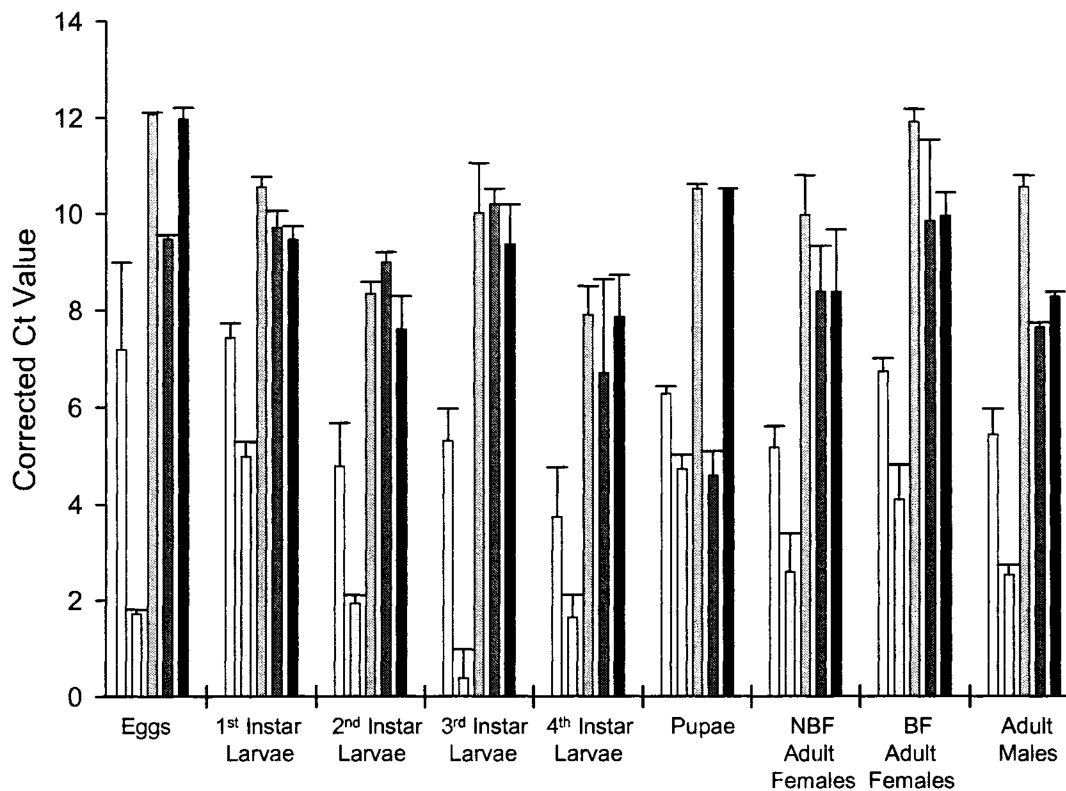

Figure 4.4. AtIAP1 mRNA variant expression profiles from *Ae. triseriatus* life stages. All five AtIAP1 mRNA variants and actin were amplified from each *Ae. triseriatus* life stage. ΔCT values were calculated by subtracting the actin CT values from the mRNA variant CT values of the same sample. ΔCT values were averaged among all samples for each mRNA variant at each life stage. The bar on the left of each life stage indicates the relative frequency of mRNA variant 1 and proceeds to variant 5 on the right. The values indicated here are from the final two columns of Table 4.2. Sample calculations can be found in the materials and methods section of this chapter. Each CT indicates a twofold increase in initial transcript.

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Table 4.3. Analysis of variance to determine statistical differences in AtIAP1 mRNA variant expression following different treatments.

	mRNA Variant 1	mRNA Variant 2	mRNA Variant 3	mRNA Variant 4	mRNA Variant 5
Variable	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
Life Stage	0.0005*	<.0001*	<.0001*	<.0001*	<.0001*
	0.2132	0.0004*	0.0272*	0.6371	0.0004*
	<.0001*	<.0001*	0.0004*	<.0001*	0.8989
	0.9608	0.4252	0.7778	0.2982	0.2388
LACV+ or LACV-	0.0611	0.1329	0.0141*	0.9700	0.1169
TOT Perm. or TOT Ref.	0.0544	N/A	0.0460*	0.1421	0.3093
Midgut or Salivary Gland	<.0001*	0.0144*	<.0001*	<.0001*	<.0001*
LACV and TOT	0.8356	0.8792	0.8700	0.0688	0.6951
LACV and Tissue	0.8083	0.0969	0.4366	0.7494	0.0448*
TOT and Tissue	0.9157	0.7220	0.5104	0.8457	0.4295
LACV and TOT and Tissue	0.3488	N/A	0.2570	N/A	0.7511

*Indicates significant differences in AtIAP1 mRNA variant expression profile between groups. Results highlighted in yellow were calculated using data from the life stage specific AtIAP1 mRNA variant expression experiment. Results highlighted in blue were calculated using data from the tissue specific AtIAP1 mRNA variant expression experiment. Results highlighted in grey were calculated using data from the TOT permissive and TOT refractory AtIAP1 mRNA variant expression experiment.

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Table 4.4. Relative frequency of AtIAP1 mRNA variants in *Ae. triseriatus* tissues.

Ae. triseriatus Tissue Type	AtIAP1 mRNA Variant	ΔCt Sample 1 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 2 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 3 (Ave. variant duplicates - Ave. Actin duplicates)	Average ΔCt (Ave ΔCt for each variant of each life stage)	Corrected ΔCt ((-1 * Average ΔCt)+15)	Standard Deviation of Corrected ΔCt
Pre-BF Midguts	Variant #1	4.88	5.58	5.65	5.37	9.63	0.432
	Variant #2	10.57	11.54	10.92	11.01	3.99	0.496
	Variant #3	3.34	3.35	3.75	3.48	11.52	0.237
	Variant #4	0.75	1.57	1.25	1.19	13.81	0.419
	Variant #5	4.79	4.86	5.18	4.94	10.06	0.208
Post-BF Midguts	Variant #1	4.39	5.31	5.30	5.00	10.00	0.528
	Variant #2	10.33	10 27	9.59	10.06	4.94	0.414
	Variant #3	3.10	2.91	2.51	2.84	12.16	0.301
	Variant #4	1.81	1.66	1 54	1.67	13.34	0.140
	Variant #5	4.87	4.59	4.32	4.59	10.41	0.280
Pre-BF Ovaries	Variant #1	8.88	8.75	9.52	9.05	5.95	0.414
	Variant #2	12.95	13.77	12.63	13.11	1.88	0.588
	Variant #3	2.26	1.48	2.33	2.02	12.98	0.472
	Variant #4	12.05	12.68	12.69	12.47	2.53	0.367
	Variant #5	5.06	5.04	5.52	5.21	9.79	0.272
Post-BF Ovaries	Variant #1	8.37	9.27	8.74	8.79	6.21	0.457
	Variant #2	12.10	12.33	11.53	11 98	3.02	0.411
	Variant #3	1.20	0.93	1.79	1.30	13.70	0.441
	Variant #4	11.83	11.44	13.54	12.27	2.73	1.119
	Variant #5	4.01	4 22	4.46	4.23	10.77	0.225
Pre-BF Salivary Glands	Variant #1	6.49	6.21	6.95	6.55	8.45	0.378
	Variant #2	10.25	12.01	11.56	11.27	3.73	0.920
	Variant #3	2.57	2.59	3.88	3.01	11.99	0.751
	Variant #4	1.81	1.45	2.53	1.93	13.07	0.554
	Variant #5	4.95	5.09	5.44	5.16	9.84	0.252
Post-BF Salivary Glands	Variant #1	4.96	6.75	6.60	6.10	8.90	0.993
	Variant #2	9.23	9.98	9.19	9.46	5.53	0.445
	Variant #3	2.17	3.16	2.68	2.67	12 33	0.495
	Variant #4	0.54	1.26	1.88	1.23	13.77	0.666
	Variant #5	3.92	4.87	4.52	4.43	10.56	0.480

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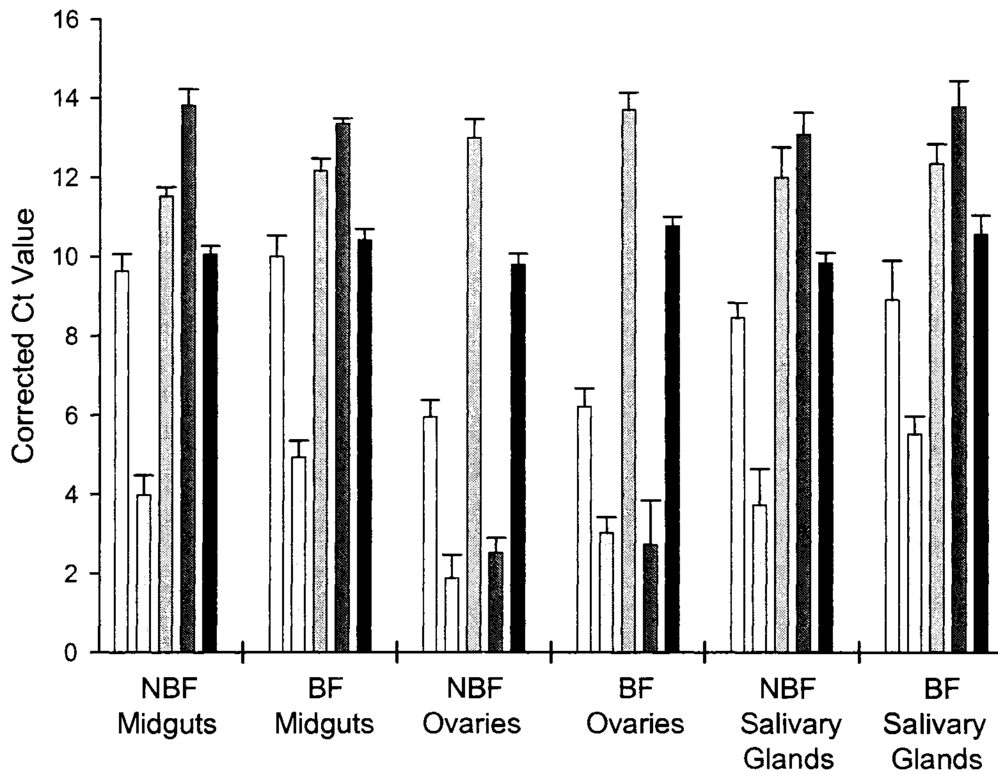

Figure 4.5. AtIAP1 mRNA variant expression profiles from *Ae. triseriatus* tissue types. All five AtIAP1 mRNA variants and actin were amplified from salivary glands, midguts, and ovaries of bloodfed and non-bloodfed *Ae. triseriatus* mosquitoes. ΔCT values were calculated by subtracting the actin CT values from the mRNA variant CT values of the same sample. ΔCT values were averaged among all samples for each mRNA variant of each tissue type. The bar on the left of each life stage indicates the relative frequency of mRNA variant 1 and proceeds to variant 5 on the right. The values indicated here are from the final two columns of Table 4.4. Sample calculations can be found in the materials and methods section of this chapter. Each CT indicates a twofold increase in initial transcript.

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abundant in salivary glands and midguts, while variant three was the most abundant in ovarian tissue. In all tissues, expression of each variant increased following a bloodmeal with the exception of mRNA variant four in the midguts. Finally, mRNA variant three was the most abundant in ovaries followed by salivary glands and then midguts. This represents the same expression pattern found in AtIAP1 protein. ANOVA determined that expression of AtIAP1 mRNA variants 2, 3, and 5 significantly increases following a bloodmeal ($p = 0.0004$, 0.0272, and 0.0004, respectively). Also, there are significant differences between tissue expression profiles of AtIAP1 mRNA variants 1 – 4 ($p < 0.0001$, < 0.0001, $= 0.0004$, and < 0.0001 respectively), while there is no statistical difference between the expression profile of AtIAP1 mRNA variant 5 in midguts, ovaries, and salivary glands (Table 4.3).

G. Relative Frequency of AtIAP1 mRNA Variants in TOT Permissive and Refractory *Ae. triseriatus* Midguts and Ovaries

Analysis of TOT permissive and refractory *Ae. triseriatus* midguts and ovaries showed some significant differences in AtIAP1 mRNA variant expression (Figs. 4.6 and 4.7 and Tables 4.3 and 4.5). In the ovaries of both LACV+/- females of the TOT permissive and refractory strains, mRNA variant three was the most abundant as it was for colony mosquitoes. In the midguts, AtIAP1 mRNA variant four was the most abundant in all groups except the TOT permissive, LACV+ samples. In this group mRNA variant one was the most abundant; however, variant four was the second most abundant and not significantly less than variant one in this case. Again, these results are similar to those obtained in the *Ae. triseriatus* colony mosquitoes. Analysis of variance

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Table 4.5. Relative frequency of AtIAP1 mRNA variants in LACV+/- *Ae. triseriatus* TOT permissive and refractory midguts and ovaries.

LACV Infection Status/ Strain/ Tissue Type	AtIAP1 mRNA Variant	ΔCt Sample 1 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 2 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 3 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 4 (Ave. variant duplicates - Ave. Actin duplicates)	ΔCt Sample 5 (Ave. variant duplicates - Ave. Actin duplicates)	Average ΔCt (Ave ΔCt for each variant of each life stage)	Corrected ΔCt ((-1 * Average ΔCt)+15)	Standard Deviation of Corrected ΔCt
TOT perm LACV+ BF Ov	Variant #1	6.43	6.02	8.05	6.61	5.87	6.59	10.77	0.866
	Variant #2	12.08	11.29	N/A	11.73	N/A	11.70	5.67	0.401
	Variant #3	1.35	2.55	2.48	1.24	2.48	2.02	15.35	0.665
	Variant #4	N/A	N/A	N/A	N/A	N/A	N/A	0.00	0.000
	Variant #5	3.96	4.11	6.99	4.61	4.50	4.83	12.53	1.234
TOT ref LACV+ BF Ov	Variant #1	5.53	8.26	8.68	8.61	6.36	7.49	9.88	1.448
	Variant #2	12.12	13.25	N/A	11.32	11.63	12.08	5.29	0.844
	Variant #3	1.60	1.92	2.15	1.62	2.28	1.91	15.45	0.301
	Variant #4	10.68	N/A	N/A	12.57	N/A	11.62	5.75	1.336
	Variant #5	4.23	6.93	N/A	6.80	4.62	5.64	11.73	1.419
TOT perm LACV- BF Ov	Variant #1	5.76	N/A	8.02	7.53	8.37	7.42	9.95	1.162
	Variant #2	14.16	N/A	11.30	12.76	11.32	12.38	5.57	0.840
	Variant #3	1.88	N/A	1.41	2.78	1.20	1.81	15.55	0.706
	Variant #4	N/A	N/A	N/A	N/A	12.16	12.16	4.58	0.898
	Variant #5	4.81	N/A	4.93	5.49	5.61	5.21	12.16	0.404
TOT ref LACV- BF Ov	Variant #1	8.01	6.91	8.97	7.69	7.46	7.81	9.56	0.767
	Variant #2	12.57	9.48	13.44	12.24	12.17	11.98	5.39	1.486
	Variant #3	1.27	0.00	2.24	1.62	1.01	1.23	16.14	0.827
	Variant #4	N/A	N/A	12.26	N/A	N/A	12.26	5.11	0.000
	Variant #5	6.34	5.28	7.22	4.37	4.63	5.57	11.80	1.200
TOT perm LACV+ BF Mg	Variant #1	4.20	3.36	5.86	3.76	3.44	4.12	13.24	1.023
	Variant #2	N/A	N/A	N/A	N/A	N/A	N/A	0.00	0.000
	Variant #3	6.23	6.59	5.52	5.81	6.24	6.08	11.29	0.418
	Variant #4	6.76	7.32	4.96	5.64	3.99	5.73	11.64	1.343
	Variant #5	7.80	7.71	6.73	7.14	7.38	7.35	10.02	0.436
TOT ref LACV+ BF Mg	Variant #1	3.03	5.36	5.43	5.47	2.93	4.44	12.92	1.339
	Variant #2	N/A	N/A	N/A	N/A	7.91	N/A	9.46	0.000
	Variant #3	4.24	5.08	5.84	5.87	4.37	5.08	12.29	0.774
	Variant #4	3.08	3.34	2.66	4.05	1.36	2.89	14.47	0.997
	Variant #5	7.24	6.77	8.96	7.84	6.44	7.45	9.92	0.999
TOT perm LACV- BF Mg	Variant #1	3.78	3.29	4.76	5.05	5.49	4.47	12.90	0.912
	Variant #2	11.05	N/A	N/A	N/A	N/A	11.05	6.32	0.000
	Variant #3	5.54	2.99	5.02	4.93	6.14	4.92	12.45	1.184
	Variant #4	4.25	2.02	4.18	4.01	7.25	4.34	13.03	1.870
	Variant #5	6.82	4.64	6.90	5.78	6.87	6.20	11.17	0.992
TOT ref LACV- BF Mg	Variant #1	5.05	5.06	5.97	5.18	6.63	5.58	11.79	0.705
	Variant #2	N/A	10.85	12.41	8.42	10.88	10.64	6.73	1.650
	Variant #3	4.80	4.20	5.72	3.35	4.79	4.57	12.80	0.874
	Variant #4	5.27	2.38	5.85	1.66	6.03	4.23	13.13	2.060
	Variant #5	5.56	6.20	7.16	6.28	6.04	6.25	11.12	0.577

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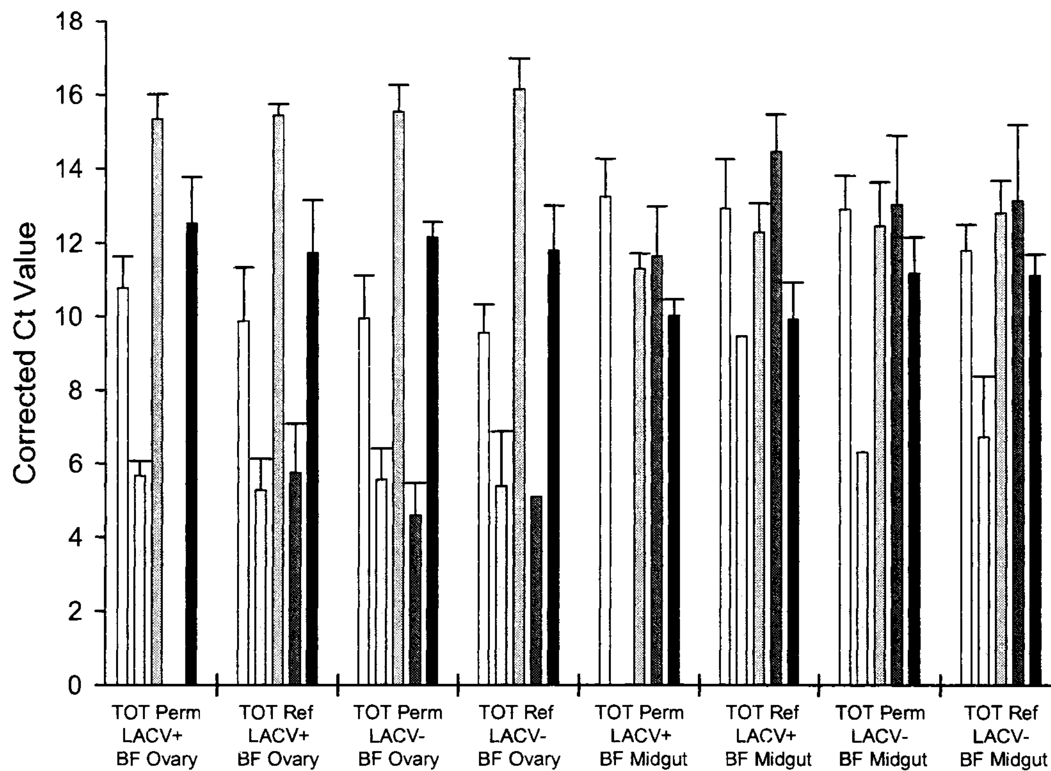

Figure 4.6. AtIAP1 mRNA variant expression profiles from bloodfed ovaries and midguts of LACV TOT permissive and refractory *Ae. triseriatus* mosquitoes. All five AtIAP1 mRNA variants and actin were amplified from ovaries and midguts of individual bloodfed *Ae. triseriatus* LACV TOT permissive and refractory mosquitoes. Both infected and uninfected mosquitoes from each strain were used to determine if differences exist between strains as well as within strains. ΔCT values were calculated by subtracting the actin CT values from the mRNA variant CT values of the same sample. ΔCT values were averaged among all samples for each mRNA variant in each group. The bar on the left of each life stage indicates the relative frequency of mRNA variant 1 and proceeds to variant 5 on the right. The values indicated here are from the final two columns of Table 4.5. Sample calculations can be found in the materials and methods section of this chapter. Each CT indicates a twofold increase in initial transcript.

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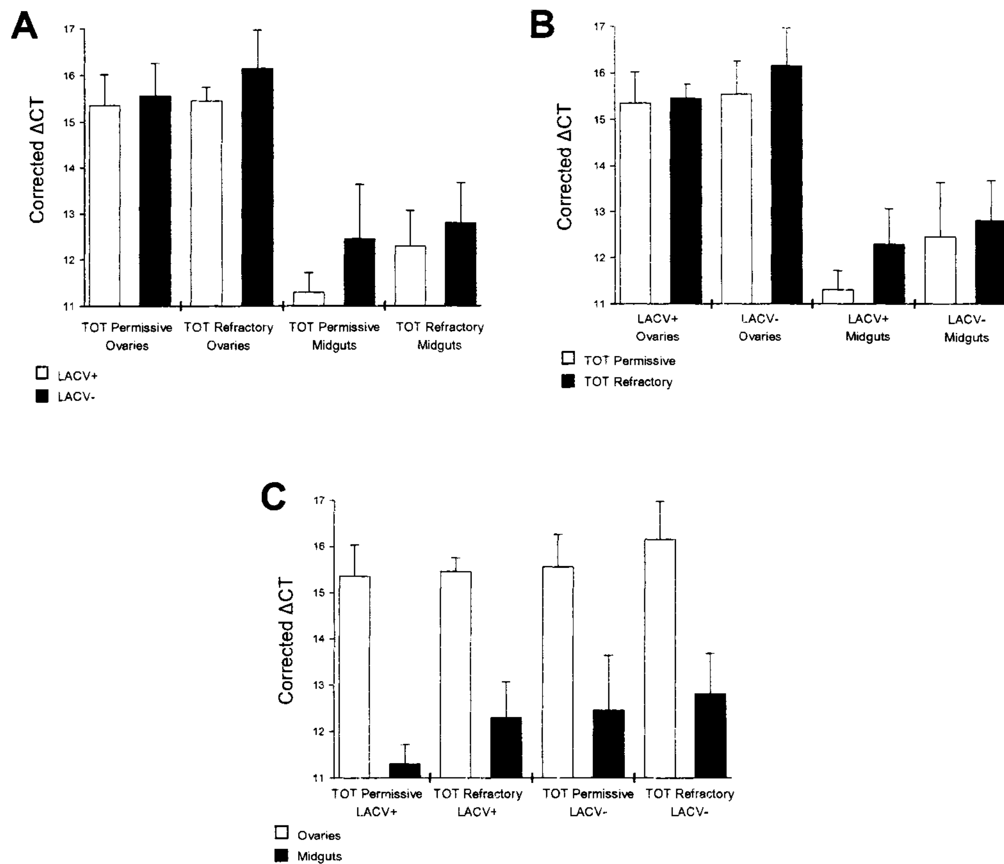

Figure 4.7. AtIAP1 mRNA variant 3 expression profiles from bloodfed ovaries and midguts of LACV TOT permissive and refractory *Ae. triseriatus* mosquitoes. All five AtIAP1 mRNA variants and actin were amplified from ovaries and midguts of individual bloodfed TOT permissive and refractory *Ae. triseriatus* mosquitoes. Both LACV infected and uninfected mosquitoes from each strain were used to determine if differences exist between strains as well as within strains. Corrected ΔCT values were calculated as described in the experimental procedures section. A) Shows the expression profiles of AtIAP1 mRNA variant 3 in LACV infected samples compared to uninfected samples. B) Shows the expression profiles of AtIAP1 mRNA variant 3 in TOT permissive samples compared to TOT refractory samples. C) Shows the expression profile of AtIAP1 mRNA variant 3 in ovary samples compared to midgut samples. The values indicated here are from the final two columns of Table 4.5. Each CT indicates a twofold increase in initial transcript.

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revealed that there is significantly less AtIAP1 mRNA variant 3 in LACV infected mosquitoes (p = 0.0141) (Table 4.3 and Fig. 4.7A). Similarly, there is significantly less AtIAP1 mRNA variant 3 in TOT permissive mosquitoes than in TOT refractory mosquitoes (p = 0.0460) (Table 4.3 and Fig 4.7B). A significantly different expression profile was seen for all five mRNA variants between midguts and ovaries (Table 4.3). This same result, with the exception of variant 5, was seen in *Ae. triseriatus* colony mosquitoes.

H. Overview of Relative Frequency of AtIAP1 mRNA Variants in *Ae. triseriatus* Mosquitoes

All of the studies show that AtIAP1 mRNA variant three is the most abundant in tissues and life stages that express AtIAP1 protein except for salivary glands. Similarly all studies revealed that AtIAP1 mRNA variant expression increases following a bloodmeal except for variant four in midguts. Another interesting observation is that all life stages and tissues tested show the presence of at least two mRNA variants expressed at similar levels except for the ovarian tissue, which always predominantly expresses mRNA variant three. Collectively these results indicate that mRNA variant three is the most important terms of AtIAP1 protein expression.

4. Discussion

Apoptotic response is a major difference between LACV infection of mammalian and insect cells. Inhibitor of apoptosis genes may play a key role in regulating apoptosis in LACV infected insect cells. The *AtIAP1* gene has been previously shown to have several mRNA splice variants (Blitvich et al., 2002), which may be involved in responding to different apoptotic stimuli, including viral infection. This study was

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performed to determine if AtIAP1 mRNA variants are the result of alternative splicing and to characterize life-stage and tissue-specific expression profiles of each variant. Finally, previous studies have shown that LACV mRNAs commonly contain a host sequence that can be found in the homogeneous region of the AtIAP1 5'UTR. Since LACV exhibits cap snatching preference from mRNAs bearing sequence similarity to *AtIAP1*, it is possible that AtIAP1 mRNA secondary structure, or some other mechanism, makes certain AtIAP1 mRNA variants more vulnerable to cap snatching. To this end, expression profiles of AtIAP1 mRNA variants from TOT permissive and TOT refractory strains of *Ae. triseriatus* were examined to determine whether differences exist between the two strains or between LACV+ and LACV- mosquitoes within the same strain.

Using genomic walking, I sequenced ~6 kb of the *Ae. triseriatus* genome. This region contains 3 of the 5 heterogeneous sequences at the distal end of the 5'UTR of AtIAP1 mRNA (Fig. 4.1). The genomic locations of the two other heterogeneous sequences were not identified. The unidentified sequences may be located too far upstream to be detected using our PCR-based approach. Indeed, as already noted, one of the heterogeneous sequences in the AeIAP1 mRNA was located ~120 kb upstream of the homogeneous 5'UTR sequence.

Similarly, I searched the available *Ae. aegypti* genome sequence for the 3 AeIAP1 5'UTR heterogeneous sequences that have been identified. Two of these heterogeneous sequences were found upstream of the *AeIAP1* open reading frame (Fig. 4.1). The *AeIAP1* genomic sequence is located on supercontig 1.368, which is made up of many smaller contig sequences. There are still gaps between some of the sequences which may be why the heterogeneous sequence from AeIAP1 mRNA variant 3 could not be found.

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The genomic location of *AtIAP1* and *AeIAP1* variable sequences upstream of the ORF suggest that mosquito IAP1 mRNA variants are produced via alternative splicing.

Approximately 95% of intron boundaries in *D. melanogaster* can be predicted using computer programs. Genomic studies have determined the most frequently occurring sequences found at the 5' splice site, the 3' splice site, and the 3' branch site of introns (Lim & Burge, 2001). These computer predictions reveal that the most common *D. melanogaster* splice signals are quite similar to sequences found in both *Ae. triseriatus* and *Ae. aegypti* genomes. Computer analysis shows that the sequence "AAGGTAAGTTT" is the most common splice site for the 5' end of an intron in *D. melanogaster*. With the exception of the underlined base pairs in this sequence, there is some variability found at each site, particularly the first and last two bases of the sequence (Lim & Burge, 2001). Similar sequences were found directly following all of the IAP1 variable 5'UTR sequences in the genomes of *D. melanogaster, Ae. aegypti* and *Ae. triseriatus* (Fig. 4.1). The sequence "TACTAAT" is the most common in the branch site generally located near the 3' end of *D. melanogaster* introns. Again, there is some variability, most of which is seen in the first two and the last base of the sequence (Lim & Burge, 2001). Sequences similar to this one were found 70 - 135 base pairs upstream from the homogeneous region of the 5'UTR in the *D. melanogaster, Ae. aegypti,* and *Ae. triseriatus* genomes (Fig. 4.1). Finally, the 3' splice site sequence of *D. melanogaster* is generally "CAGAT" and is preceded by approximately 17 thymidines (Lim & Burge, 2001). This "CAGAT" sequence is found at the beginning of the homogeneous 5'UTR sequence (i.e. the 3' end of the intron separating the heterogeneous and homogeneous 5'UTR sequences) in the genomes of *D. melanogaster, Ae. aegypti,* and *Ae. triseriatus*

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(Fig. 4.1). The *D. melanogaster* genome also has several thymidines preceding the 3' splice sequence. While the *Ae. aegypti* and *Ae. triseriatus* sequences are not directly preceded by a stretch of thymidines, they are in a fairly thymidine-rich area of the genome. These sequence motifs further indicate that *AeIAP1* and *AtIAP1* genes undergo alternative splicing to create mRNA variants, which is interesting because alternative splicing has been reported infrequently in mosquitoes.

Previous studies show that AtIAP1 mRNA was detected in all developmental stages (embryos, first-fourth instar larvae, early and late pupae, and adults) and adult tissues (midguts, ovaries, and salivary glands) examined (Blitvich et al., 2002). Here, earlier observations were extended and show that each AtIAP1 mRNA variant is also detectable, although at different concentrations, in all of these life-stages and tissues, indicating that there is constitutive expression of each IAP1 mRNA variant (Figs. 4.4 and 4.5).

While each AtIAP1 mRNA variant may be present in all life-stages and tissues it is possible that each one may only be translated in response to a particular apoptosis-inducing stimulus or in a certain life-stage or tissue. For example, our previous studies show that AtIAP1 protein is predominantly found in the ovaries and to a lesser extent in the salivary glands and is undetectable in midguts (Blitvich et al., 2002). The current studies show that the majority of AtIAP1 mRNA variant 3 expression occurs in the ovaries followed by the salivary glands and then the midgut (Fig. 4.5). It is possible that in adult females only AtIAP1 mRNA variant 3 is translated to make protein. It is also interesting to note that mRNA variants 3 and 5 are the two most prominent mRNA variants in the life stages where the mosquitoes are transitioning to the next stage of life

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(i.e. embryos, L4 larvae, and pupae) (Fig. 4.4). These variants could play a role in AtIAP1 mRNA translation during critical periods of cellular lysis and organogenesis during metamorphosis.

Analysis of the 5'UTR sequence of all AtIAP1 mRNA variants using the DINAMelt web server (Markham & Zuker, 2005), revealed that each 5'UTR differed considerably in respect to predicted secondary structure. While the homogeneous region of most variants looked nearly identical, the heterogeneous region of each sequence created large differences in the predicted secondary structure of the UTR (Fig. 4.3). Particular secondary structures may be translated more efficiently by translational activators found in particular tissues or life stages. Differential translational regulation has been reported for other mRNA variants that contain distinct 5'UTRs; examples include human surfactant protein A, estrogen receptor-α and insulin (Kos et al., 2002, Shalev et al., 2002, Wang et al., 2005). The secondary structure predictions reveal that the host-derived "CCACTCGCCACT" sequence found in the mRNAs of LACV is found in different structural contexts based on the heterogeneous sequence of the 5'UTR. Differing structure may make it easier for LACV to scavenge this sequence from particular AtIAP1 mRNA variants. If LACV is predominantly scavenging the cap from one AtIAP1 mRNA variant then that variant would likely be less abundant in TOT permissive mosquitoes compared to TOT refractory mosquitoes or it is even more likely that this variant would be less abundant in LACV+ mosquitoes compared to LACV- mosquitoes. Only AtIAP1 mRNA variant 3 showed a significant difference between LACV infected and uninfected mosquitoes and between TOT permissive and refractory mosquitoes (Figs. 4.6 and 4.7 and Tables 4.3 and 4.5). The LACV infected samples and

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TOT permissive samples have significantly less variant 3 mRNA than LACV uninfected and TOT refractory samples, respectively. This result suggests that LACV is either predominantly scavenging the cap from AtIAP1 mRNA variant 3 or that lower initial amounts of AtIAP1 mRNA lead to increased infection.

Reducing AtIAP1 mRNA levels appears to be counterintuitive because diminished quantities of AtIAP1 would theoretically lead to more apoptosis and LACV infected mosquito cells generally show little or no pathogenic effects (including apoptosis). It is possible that decreased AtIAP1 mRNA leads to a small amount of apoptosis in LACV infected cells which in turn leads to increased infection, but goes relatively unnoticed in pathogenesis studies. Alternatively, initial studies of the AtIAP1 gene hypothesized that a portion of the highly structured 5'UTR may act as an internal ribosome entry site (IRES) (Blitvich et al., 2002). Even if the cap is being scavenged from AtIAP1 mRNA variant 3 the remaining portion of the 5'UTR may contain an IRES element that can be used for translation initiation. Under this circumstance removing a portion of the 5'UTR may actually create an mRNA which is better suited to cap-independent translation. Cellular IRES elements are most commonly found on mRNAs that are translated during times of stress, apoptosis, and mitosis (all of which can be found in developing ovaries) (Komar & Hatzoglou, 2005). If the cap is indeed being scavenged from AtIAP1 mRNA variant 3 then the lack of apoptosis generally associated with LACV infection supports the conclusion that AtIAP1 mRNA variant 3 can be translated in a cap-independent manner.

This study shows that AtIAP1 and AeIAP1 mRNAs contain variations in the 5'UTR that are produced through alternative splicing, but this splicing does not affect

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protein sequence. Constitutive expression of all mRNA variants can be seen in all *Ae. triseriatus* life stages and tissue types. However, it is possible that specific AtIAP1 mRNA variants are predominantly translated following different apoptosis-inducing stimuli or in different mosquito life-stages or tissue types. The data from this chapter combined with data from the previous chapter indicate that the *AtIAP1* gene is highly polymorphic and complex. While polymorphisms in the coding sequence do not appear to condition TOT of LACV, differences in the expression of AtIAP1 mRNA variant 3 may play a role in conditioning TOT of LACV in *Ae. triseriatus*.

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Chapter 5:

Quantification of La Crosse Virus RNA in Midguts and Ovaries of Transovarially Infected *Aedes triseriatus* Mosquitoes

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1. Introduction

La Crosse virus (LACV) is the leading cause of pediatric arboviral encephalitis in the United States (McJunkin et al., 1998, Rust et al., 1999, Thompson et al., 1965). The primary vector of LACV is the mosquito *Aedes triseriatus*, which can be found throughout the eastern half of the United States (Darsie & Ward, 2005, Watts et al., 1972). In addition to transmitting LACV to mammalian hosts while taking a bloodmeal, *Ae. triseriatus* mosquitoes have the ability to transovarially transmit (TOT) LACV to their offspring (Pantuwatana et al., 1974). While TOT can amplify LACV in nature, its function as the overwintering mechanism of LACV is most important (Watts et al., 1974).

In their northern range, *Ae. triseriatus* mosquitoes survive the winter as eggs in a diapause state (Shroyer & Craig, 1983) and LACV can persist through the winter in transovarially infected eggs (Watts et al., 1973; 1974). In fact LACV has been detected in larvae from the same mesh-covered treehole in two consecutive seasons. The mesh covering prevented additional oviposition events and installment hatching permitted collection of larvae in the spring months of two consecutive years. LACV infected progeny were obtained in both years indicating that LACV has the ability to survive at least two seasons in diapausing *Ae. triseriatus* eggs (Beaty & Thompson, 1975, Watts et al., 1974).

In the laboratory, TOT rates (percent of infected females producing at least one infected offspring) and filial infection rates (FIR) (percent of LACV infected eggs laid by an infected female) can both be as high as 70% (Miller et al., 1977, Woodring et al., 1998). Furthermore, laboratory studies show that FIRs can remain greater than 70% over

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at least eight generations without any subsequent horizontal transmission events (Miller et al., 1977).

In nature FIRs are much lower and the reasons remain unknown. In one study the FIR was 0.6% of all mosquitoes collected. Larval collections from individual treeholes rarely yield more than one infected individual and infection rates in individual treeholes ranged from 0.0 – 16.7% for those treeholes containing more than twenty larvae (Beaty & Thompson, 1975). In overwintering *Ae. triseriatus* mosquitoes from highly endemic areas, the LACV minimum field infection rate usually ranges from 0.003 to 0.006 (Lisitza et al., 1977). It has been demonstrated that LACV infection does affect the fitness of overwintering *Ae. triseriatus* embryos. LACV infected embryos have a significantly higher mortality rate than uninfected embryos (16% greater mortality) after breaking diapause. This decrease in embryo survival equates to a reduction in FIR over time and could be the reason for the discrepancy between laboratory and field FIRs (McGaw et al., 1998). However, it is important to note that even though LACV confers a fitness cost on transovarially infected *Ae. triseriatus* eggs, the majority of infected embryos survive and allow LACV to persist in the next year (McGaw et al., 1998). While the McGaw study shows that LACV can persist from one season to the next, it suggests that TOT is not a successful method for long-term persistence of LACV in the field (McGaw et al., 1998). One model for transovarial transmission of Keystone virus (KEYV), another virus in the California encephalitis serogroup, suggested that the FIR must be at least 5% in order to maintain constant virus prevalence from year to year (Fine & LeDuc, 1978). While there are some differences between the LACV – *Ae. triseriatus* life cycle compared to the KEYV – *Ae. atlanticus* life cycle, the model suggests that

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LACV FIRs are not sufficient to maintain a constant LACV prevalence in nature (Fine & LeDuc, 1978).

One explanation for the discrepancy between LACV FIRs and successful virus persistence is that some *Ae. triseriatus* mosquitoes may develop high titered, stabilized infections and act more like laboratory infected mosquitoes. Stabilized infections have been defined as infections of the germarium that result in infection of the majority of progeny and were initially described in Sigma virus infection of *D. melanogaster* (Tesh & Shroyer, 1980). Non-stabilized infections can still infect a large percentage of progeny, but infection occurs by invading the oocytes (located in the follicles) rather than the germ cells (Tesh & Shroyer, 1980). Stabilized infections are maintained indefinitely by maternal transmission and have been demonstrated in *Aedes dorsalis* mosquitoes infected with California encephalitis virus (Turell et al., 1982). Stably infected mosquitoes may play the most important epidemiological role in natural LACV persistence (Tesh & Beaty, 1983). However, very little work has been done with field collected specimens to determine if stabilized infections can occur and whether this phenotypic difference in permissiveness to LACV infection consequently affects TOT efficiency.

Ae. triseriatus mosquitoes develop high titered, stabilized infections in the laboratory, potentially making them a valid model for the proposed high titered, stabilized infections in some field specimens. Laboratory colonized *Ae. triseriatus* Walton mosquitoes have an average LACV titer of 3.1 Log_{10} PFUs in ovaries, which were dissected from mosquitoes 21 days after an infectious bloodmeal with a $TCID_{50}$ titer of $10^{5.1}$/0.025 mL (Paulson & Grimstad, 1989). Studies using reverse-transcriptase PCR

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demonstrated increased LACV replication in *Ae. triseriatus* ovaries following a non-infectious bloodmeal (Chandler et al., 1996, Kempf et al., 2006). Increases in LACV replication were most likely due to greater metabolic activity associated with quiescent ovaries becoming active following a bloodmeal (Banks et al., 1994, Clements & Boocock, 1984). Additionally, it has been shown that FIR increases with each subsequent gonotrophic cycle (Miller et al., 1979).

Ae. triseriatus mosquitoes can be selected for high and low TOT rates (termed TOT permissive and TOT refractory, respectively) (Graham et al., 1999). However, studies have never been undertaken to characterize the virus loads in the ovaries of these strains to determine if differences in infectious virus titer play a role in the TOT phenotype. Following a bloodmeal, LACV titers may increase in the ovaries resulting in a greater embryo infection rate in the TOT permissive strain. Infection of the ovaries in the TOT refractory strain could be reduced because of embryo death or the nonpermissive mosquitoes may be able to clear LACV infection by innate immunity or other mechanisms. Finally, high virus titers may overwhelm barriers to TOT in refractory mosquitoes and cause a small percentage of the progeny to become infected.

Transovarial transmission of LACV is a complex process and has several genetic components. However, stochastic events such as differences in ovarian LACV titers may also play a role in TOT efficiency. LACV loads in the ovaries of *Ae. triseriatus* mosquitoes may have a direct correlation to TOT efficiency. These studies tested the hypothesis that the amount of LACV S-segment genome equivalents in tissues of transovarially infected TOT permissive, TOT refractory, and AIDL colony mosquitoes differed. Viral genome equivalents were measured in the ovaries and midguts of

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individual mosquitoes by quantitative-reverse-transcriptase PCR to determine levels prior to and following bloodfeeding. In addition, LACV genome equivalents in the midguts and ovaries of field infected mosquitoes were determined. Detection of major differences in titers of ovaries of field specimens would suggest that there are differences in TOT permissiveness and that stabilized infections may occur.

2. Materials and Methods

A. Mosquitoes

Transovarial Transmission Permissive Ae. triseriatus Mosquitoes

AIDL colony mosquitoes were previously selected for their ability to efficiently transovarially transmit LACV (TOT+) (Graham et al., 1999). The FIR of the selected LACV infected females was 60 – 70%. TOT+ hatched larvae were reared to adults in the laboratory. Legs were removed from individual mosquitoes and tested for LACV antigen by immunofluorescence (Beaty & Thompson, 1975). LACV negative mosquitoes were discarded. Midguts and ovaries were dissected from LACV infected mosquitoes at 4 days post eclosion (pre-bloodfed) or 3 days post ingestion of artificial bloodmeals consisting of defibrinated sheep blood in a water jacketed membrane feeder that was provided at 4 days post eclosion (Rutledge et al., 1964).

Field Collected Ae. triseriatus Mosquitoes

Ae. triseriatus eggs were collected from the field by the La Crosse County Health Department. The LACV infection rate from this study area is roughly 2 – 5% (unpublished data). Field samples were reared to adults in the laboratory and legs were removed from individual specimens and tested for the presence of LACV antigen by immunofluorescence (Beaty & Thompson, 1975). Because IFA results in field collected

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mosquitoes are often hard to interpret, due to the low level of LACV antigen present, weakly positive results were confirmed by several laboratory members. LACV negative mosquitoes were discarded, and midguts and ovaries from LACV infected samples were dissected at 4 days post eclosion (non-bloodfed) or 3 days post bloodfeed (same bloodfeeding conditions as the TOT permissive mosquitoes).

Transovarial Transmission Refractory Ae. triseriatus Mosquitoes

AIDL colony mosquitoes were previously selected for inefficient transovarial transmission of LACV (TOT-) (Graham et al., 1999). The selection process produced female mosquitoes that lay only uninfected eggs. TOT- eggs were reared to adults and provided an artificial bloodmeal consisting of a 1:1 mixture of LACV infected BHK-21 cells suspended in cell culture medium and defibrinated sheep blood. The bloodmeal titered $10^{7.4}$ $TCID_{50}$ per mL. Bloodfed mosquitoes were separated from the unfed mosquitoes and placed in a colony cage with TOT- males for mating. Eggs from the primary gonotrophic cycle were discarded because they are infrequently infected with LACV (Miller et al., 1979). Mosquitoes were provided a non-infectious bloodmeal 14 days after LACV infection and then allowed to oviposit. This secondary egg batch was collected and held at normal insectary temperatures for two weeks. The matured eggs were hatched, and progeny were reared to adults, which were tested for the presence of LACV antigen by immunofluoresence assay of a leg. Midguts and ovaries were dissected from LACV infected samples as described for the previous mosquitoes.

Laboratory Colony Ae. triseriatus Mosquitoes

AIDL colony mosquitoes (originally from La Crosse, WI) were maintained using uninfected individuals. Eggs were reared to adults and infected with LACV in the same

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manner as the TOT refractory mosquitoes. Bloodfed mosquitoes were separated from the unfed mosquitoes and placed in a colony cage with uninfected AIDL colony males for mating. Eggs from the primary gonotrophic cycle were discarded because they are infrequently infected with LACV (Miller et al., 1979). Mosquitoes were given a non-infectious bloodmeal 14 days after the initial LACV infection and allowed to oviposit. Again, the secondary egg batch was collected and held at normal insectary temperatures for two weeks. The matured eggs were hatched, progeny were reared to adults, and tested for the presence of LACV antigen by immunofluoresence assay of a leg. Midguts and ovaries were dissected from LACV infected samples under the same conditions as the three previous mosquito strains.

B. RNA Extraction

Tissue samples were dissected from individual mosquitoes and placed into 50 µL of Leibowitz-15 (L-15) cell culture medium and frozen at -80°C until RNA extraction. Each sample was thawed and triturated. Then 300 µL of TRIzol reagent (Invitrogen, Carlsbad, CA) was added and RNA was extracted according to the manufacturer's directions. The RNA pellet was initially resuspended in 20 µL of diethyl pyrocarbonate (DEPC)-treated ddH_2O, and an aliquot of the sample was removed to determine concentration using a spectrophotometer. After quantification the appropriate amount of DEPC-treated ddH_2O was added to each sample to dilute the concentration to 20 ng/µL.

C. Detection of La Crosse Virus RNA with Quantitative Reverse Transcriptase PCR

The S-segment of the LACV genome was reverse transcribed and quantified using a reverse transcriptase and quantitative PCR assay (Kempf et al., 2006). Specifically, the LNFLAC primer was used for reverse transcription and LACV primer

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and probe set #2 was used for Q-PCR of the S-segment. This assay only detects the genomic RNA and does not measure S-segment messenger RNA or viral complement RNA. For each ovary and midgut sample, 100 ng of total RNA was used for reverse transcription (in a 20 μL reaction). Two μL of the resulting cDNA was used for Q-PCR with each reaction being run in duplicate. Genome equivalents were determined per 100 ng of RNA by comparing the CT value of each sample with a standard curve generated using known copy numbers of a plasmid containing a portion of the LACV S-segment (base pairs 186-246 Genbank accession number NC_004110).

D. Determination of Q-RT-PCR Sensitivity and Correlation with Infectivity ($TCID_{50}$)

In order to determine the minimum detection limits of the assay, uninfected *Ae. triseriatus* midgut and ovary samples were dissected and placed in 50 μL of L-15 medium that was spiked with known doses of LACV ($TCID_{50}$). Initially, three aliquots of LACV (H78 strain) were titrated by $TCID_{50}$. The average $TCID_{50}$ titer from these three aliquots was taken as the titer for a fourth aliquot of the same virus from the same stock. This virus was serially diluted in L-15 to obtain aliquots with known $TCID_{50}$ titers. These were added to ovaries and midguts from individual mosquitoes, triturated, and extracted with 300 μL of TRIzol according to the manufacturer's protocol. RNA was quantified and 100 ng of each sample was used in the reverse transcription reaction. Two μL of the resulting cDNA was used for Q-PCR to determine the lowest amount of infectious virus necessary in a tissue sample to provide an accurate Q-RT-PCR signal. The number of LACV S-segment genome equivalents detected in a known $TCID_{50}$ dilution series provided a model between the titer of infectious virus and Q-RT-PCR results.

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3. Results

A. Detection Limits of LACV Quantitative Reverse Transcriptase PCR

Ovarian and midgut tissues were dissected and spiked with known $TCID_{50}$ titers of LACV. RNA extractions were performed and 100 ng was reverse transcribed for analysis by quantitative PCR. Signal was detected in most of the samples containing LACV; however the assay appeared to lose its quantitative ability at titers lower than 10^1 $TCID_{50}$ for both midguts and ovaries (Table 5.1).

B. Correlation of LACV Genome Equivalents to Infectious Virus

As noted in A, the Q-RT-PCR assay functioned poorly when the titer in either the midgut or ovary was less than 10^1 $TCID_{50}$/tissue sample. As a result, the samples spiked with $TCID_{50}$ titers of 10^0 and 10^{-1} were not used to correlate LACV GE/100 ng RNA to $TCID_{50}$ titer. For midguts the regression equation is: Log $TCID_{50}$ titer = 1.2859(Log LACV GE) – 1.0991; $R^2 = 0.982$ (Fig. 5.1A). In ovarian tissue the regression equation is: Log $TCID_{50}$ titer = 1.0512(Log LACV GE) – 0.8258; $R^2 = 0.964$ (Fig. 5.1B). These curves were made using results obtained with midguts and ovaries from non-bloodfed mosquitoes and would likely differ slightly in their bloodfed counterparts.

C. Quantification of La Crosse Virus Genome Equivalents in Midguts of Non-Bloodfed Mosquitoes

The average number of LACV S-segment genome equivalents/100 ng total RNA (LACV GE) was determined for midgut samples from non-bloodfed transovarially infected *Ae. triseriatus* mosquitoes (Table 5.2 and Fig. 5.2). The TOT permissive strain midguts contained a mean titer of $10^{4.52}$ LACV GE. The midguts of the TOT refractory strain had a mean titer of $10^{4.26}$ LACV GE. The midguts of the AIDL colony strain had a

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Table 5.1. Comparison of LACV $TCID_{50}$ titer to LACV genome equivalents in midgut and ovarian tissues.

Tissue Type	Total $TCID_{50}$ Titer in Sample	Average GE/100 ng RNA	Std. Deviation
Ovaries	10^{-1}	3.90	2.19
Ovaries	10^{0}	2.03	3.52
Ovaries	10^{1}	1.42	2.46
Ovaries	10^{2}	8.65	9.41
Ovaries	10^{3}	342.18	333.77
Ovaries	10^{4}	1067.71	931.03
Ovaries	10^{5}	4898.07	4474.35
Midgut	10^{-1}	32.47	31.23
Midgut	10^{0}	24.95	23.91
Midgut	10^{1}	57.66	40.62
Midgut	10^{2}	283.71	106.92
Midgut	10^{3}	1073.28	467.00
Midgut	10^{4}	5979.26	1921.11
Midgut	10^{5}	82641.76	7011.84

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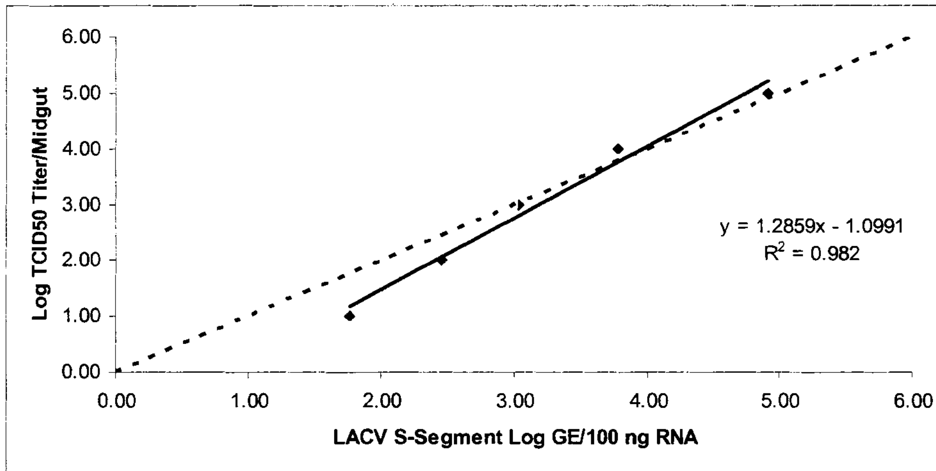
A



B

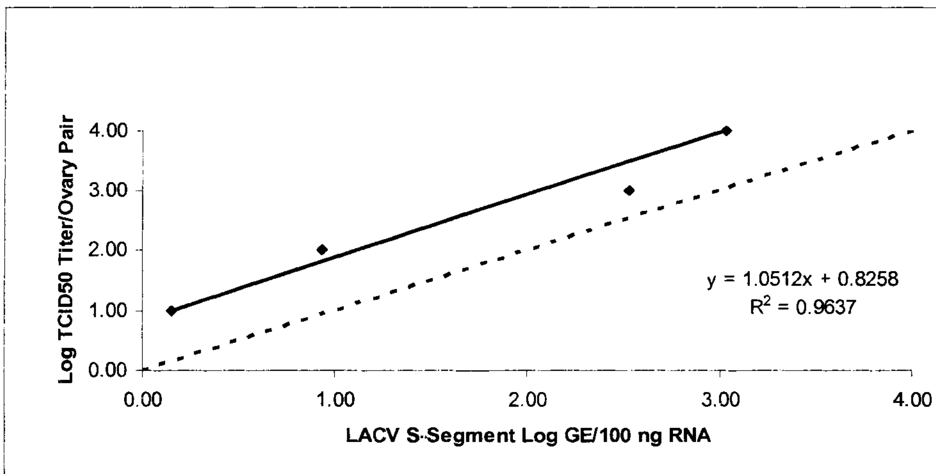


Figure 5.1. Correlation of $TCID_{50}$ titer with LACV GE/100 ng RNA. Midgut (Panel A) and Ovary (Panel B) samples were spiked with known $TCID_{50}$ titers of LACV. RNA extractions were performed and analyzed by Q-RT-PCR to determine the number of LACV GE present in 100 ng of RNA. Three samples were spiked with the same $TCID_{50}$ titer and the Q-RT-PCR results were averaged, plotted, and correlated to the starting $TCID_{50}$ titer. The number of LACV GE found in the samples containing $TCID_{50}$ titers of 10^0 and 10^{-1} were not used for the correlation because the detection does not appear to be accurate (Table 5.2). The dotted line represents the y = x line (a 1:1 correlation between S-segment GE/$TCID_{50}$) and the solid line indicates the observed results.

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Table 5.2. LACV S-segment genome equivalents in the midguts and ovaries of several *Ae. triseriatus* mosquito strains.

Sample	Samples Tested	Positive Samples used in Analyses	Average Genome Eqs/100 ng/RNA	Log Average Genome Eqs/100 ng RNA	P-value from Two-Tailed T-Test of BF and NBF Sample
TOT- NBF Midguts	10	9	18069.16	4.26	0.698
TOT- BF Midguts	10	9	13143.83	4.12	
TOT- NBF Ovaries	10	9	9437.97	3.97	0.093
TOT- BF Ovaries	10	10	4695.79	3.67	
TOT+ NBF Midguts	10	10	33180.76	4.52	0.300
TOT+ BF Midguts	10	10	77298.70	4.89	
TOT+ NBF Ovaries	10	10	52100.74	4.72	0.290
TOT+ BF Ovaries	10	10	86440.30	4.94	
AIDL NBF Midguts	10	9	3025.34	3.48	0.026
AIDL BF Midguts	10	10	16506.32	4.22	
AIDL NBF Ovaries	10	10	3469.98	3.54	0.044
AIDL BF Ovaries	10	10	15699.15	4.20	
Field NBF Midguts	25	24	36.09	1.56	0.000
Field BF Midguts	29	22	14.92	1.17	
Field NBF Ovaries	25	7	31.37	1.50	0.048
Field BF Ovaries	29	28	82.51	1.92	

* indicates significant differences between non-bloodfed and bloodfed mean LACV GE

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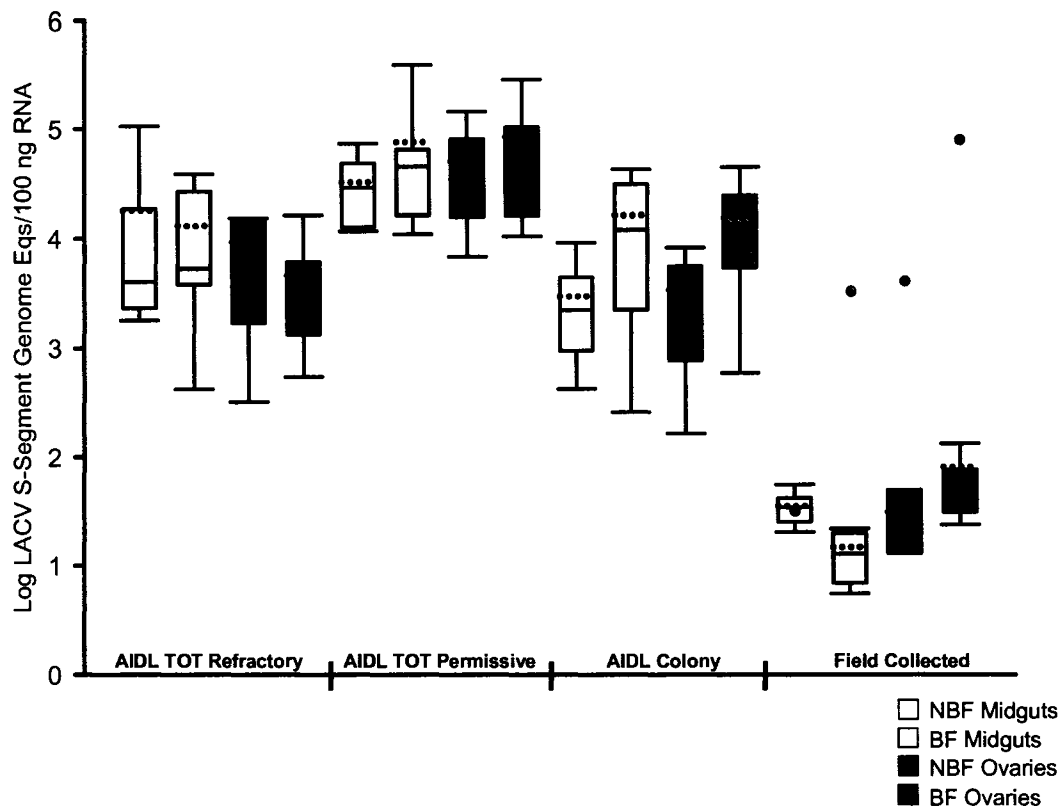

Figure 5.2. LACV S-segment genome equivalents in the midguts and ovaries of several *Ae. triseriatus* mosquito strains infected transovarially with LACV. This graph depicts the log value of LACV S-segment genome equivalents found in midguts and ovaries from TOT refractory, TOT permissive, AIDL colony, and field collected samples of *Ae. triseriatus*. The green dots represent the LACV GE found in the midgut and ovaries of one particular non-bloodfed mosquito collected in the field. The purple dots represent the LACV GE found in the midgut and ovaries of one particular bloodfed mosquito collected in the field. Neither the green or purple samples were used to calculate the average LACV GE because they are obvious outliers and could possibly represent stably infected mosquitoes. The boxes show the 25th – 75th percentiles for each group with a solid line at the median value and a dotted line at the mean value. The bars extend to the 10th and 90th percentiles for each group.

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mean titer of $10^{3.48}$ LACV GE. The midguts of the field strain had a mean titer of $10^{1.56}$ LACV GE, which is significantly lower than the mean LACV GE titers of the midguts of the TOT permissive ($p = 0.001$) and AIDL colony strains ($p = 0.016$), but not the TOT refractory strain ($p = 0.150$) (Table 5.3).

D. Quantification of La Crosse Virus Genome Equivalents in Midguts of Bloodfed Mosquitoes

The mean LACV GE titers were determined in the midguts of four bloodfed strains of *Ae. triseriatus* mosquitoes. The infection pattern was slightly different from that seen in the midguts of non-bloodfed mosquito of the same strains (Table and Fig. 5.2). The greatest mean midgut titer was $10^{4.89}$ LACV GE in the TOT permissive strain. Midguts from bloodfed AIDL colony mosquitoes had the second greatest mean titer averaging $10^{4.22}$ LACV GE. Midguts from the bloodfed TOT refractory strain had a mean titer of $10^{4.12}$ LACV GE, and the midguts from the bloodfed field collected mosquitoes had a mean titer of $10^{1.17}$ LACV GE. There was one exception in the bloodfed field collected mosquitoes – one midgut contained $10^{3.5}$ LACV GE (indicated by purple dot in Fig. 5.2). Because the titer was significantly greater, this sample was not included in the calculation of the mean. The midguts from bloodfed field collected mosquitoes had a significantly lower mean LACV GE titer than the midguts from the TOT refractory ($p = 0.029$) and AIDL colony strain ($p = 0.010$), but not the TOT permissive strain ($p = 0.083$). However, there was a large variance in the TOT permissive group) (Table 5.3).

E. Quantification of La Crosse Virus Genome Equivalents in Ovaries of Non-Bloodfed Mosquitoes

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Table 5.3. Mean LACV GE titers in tissues of TOT permissive, TOT refractory, AIDL colony, and field collected *Ae. triseriatus* mosquito strains.

Mean LACV GE from Non-Bloodfed Mosquito Midguts (P-values between groups)				
	TOT Refractory	TOT Permissive	AIDL Colony	Field Collected
TOT Refractory	N/A	0.278	0.223	0.150
TOT Permissive	0.278	N/A	0.002*	0.001*
AIDL Colony	0.223	0.002*	N/A	0.016*
Field Collected	0.150	0.001*	0.016*	N/A

Mean LACV GE from Bloodfed Mosquito Midguts (P-values between groups)				
	TOT Refractory	TOT Permissive	AIDL Colony	Field Collected
TOT Refractory	N/A	0.143	0.641	0.029*
TOT Permissive	0.143	N/A	0.163	0.083
AIDL Colony	0.641	0.163	N/A	0.010*
Field Collected	0.029*	0.083	0.010*	N/A

Mean LACV GE from Non-Bloodfed Mosquito Ovaries (P-values between groups)				
	TOT Refractory	TOT Permissive	AIDL Colony	Field Collected
TOT Refractory	N/A	0.015*	0.026*	0.002*
TOT Permissive	0.015*	N/A	0.007*	0.005*
AIDL Colony	0.026*	0.007*	N/A	0.006*
Field Collected	0.002*	0.005*	0.006*	N/A

Mean LACV GE from Bloodfed Mosquito Ovaries (P-values between groups)				
	TOT Refractory	TOT Permissive	AIDL Colony	Field Collected
TOT Refractory	N/A	0.017*	0.069	0.017*
TOT Permissive	0.017*	N/A	0.031*	0.013*
AIDL Colony	0.069	0.031*	N/A	0.015*
Field Collected	0.017*	0.013*	0.015*	N/A

* Indicates significant differences ($\alpha = 0.05$) in mean LACV GE titer between strains using a T-test that assumes unequal variance.

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The mean number of LACV GE was also determined in ovaries of non-bloodfed TOT permissive, TOT refractory, AIDL colony, and field collected *Ae. triseriatus* mosquitoes (Table and Fig. 5.2). The ovaries were dissected from the same non-bloodfed mosquitoes as the midguts that were assayed in section C, and the results were virtually identical. The non-bloodfed TOT permissive mosquitoes contained the largest LACV GE titer ($10^{4.76}$) in the ovaries. The non-bloodfed TOT refractory mosquitoes had a mean of $10^{3.97}$ LACV GE in the ovaries. Ovaries of AIDL colony mosquitoes had a mean of $10^{3.54}$ LACV GE, the lowest for laboratory mosquito strains. Finally, ovaries from the field collected strain had a mean LACV GE titer of $10^{1.50}$.

The mean LACV GE titer in ovaries from non-bloodfed field collected samples was significantly lower than that of all of laboratory groups ($p = 0.005$, $p = 0.002$, and $p = 0.006$ for TOT permissive, TOT refractory, and AIDL colony groups respectively) (Table 5.3). The ovaries from one field collected non-bloodfed mosquito contained $10^{3.62}$ LACV GE; the midgut of that same mosquito only contained $10^{1.50}$ LACV GE (indicated by green dots on Fig. 5.2). This mosquito was not used in determining average LACV GE for the field strain because it was an outlier.

F. Quantification of La Crosse Virus Genome Equivalents in Ovaries of Bloodfed Mosquitoes

The mean number of LACV GE was determined in the ovaries from all four strains of *Ae. triseriatus* mosquitoes following a bloodmeal. Ovary and midgut samples were dissected from the same bloodfed mosquitoes. Bloodfed TOT permissive mosquitoes averaged $10^{4.94}$ LACV GE in the ovaries. Bloodfed AIDL colony mosquitoes had $10^{4.20}$ LACV GE in the ovaries. TOT refractory mosquitoes averaged $10^{3.67}$ LACV

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GE in ovaries following a bloodmeal. Finally, bloodfed field collected samples had the lowest mean LACV GE titer in ovaries with $10^{1.92}$ LACV GE. The ovaries of bloodfed field collected mosquitoes had a significantly lower mean LACV GE titer than all three laboratory strains ($p = 0.013$, $p = 0.015$, and $p = 0.017$ for TOT permissive, AIDL colony, and TOT refractory groups, respectively) (Table 5.3).

The field collected bloodfed mosquitoes had one obvious outlier that was not included in calculating the mean LACV GE titer for this group. Ovaries of this mosquito contained $10^{4.89}$ LACV GE. These ovaries came from the same mosquito as the midgut outlier in the group of bloodfed field collected mosquitoes (both the ovary and midgut samples are indicated by purple dots in Fig. 5.2).

G. Quantification of La Crosse Virus Genome Equivalents Pre- and Post-Bloodmeal

The mean LACV GE titers were compared in ovaries and midguts of all four *Ae. triseriatus* strains before and after a bloodmeal (Table and Fig. 5.2). In both the midguts and ovaries of the TOT refractory strain, there was a decrease in LACV GE following a bloodmeal, however, this change was not significant ($p = 0.698$ and $p = 0.093$ respectively). Conversely, in midguts and ovaries of the TOT permissive strain the LACV GE titer increased after a bloodmeal, but again neither was significantly different from its unfed counterpart ($p = 0.300$ and $p = 0.290$ respectively). The LACV GE titer significantly increased following a bloodmeal in both the midguts and ovaries of the AIDL colony strain ($p = 0.026$ and $p = 0.044$ respectively). Finally, the LACV GE titer in the midguts of field collected mosquitoes significantly decreased following a bloodmeal ($p = 0.000$), while the mean LACV GE titer of the ovaries significantly increased ($p = 0.048$).

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H. Trends Affecting LACV GE in Transovarially Infected *Ae. triseriatus* Mosquitoes

A comparison of all samples used in this study revealed that strain has the most significant effect on the LACV GE in a tissue sample ($p > 0.0001$) (Table 5.4). Bloodmeal status also plays a role in determining the LACV GE titer in a tissue sample. However, bloodfeeding only has a significant effect on LACV GE titer when coupled with the strain ($p = 0.0372$) (Table 5.4). Interestingly, tissue type does not play a significant role in determining the LACV GE titer found in these samples (Table 5.4).

4. Discussion

This study lends new insight into transovarial transmission of LACV infection in different strains of *Ae. triseriatus* mosquitoes. That laboratory infected mosquitoes have significantly increased viral loads from field mosquitoes is not a novel idea. However, this study focused on epidemiologically relevant organs of both laboratory and field collected mosquitoes. Q-RT-PCR results demonstrated that there are significant differences in LACV GE between transovarially infected field and laboratory strains of *Ae. triseriatus*. These results also suggested that some field mosquitoes can develop high titered, stabilized LACV infections.

The TOT permissive laboratory mosquitoes contained the greatest average number of LACV GE in the ovaries and midguts of both non-bloodfed and bloodfed mosquitoes. This was expected because this strain of mosquitoes has been highly selected for the ability to efficiently TOT LACV. Greater LACV GE titers may also result in greater filial infection rates. In this strain LACV GE did not significantly increase in either tissue following a bloodmeal (Table 5.2). This may be due to the highly selected nature of this strain. Virus titers are high before feeding and probably

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Table 5.4. Analysis of variance comparing the effects of tissue type, bloodfeeding status, and *Ae. triseriatus* strain on LACV GE titer.

Variable	F-Value	Pr > F
Strain	25.31	<.0001*
Tissue Type	0.05	0.8301
Strain and Tissue Type	0.61	0.6073
Bloodmeal Status	3.52	0.0622
Strain and Bloodmeal	2.88	0.0372*
Tissue and Bloodmeal	0.05	0.8271
Strain and Tissue and Bloodmeal	0.04	0.9888

*Indicates that the given variable had a significant effect on LACV GE titer in this study

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have little room for increase before causing deleterious effects to the mosquito.

The TOT refractory mosquitoes are also a highly selected line; however, they were selected for a decreased ability to TOT LACV. Mosquitoes of this strain that are infected via TOT develop high titered LACV infections. Interestingly, mean titers of LACV GE decreased in both the midgut and ovaries after a bloodmeal (Table 5.2). While these decreases are not statistically significant ($p = 0.698$ and $p = 0.093$ in midguts and ovaries respectively), the trend is interesting and could become significant with a greater sample size (at least in the ovaries). It is possible that upregulation of some translational products in the ovaries (and to a lesser extent the midguts) following bloodfeeding condition the ability of LACV to replicate effectively. One possibility is that the RNA interference response is more robust in TOT refractory mosquitoes compared to TOT permissive mosquitoes following a bloodmeal. This increased innate immune response may decrease the amount of LACV RNA present in the ovaries and midguts of TOT refractory mosquitoes following a bloodmeal. Increased expression of *Ae. triseriatus* inhibitor of apoptosis 1 (AtIAP1) mRNA variant three may also play a role in reduced LACV GE titers following a bloodmeal in TOT refractory mosquitoes. As described in the previous chapter there is significantly more AtIAP1 mRNA variant 3 in the ovaries of TOT refractory mosquitoes than TOT permissive mosquitoes. Increased AtIAP1 may hinder LACV's ability to replicate effectively following a bloodmeal and cause lower LACV GE titers.

In the AIDL colony mosquitoes there was a significant increase in LACV GE after a bloodmeal in both the midguts and the ovaries. This was expected and confirms previous results with LACV (Chandler et al., 1996, Kempf et al., 2006, Paulson &

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Grimstad, 1989). Increased metabolic activity resulting from a bloodmeal likely provides a permissive environment for virus replication.

The field collected mosquitoes generally had significantly lower mean LACV GE titers than the laboratory strains of *Ae. triseriatus* (Table 5.3). Furthermore, the LACV GE titers decreased in the midguts of field collected mosquitoes after a bloodmeal. However, it is important to note that these values fall within the lower detection limits of the Q-RT-PCR assay, where GE titers can't be accurately determined. Thus this difference may not be valid. LACV GE titers in the ovaries significantly increased ($p = 0.048$) following a bloodmeal. However, in this case the difference may be even greater because only 7 of 25 mosquitoes were used to calculate the average LACV GE titer in the ovaries of non-bloodfed mosquitoes. If the remaining 18 samples, those with undetectable LACV GE titers by Q-PCR, had been included the mean LACV GE titers in the ovaries of non-bloodfed mosquitoes would be even lower. Again, increased levels of LACV genomic RNA in the ovaries of bloodfed mosquitoes has been reported previously and was expected (Kempf et al., 2006).

The most interesting result of this study was the variability in LACV GE titers found in the field collected mosquitoes. One of the non-bloodfed mosquitoes had $10^{3.62}$ and $10^{1.50}$ LACV GE in the ovaries and midgut, respectively. The LACV titer in this midgut was about average for field samples while the level found in the ovaries was much greater than average. In this field sample, ovarian LACV GE titer was actually greater than the mean for AIDL colony mosquitoes. Thus this mosquito would likely have a TOT potential as great as the laboratory colony (upwards of 50% filial infection rate) (data not shown). Similarly, one of the bloodfed field samples had LACV GE titers

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of $10^{4.89}$ and $10^{3.52}$ in the ovaries and midguts respectively. The midgut titer of $10^{3.52}$ LACV GE was lower than average for midguts from all bloodfed laboratory strains. However, a $10^{4.89}$ LACV GE titer in the ovaries was greater than the average for both TOT refractory and AIDL colony mosquito strains and only slightly lower than the average for the TOT permissive strain. This mosquito would seem to have the same TOT potential as the TOT permissive strain (60 – 70% filial infection rate) (data not shown).

Previous investigators have postulated that some *Ae. triseriatus* mosquitoes develop high titered, stabilized infections and are more epidemiologically relevant in LACV persistence (Tesh & Beaty, 1983). It is possible that these few mosquitoes contribute the majority of transovarially infected, overwintering offspring and play the most important role in LACV persistence in nature. These studies appear to reinforce at least part of this hypothesis and demonstrated that some *Ae. triseriatus* mosquitoes can develop significantly higher titered LACV infections than most others. Of 54 field collected mosquitoes (25 non-bloodfed and 29 bloodfed) only two samples (one non-bloodfed and one bloodfed) had LACV GE levels similar to laboratory colonies. These mosquitoes may have had stabilized infections and significantly greater TOT potential than the remaining LACV positive field samples. Obviously, removal of the ovaries precludes testing whether there was any relevance to this observation in terms of TOT, but the observation is provocative.

A previous study indicates that the true LACV larval field infection of *Ae. triseriatus* mosquitoes is 0.6% (Beaty & Thompson, 1975) while another study also based on virus isolation reported a minimum field infection rate of 0.9% (Watts et al., 1974). *Ae. triseriatus* collections from the same area made in 2000 -- 2006 were analyzed at

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AIDL using immunofluorescence and RT-PCR. The larval infection rate has ranged from 2 – 5% (unpublished data).

The epidemiological relevance of estimates based on LACV prevalence in larvae remains to be determined. Theoretically, only a limited number of field-infected mosquitoes have sufficient ovarian titers to transovarially transmit LACV. If this is the case, only mosquitoes with relevant LACV titers should be used to determine field infection rates. The current study did not measure the amount of LACV in the salivary glands of mosquitoes, but it would also be interesting to determine if only the field samples with high titered, stabilized infections have sufficient salivary titers for horizontal transmission of LACV.

This study determined levels of LACV S-segment genomic RNA (LACV GE) in the midguts and ovaries of four different *Ae. triseriatus* mosquito strains. While the results based on detection of LACV GE are interesting they do not necessarily indicate levels of infectious virus. The correlations made between LACV GE and $TCID_{50}$ titer were made using LACV spiked AIDL colony mosquito tissues and may not accurately correlate infectious virus to LACV GE in different strains of *Ae. triseriatus* mosquitoes. For example, the same level of LACV GE in TOT refractory and AIDL colony tissues may not correlate to the same amount of infectious virus in these samples. Similarly, these studies do not detect the amount of transcription/replication that is occurring in each tissue, because they do not detect LACV mRNA or viral complement RNA (replicative intermediate). While previous studies with AIDL colony mosquitoes demonstrated that increases in ovarian LACV GE titer correlated to increases in mRNA and viral complement RNA (Kempf et al., 2006), the same is not necessarily true for

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other *Ae. triseriatus* strains. Previous studies also showed that persistently infected AIDL colony mosquitoes downregulate LACV mRNA and vcRNA during times of ovarian quiescence. Meanwhile LACV GE are detectable by RT-PCR at similar levels to those in metabolically active ovaries (Chandler et al., 1996). Again, these results may differ for different *Ae. triseriatus* strains. For example, in TOT permissive mosquito ovaries, LACV may continue to replicate during times of quiescence leading to increased LACV titers and greater TOT efficiency. Correlating infectious LACV and the replicative forms of LACV RNA in these *Ae. triseriatus* strains could provide insight into LACV replication efficiency in different TOT phenotypes.

Two findings in this study deserve further discussion. One is that laboratory mosquitoes selected for their limited TOT potential actually seem to decrease LACV levels in the ovaries following a bloodmeal. This contradicts the dogma that increased metabolic activity following a bloodmeal will lead to an increase in LACV replication. It is also possible that LACV RNA levels initially increase, but then innate immunity, such as RNA inhibition, controls the infection. Another interesting result is that a small percentage of transovarially infected mosquitoes from the field have LACV levels similar to those observed in transovarially infected laboratory mosquitoes. Little is known about the amount of virus that is necessary for a mosquito to successfully TOT LACV. Logically, some ovarian titer threshold must be crossed for successful TOT of LACV otherwise innate immunity in the eggs may be capable of clearing the infection.

This study corroborated the existence of *Ae. triseriatus* mosquitoes with high titered, stabilized infections. Future studies will need to be performed to compare TOT potential of field mosquitoes with stabilized infections vs. those that have very low

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LACV titers in the ovaries. Identifying genetic differences between high and low titer LACV infected field samples may be the key to determining what genes, metabolic pathways, or immune pathways are involved in conditioning high titer LACV infections in field mosquitoes and could provide markers for risk assessment.

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Chapter 6:

Summary

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La Crosse virus is an important cause of arboviral encephalitis in children of the Midwestern United States (McJunkin et al., 1998, Rust et al., 1999, Thompson et al., 1965). Transovarial transmission (TOT) is an important process in overwintering of LACV (Watts et al., 1973; 1974). Previous studies have shown that the ability of *Aedes triseriatus* mosquitoes to effectively TOT LACV has a genetic component that is marked by at least three quantitative trait loci (Graham et al., 2003). A better understanding of the genes conditioning TOT will allow mosquito control efforts to be focused in areas where TOT permissive genotypes are present. Previous hypotheses proposed that in nature there are a limited number of *Ae. triseriatus* mosquitoes that develop high titer stabilized infections and that these mosquitoes are most important in persistence of LACV (Tesh & Beaty, 1983).

These dissertation studies have shown that over a 14,000 km^2 collection area in the Midwestern United States, *Ae. triseriatus* mosquitoes exist as a panmictic population (Chapter 2). This information confirms previous studies showing that this mosquito has high levels of gene flow in this area of the United States. Perhaps the more important outcome is that researchers can analyze mosquitoes from throughout the study area as if they were from the same collection. This will increase the power of finding genetic differences between mosquitoes that effectively TOT LACV and those that do not.

The *Ae. triseriatus* inhibitor of apoptosis 1 gene (*AtIAP1*) is very complex (Chapter 3). The analysis of the *AtIAP1* gene in field collected *Ae. triseriatus* mosquitoes reveals substantial variability in this gene (roughly 1/6 nucleotides are polymorphic), but the baculovirus inhibitor of apoptosis repeat motifs are highly conserved, likely due to their importance in protein-protein interactions. Despite the large number of single

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nucleotide polymorphisms (SNPs) in the homogeneous portion of the 5'UTR and the open reading frame, there does not appear to be any association between individual SNPs and the ability to TOT LACV. While preliminary studies suggested as many as five SNPs were involved in the TOT phenotype, a follow-up study failed to verify these results. A major drawback of this study is that there is no way of knowing if LACV- mosquitoes were never exposed or if they were exposed, but didn't get infected. While this type of association mapping study has been successfully performed in the past, it is difficult to detect any associations that only play a small role in conditioning the phenotype that is under investigation. These results suggest that SNPs in the *AtIAP1* homogenous 5'UTR and open reading frame do not play a major, if any, role in conditioning TOT of LACV in the mosquito *Ae. triseriatus.*

Further analyses of the *AtIAP1* gene revealed that there are at least five different mRNA splice variants that encode the same protein, but differ only in a portion of the 5' untranslated region (5'UTR) (Chapter 4). Analysis of the *Ae. triseriatus* genome showed the presence of common *Drosophila* intron splice site motifs indicating that alternative splicing is responsible for creating AtIAP1 mRNA variants. Secondary structure predictions were made for the 5'UTR of all five AtIAP1 mRNA variants. These predictions revealed noticeable structural differences in the variable portion of the UTR, but the conserved region remained structurally similar. These structural differences may be important in responding to different apoptotic stimuli or to different translational factors. In concordance with this hypothesis, different mRNA variants are more abundant in certain tissues and developmental stages of the *Ae. triseriatus* mosquito life cycle. Most interestingly, AtIAP1 mRNA variant 3 expression is significantly lower in

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LACV infected samples compared to uninfected ones and in TOT permissive samples compared to TOT refractory samples of *Ae. triseriatus*. This result indicates that AtIAP1 mRNA variant 3 may play a role in conditioning TOT of LACV. Further experiments are needed to determine what this role may be and why decreased levels of AtIAP1 mRNA variant 3 seems to lead to greater LACV infection of *Ae. triseriatus* mosquitoes. Regulation of AtIAP1 expression is obviously complex, and there is still much to learn about the purpose of each mRNA variant and the role each of them play in inhibiting apoptosis.

Finally, the last study in this dissertation confirms that in nature some *Ae. triseriatus* females have the ability to develop significantly higher titer LACV infections than most others (Chapter 5). These few mosquitoes may play a major role in LACV persistence in the environment. Future experiments to determine whether only these stably infected field mosquitoes can TOT LACV efficiently would lead to a better understanding of LACV epidemiology. In light of these results it would be interesting to determine the average LACV titers of salivary glands to see if the pattern is the same as that seen in the ovaries. It is possible that females with high titered stabilized infections are not only the most important in terms of TOT of LACV, but also play the most important role in salivary transmission to vertebrate hosts. These same mosquitoes may also play a major role in successful overwintering of LACV. If only those mosquitoes with high titered, stabilized infections are epidemiologically relevant, then the laboratory model is likely a very accurate portrayal of field infections because stably infected field samples have virus loads similar to those of laboratory infected mosquitoes. This study

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has laid the groundwork for future experiments to better understand the role played by stably infected *Ae. triseriatus* mosquitoes in transmission of LACV.

The overall body of work presented here has shown that mosquitoes from different field populations of *Ae. triseriatus* mosquitoes can become infected transovarially; however, some of these mosquitoes develop much higher titer virus loads than others. Population genetic studies showed that the genetics controlling infection potential will likely be present over a wide range due to large gene flow rates in Midwestern *Ae. triseriatus* mosquitoes. Association mapping studies have determined that the homogeneous portion of the *AtIAP1* 5' UTR and the coding region of this gene are very polymorphic, however these polymorphisms do not play a role in conditioning TOT. While the *AtIAP1* coding regions appears to have no effect on TOT potential, expression of AtIAP1 mRNA variant 3 is significantly lower in LACV infected and TOT permissive mosquitoes. Future association mapping studies regarding genes involved with TOT of LACV may be more fruitful if mosquitoes with high titered, stabilized LACV infections are compared to those with low titer infections.

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REFERENCES CITED

Abraham, E. G., Islam, S., Srinivasan, P., Ghosh, A. K., Valenzuela, J. G., Ribeiro, J. M. C., Kafatos, F. C., Dimopoulos, G. & Jacobs-Lorena, M. (2004). Analysis of the *Plasmodium* and *Anopheles* transcriptional repertoire during ookinete development and midgut invasion. *J Biol Chem* **279**, 5573-5580.

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F., George, R. A., Lewis, S. E., Richards, S., Ashburner, M., Henderson, S. N., Sutton, G. G., Wortman, J. R., Yandell, M. D., Zhang, Q., Chen, L. X., Brandon, R. C., Rogers, Y. H. C., Blazej, R. G., Champe, M., Pfeiffer, B. D., Wan, K. H., Doyle, C., Baxter, E. G., Helt, G., Nelson, C. R., Miklos, G. L. G., Abril, J. F., Agbayani, A., An, H. J., Andrews-Pfannkoch, C., Baldwin, D., Ballew, R. M., Basu, A., Baxendale, J., Bayraktaroglu, L., Beasley, E. M., Beeson, K. Y., Benos, P. V., Berman, B. P., Bhandari, D., Bolshakov, S., Borkova, D., Botchan, M. R., Bouck, J., Brokstein, P., Brottier, P., Burtis, K. C., Busam, D. A., Butler, H., Cadieu, E., Center, A., Chandra, I., Cherry, J. M., Cawley, S., Dahlke, C., Davenport, L. B., Davies, A., de Pablos, B., Delcher, A., Deng, Z. M., Mays, A. D., Dew, I., Dietz, S. M., Dodson, K., Doup, L. E., Downes, M., Dugan-Rocha, S., Dunkov, B. C., Dunn, P., Durbin, K. J., Evangelista, C. C., Ferraz, C., Ferriera, S., Fleischmann, W., Fosler, C., Gabrielian, A. E., Garg, N. S., Gelbart, W. M., Glasser, K., Glodek, A., Gong, F. C., Gorrell, J. H., Gu, Z. P., Guan, P., Harris, M., Harris, N. L., Harvey, D., Heiman, T. J., Hernandez, J. R., Houck, J., Hostin, D., Houston, D. A., Howland, T. J., Wei, M. H., Ibegwam, C., et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-2195.

Apostol, B. L., Black IV, W. C., Reiter, P. & Miller, B. R. (1994). Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. *Am J Trop Med Hyg* **51**, 89-97.

Apostol, B. L., Black, W. C., Miller, B. R., Reiter, P. & Beaty, B. J. (1993). Estimation of the number of full sibling families at an oviposition site using RAPD-PCR markers: Applications to the mosquito *Aedes aegypti*. *Theor Appl Genet* **86**, 991-1000.

Arnheiter, H., Davis, N. L., Wertz, G., Schubert, M. & Lazzarini, R. A. (1985). Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell* **41**, 259-267.

Ashhab, Y., Alian, A., Polliack, A., Panet, A. & Ben Yehuda, D. (2001). Two splicing variants of a new inhibitor of apoptosis gene with different biological properties and tissue distribution pattern. *Febs Lett* **495**, 56-60.

Avise, J. C. (1994). Molecular Markers, Natural History, and Evolution. New York: Chapman and Hall.

Balfour, H. H., Jr., Siem, R. A., Bauer, H. & Quie, P. G. (1973). California arbovirus (La Crosse) infections. I. Clinical and laboratory findings in 66 children with meningoencephalitis. *Pediatrics* **52**, 680-91.

Balkhy, H. H. & Schreiber, J. R. (2000). Severe La Crosse encephalitis with significant neurologic sequelae. *Pediatr Infect Dis J* **19**, 77-80.

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Banks, G. K., Malcolm, C. A. & Clements, A. N. (1994). Temporal pattern of RNA and protein synthesis in the ovary of *Aedes aegypti*. *J Insect Physiol* **40**, 463-468.

Beaty, B. J. & Bishop, D. H. (1988). Bunyavirus-vector interactions. *Virus Res* **10**, 289-301.

Beaty, B. J., Bishop, D. H., Gay, M. & Fuller, F. (1983). Interference between bunyaviruses in *Aedes triseriatus* mosquitoes. *Virology* **127**, 83-90.

Beaty, B. J., Holterman, M., Tabachnick, W., Shope, R. E., Rozhon, E. J. & Bishop, D. H. (1981a). Molecular basis of bunyavirus transmission by mosquitoes: role of the middle-sized RNA segment. *Science* **211**, 1433-5.

Beaty, B. J., Miller, B. R., Shope, R. E., Rozhon, E. J. & Bishop, D. H. L. (1982). Molecular basis of bunyavirus per os infection of mosquitoes: Role of the middle-sized RNA segment. *Proc Natl Acad Sci USA* **79**, 1295-1297.

Beaty, B. J., Rayms-Keller, A., Borucki, M. K. & Blair, C. D. (2000). La Crosse encephalitis virus and mosquitoes: A remarkable relationship. *ASM News* **66**, 349-357.

Beaty, B. J., Rozhon, E. J., Gensemer, P. & Bishop, D. H. L. (1981b). Formation of reassortant bunyaviruses in dually infected mosquitoes. *Virology* **111**, 662-665.

Beaty, B. J., Sundin, D. R., Chandler, L. J. & Bishop, D. H. (1985). Evolution of bunyaviruses by genome reassortment in dually infected mosquitoes (*Aedes triseriatus*). *Science* **230**, 548-50.

Beaty, B. J. & Thompson, W. H. (1975). Emergence of La Crosse virus from endemic foci: Fluorescent antibody studies of overwintered *Aedes triseriatus*. *Am J Trop Med Hyg* **24**, 685-91.

Beaty, B. J. & Thompson, W. H. (1976). Delineation of La Crosse virus in developmental stages of transovarially infected *Aedes triseriatus*. *Am J Trop Med Hyg* **25**, 505-512.

Beck, E. T., Blair, C. D., Black IV, W. C., Beaty, B. J. & Blitvich, B. J. Alternative splicing generates multiple transcripts of the inhibitor of apoptosis 1 gene in *Aedes* and *Culex* spp. *Insect Biochem Mol Biol* **In Press**.

Beck, E. T., Bosio, C. F., Geske, D. A., Blair, C. D., Beaty, B. J. & Black, W. C. (2005). An analysis of gene flow among Midwestern populations of the mosquito *Ochlerotatus triseriatus*. *Am J Trop Med Hyg* **73**, 534-540.

Bergmann, A., Yang, A. Y.-P. & Srivastava, M. (2003). Regulators of IAP function: coming to grips with the grim reaper. *Curr Opin Cell Biol* **15**, 717-724.

Besansky, N. J., Lehmann, T., Fahey, G. T., Fontenille, D., Braack, L. E., Hawley, W. A. & Collins, F. H. (1997). Patterns of mitochondrial variation within and between African malaria vectors, *Anopheles gambiae* and *An. arabiensis*, suggest extensive gene flow. *Genetics* **147**, 1817-28.

Bishop, D. H. (1996). Biology and molecular biology of bunyaviruses. In *The Bunyaviridae*, pp. 19-61. Edited by R. M. Elliott. New York: Plenum Press.

Bishop, D. H., Gay, M. E. & Matsuoko, Y. (1983). Nonviral heterogeneous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA. *Nucleic Acids Res* **11**, 6409-18.

Bishop, D. H. & Shope, R. E. (1979). *Bunyaviridae*. In *Comprehensive Virology*, pp. 1-156.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Bishop, D. H. L. (1985). The genetic-basis for describing viruses as species. *Intervirology* **24**, 79-93.

Black IV, W. C. & DuTeau, N. M. (1997). RAPD-PCR and SSCP analysis for insect population genetic studies. In *The Molecular Biology of Insect Disease Vectors: A Methods Manual*, pp. 361-373. Edited by J. Crampton, C. B. Beard & C. Louis. New York: Chapman and Hall.

Black IV, W. C., Gorrochotegui-Escalante, N. & Duteau, N. M. (2006). Heated oligonucleotide ligation assay (HOLA): an affordable single nucleotide polymorphism assay. *J Med Entomol* **43**, 238-47.

Black IV, W. C., Rai, K. S., Turco, B. J. & Arroyo, D. C. (1989). Laboratory study of competition between United States strains of *Aedes albopictus* and *Aedes aegypti* (Diptera: *Culicidae*). *J Med Entomol* **26**, 260-271.

Blakqori, G., Delhaye, S., Habjan, M., Blair, C. D., Sanchez-Vargas, I., Olson, K. E., Attarzadeh-Yazdi, G., Fragkoudis, R., Kohl, A., Kalinke, U., Weiss, S., Michiels, T., Staeheli, P. & Weber, F. (2007). La Crosse bunyavirus nonstructural protein NSs serves to suppress the type I interferon system of mammalian hosts. *J Virol* **81**, 4991-4999.

Blitvich, B. J., Blair, C. D., Kempf, B. J., Hughes, M. T., Black, W. C., Mackie, R. S., Meredith, C. T., Beaty, B. J. & Rayms-Keller, A. (2002). Developmental- and tissue-specific expression of an inhibitor of apoptosis protein 1 homologue from *Aedes triseriatus* mosquitoes. *Insect Mol Biol* **11**, 431-42.

Blumberg, B. M. & Kolakofsky, D. (1981). Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. *J Virol* **40**, 568-76.

Blumberg, B. M., Leppert, M. & Kolakofsky, D. (1981). Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. *Cell* **23**, 837-45.

Borucki, M. K., Chandler, L. J., Parker, B. M., Blair, C. D. & Beaty, B. J. (1999). Bunyavirus superinfection and segment reassortment in transovarially infected mosquitoes. *J Gen Virol* **80**, 3173-3179.

Borucki, M. K., Kempf, B. J., Blitvich, B. J., Blair, C. D. & Beaty, B. J. (2002). La Crosse virus: Replication in vertebrate and invertebrate hosts. *Microbes Infect* **4**, 341-350.

Bouloy, M. (1991). *Bunyaviridae*: Genome organization and replication strategies. *Adv Virus Res* **40**, 235-75.

Bridgen, A., Weber, F., Fazakerley, J. K. & Elliott, R. M. (2001). Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc Natl Acad Sci USA* **98**, 664-669.

Briese, T., Jia, X. Y., Huang, C., Grady, L. J. & Lipkin, W. I. (1999). Identification of a Kunjin/West Nile-like flavivirus in brains of patients with New York encephalitis. *Lancet* **354**, 1261-1262.

Burkot, T. R. & DeFoliart, G. R. (1982). Bloodmeal sources of *Aedes triseriatus* and *Aedes vexans* in a southern Wisconsin forest endemic for La Crosse encephalitis virus. *Am J Trop Med Hyg* **31**, 376-381.

Caccone, A., Garcia, B. A. & Powell, J. R. (1996). Evolution of the mitochondrial DNA control region in the *Anopheles gambiae* complex. *Insect Mol Biol* **5**, 51-9.

Calisher, C. H. (1994). Medically important arboviruses of the United States and Canada. *Clin Microbiol Rev* **7**, 89-116.

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Calisher, C. H. (1996). History, classification, and taxonomy of viruses in the family *Bunyaviridae*. In *The Bunyaviridae*, pp. 1-17. Edited by R. M. Elliott. New York: Plenum Press.
Calisher, C. H., Coimbra, T. L. M., de Souza Lopes, O., Muth, D. J., de Abreu Sacchetta, L., Francy, D. B., Lazuick, J. S. & Cropp, C. B. (1983). Identification of new Guama and group C serogroup bunyaviruses and an ungrouped virus from southern Brazil. *Am J Trop Med Hyg* **32**, 424-431.
Campbell, C. C. (1997). Malaria: an emerging and re-emerging global plague. *FEMS Immunol Med Microbiol* **18**, 325-331.
Casals, J. (1963). New developments in the classification of arthropod-borne animal viruses. *An Microbiol (Rio J)* **11**, 13-34.
Chai, J. J., Du, C. Y., Wu, J. W., Kyin, S., Wang, X. D. & Shi, Y. G. (2000). Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* **406**, 855-862.
Chandler, L. J., Wasieloski, L. P., Blair, C. D. & Beaty, B. J. (1996). Analysis of La Crosse virus S-segment RNA and its positive-sense transcripts in persistently infected mosquito tissues. *J Virol* **70**, 8972-8976.
Chare, E. R., Gould, E. A. & Holmes, E. C. (2003). Phylogenetic analysis reveals a low rate of homologous recombination in negative-sense RNA viruses. *J Gen Virol* **84**, 2691-703.
Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S.-I. & Abrams, J. M. (2002). The damage-responsive *Drosophila* gene sickle encodes a novel IAP binding protein similar to but distinct from reaper, grim, and hid. *Curr Biol* **12**, 137-140.
Christophides, G. K., Zdobnov, E., Barillas-Mury, C., Birney, E., Blandin, S., Blass, C., Brey, P. T., Collins, F. H., Danielli, A., Dimopoulos, G., Hetru, C., Hoa, N., Hoffmann, J. A., Kanzok, S. M., Letunic, I., Levashina, E. A., Loukeris, T. G., Lycett, G., Meister, S., Michel, K., Muller, H. M., Osta, M. A., Paskewitz, S. M., Reichhart, J. M., Rzhetsky, A., Troxler, L., Vernick, K. D., Vlachou, D., Volz, J., von Mering, C., Xu, J. N., Zheng, L. B., Bork, P. & Kafatos, F. C. (2002). Immunity-related genes and gene families in *Anopheles gambiae*. *Science* **298**, 159-165.
Clark, G. G., Pretula, H. L., Rohrer, W. H., Harroff, R. N. & Jakubowski, T. (1983). Persistence of La Crosse virus (California encephalitis serogroup) in north-central Illinois. *Am J Trop Med Hyg* **32**, 175-184.
Clay, M. E. & Venard, C. E. (1971). Diapause in *Aedes triseriatus* (Diptera: *Culicidae*) larvae terminated by molting hormones. *Ann Entomol Soc Am* **64**, 968-&.
Clay, M. E. & Venard, C. E. (1972). Larval diapause in mosquito *Aedes triseriatus* : Effects of diet and temperature on photoperiodic induction. *J Insect Physiol* **18**, 1441-&.
Clements, A. N. & Boocock, M. R. (1984). Ovarian development in mosquitos: Stages of growth and arrest, and follicular resorption. *Physiol Entomol* **9**, 1-8.
Colon-Ramos, D. A., Irusta, P. M., Gan, E. C., Olson, M. R., Song, J., Morimoto, R. I., Elliott, R. M., Lombard, M., Hollingsworth, R., Hardwick, J. M., Smith, G. K. & Kornbluth, S. (2003). Inhibition of translation and induction of apoptosis by bunyaviral nonstructural proteins bearing sequence similarity to Reaper. *Mol Biol Cell* **14**, 4162-4172.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Conn, J. E., Mitchell, S. E. & Cockburn, A. F. (1997). Mitochondrial DNA variation within and between two species of neotropical anopheline mosquitoes (Diptera: *Culicidae*). *J Hered* **88**, 98-107.
Conn, J. E., Mitchell, S. E. & Cockburn, A. F. (1998). Mitochondrial DNA analysis of the neotropical malaria vector *Anopheles nuneztovari*. *Genome* **41**, 313-327.
Conway, E. M., Pollefeyt, S., Cornelissen, J., DeBaere, I., Steiner-Mosonyi, M., Ong, K., Baens, M., Collen, D. & Schuh, A. C. (2000). Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions. *Blood* **95**, 1435-1442.
Cramblet, H. G., Stegmill, H. & Spencer, C. (1966). California encephalitis virus infections in children. *J Am Med Assoc* **198**, 108-&.
Crook, N. E., Clem, R. J. & Miller, L. K. (1993). An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol* **67**, 2168-2174.
Darsie Jr., R. F. & Ward, R. A. (2005). Identification and geographical distribution of the mosquitoes of North America, north of Mexico, 2nd edn. Gainesville, FL: University Press of Florida.
De Merida, A. M., Palmieri, M., Yurrita, M., Molina, A., Molina, E. & Black IV, W. C. (1999). Mitochondrial DNA variation among *Anopheles albimanus* populations. *Am J Trop Med Hyg* **61**, 230-9.
Deveraux, Q. L. & Reed, J. C. (1999). IAP family proteins: Suppressors of apoptosis. *Genes Dev* **13**, 239-252.
Dobie, D. K., Blair, C. D., Chandler, L. J., Rayms-Keller, A., McGaw, M. M., Wasieloski, L. P. & Beaty, B. J. (1997). Analysis of La Crosse virus S mRNA 5' termini in infected mosquito cells and *Aedes triseriatus* mosquitoes. *J Virol* **71**, 4395-9.
Dong, Y., Taylor, H. E. & Dimopoulos, G. (2006). AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system. *PLoS Biol* **4**, e229.
Dunn, E. F., Pritlove, D. C., Jin, H. & Elliott, R. M. (1995). Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins. *Virology* **211**, 133-143.
Dushay, M. S., Asling, B. & Hultmark, D. (1996). Origins of immunity: Relish, a compound rel-like gene in the antibacterial defense of *Drosophila*. *Proc Natl Acad Sci USA* **93**, 10343-10347.
El Said, L. H., Vorndam, V., Gentsch, J. R., Clewley, J. P., Calisher, C. H., Klimas, R. A., Thompson, W. H., Grayson, M., Trent, D. W. & Bishop, D. H. L. (1979). Comparison of La Crosse virus isolates obtained from different ecological niches and an analysis of the structural components of California encephalitis serogroup viruses and other bunyaviruses. *Am J Trop Med Hyg* **28**, 364-386.
Elliott, R. M. (1989). Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family *Bunyaviridae*. *Virology* **173**, 426-436.
Elliott, R. M. (1997). Emerging viruses: the *Bunyaviridae*. *Mol Med* **3**, 572-7.
Engstrom, Y., Kadalayil, L., Sun, S.-C., Samakovlis, C., Hultmark, D. & Faye, I. (1993). Kappa B-like motifs regulate the induction of immune genes in *Drosophila*. *J Mol Biol* **232**, 327-333.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-91.

Fazakerley, J. K. & Ross, A. M. (1989). Computer analysis suggests a role for signal sequences in processing polyproteins of enveloped RNA viruses and as a mechanism of viral fusion. *Virus Genes* **2**, 223-239.

Felsenstein, J. (1993). Phylogeny Inference Package, Version 3.5C. Seattle, WA: University of Washington.

Fine, P. E. M. & LeDuc, J. W. (1978). Towards a quantitative understanding of the epidemiology of keystone virus in the eastern United States. *Am J Trop Med Hyg* **27**, 322-338.

Fu, Y. X. & Li, W. H. (1993). Statistical tests of neutrality of mutations. *Genetics* **133**, 693-709.

Fuller, F., Bhown, A. S. & Bishop, D. H. L. (1983). Bunyavirus nucleoprotein, N, and a non-structural protein, NSs, are coded by overlapping reading frames in the S-RNA. *J Gen Virol* **64**, 1705-1714.

Gabitzsch, E. S., Blair, C. D. & Beaty, B. J. (2006). Effect of La Crosse virus infection on insemination rates in female *Aedes triseriatus* (Diptera: *Culicidae*). *J Med Entomol* **43**, 850-852.

Gauld, L. W., Hanson, R. P., Thompson, W. H. & Sinha, S. K. (1974). Observations on a natural cycle of La Crosse virus (California group) in southwestern Wisconsin. *Am J Trop Med Hyg* **23**, 983-992.

Geiser, D. L., Chavez, C. A., Flores-Munguia, R., Winzerling, J. J. & Pham, D. Q.-D. (2003). *Aedes aegypti* ferritin: A cytotoxic protector against iron and oxidative challenge? *Eur J Biochem* **270**, 3667-3674.

Gerhardt, R. R., Gottfried, K. L., Apperson, C. S., Davis, B. S., Erwin, P. C., Smith, A. B., Panella, N. A., Powell, E. E. & Nasci, R. S. (2001). First isolation of La Crosse virus from naturally infected *Aedes albopictus*. *Emerg Infect Dis* **7**, 807-11.

Gerrard, S. R., Li, L., Barrett, A. D. & Nichol, S. T. (2004). Ngari virus is a Bunyamwera virus reassortant that can be associated with large outbreaks of hemorrhagic fever in Africa. *J Virol* **78**, 8922-8926.

Ghosh, S., May, M. J. & Kopp, E. B. (1998). NF-kB and rel proteins: Evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* **16**, 225-260.

Gorrochotegui-Escalante, N., Gomez-Machorro, C., Lozano-Fuentes, S., Fernandez-Salas, L., De Lourdes Munoz, M., Farfan-Ale, J. A., Garcia-Rejon, J., Beaty, B. J. & Black IV, W. C. (2002). Breeding structure of *Aedes aegypti* populations in Mexico varies by region. *Am J Trop Med Hyg* **66**, 213-222.

Gorrochotegui-Escalante, N., Lozano-Fuentes, S., Bennett, K. E., Molina-Cruz, A., Beaty, B. J. & Black IV, W. C. (2005). Association mapping of segregating sites in the early trypsin gene and susceptibility to dengue-2 virus in the mosquito *Aedes aegypti*. *Insesct Biochem Mol Biol* **35**, 771-788.

Gorrochotegui-Escalante, N., Munoz, M. L., Fernandez-Salas, I., Beaty, B. J. & Black IV, W. C. (2000). Genetic isolation by distance among *Aedes aegypti* populations along the northeastern coast of Mexico. *Am J Trop Med Hyg* **62**, 200-209.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Graham, D. H., Holmes, J. L., Beaty, B. J. & Black IV, W. C. (2003). Quantitative trait loci conditioning transovarial transmission of La Crosse virus in the eastern treehole mosquito, *Ochlerotatus triseriatus*. *Insect Mol Biol* **12**, 307-18.

Graham, D. H., Holmes, J. L., Higgs, S., Beaty, B. J. & Black IV, W. C. (1999). Selection of refractory and permissive strains of *Aedes triseriatus* (Diptera: *Culicidae*) for transovarial transmission of La Crosse virus. *J Med Entomol* **36**, 671-8.

Graveley, B. R., Kaur, A., Gunning, D., Zipursky, S. L., Rowen, L. & Clemens, J. C. (2004). The organization and evolution of the dipteran and hymenopteran down syndrome cell adhesion molecule (Dscam) genes. *RNA* **10**, 1499-1506.

Grimstad, P. R., Garry, C. E. & DeFoliart, G. R. (1974). *Aedes hendersoni* and *Aedes triseriatus* (Diptera: *Culicidae*) in Wisconsin: Characterization of larvae, larval hybrids, and comparison of adult and hybrid mesoscutal patterns. *Ann Entomol Soc Am* **67**, 795-804.

Grimstad, P. R., Kobayashi, J. F., Zhang, M. & Craig, G. B. (1989). Recently introduced *Aedes albopictus* in the United-States: Potential vector of La Crosse virus (*Bunyaviridae*, California serogroup). *J Am Mosq Control Assoc* **5**, 422-427.

Gubler, D. J. (2002). The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* **33**, 330-42.

Gubler, D. J. (2006). Dengue/dengue haemorrhagic fever: history and current status. *Novartis Found Symp* **277**, 3-16; discussion 16-22, 71-3, 251-3.

Hacker, J. K., Volkman, L. E. & Hardy, J. L. (1995). Requirement for the G1 protein of California encephalitis virus in infection *in vitro* and *in vivo*. *Virology* **206**, 945-953.

Haramis, L. D. & Foster, W. A. (1990). Gonotrophic cycle duration, population age structure, and onset of sugar feeding and insemination of *Aedes triseriatus* (Diptera: *Culicidae*). *J Med Entomol* **27**, 421-8.

Hay, B. A., Wassarman, D. A. & Rubin, G. M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**, 1253-1262.

Hemingway, J., Hawkes, N., Prapanthadara, L., Jayawardenal, K. G. & Ranson, H. (1998). The role of gene splicing, gene amplification, and regulation in mosquito insecticide resistance. *Philos Trans R Soc Lond B Biol Sci* **353**, 1695-9.

Hewlett, M. J., Pettersson, R. F. & Baltimore, D. (1977). Circular forms of uukuniemi virion RNA: Electron-microscopic study. *J Virol* **21**, 1085-1093.

Holcik, M. & Korneluk, R. G. (2000). Functional characterization of the X-linked inhibitor of apoptosis (XIAP) internal ribosome entry site element: Role of La autoantigen in XIAP translation. *Mol Cell Biol* **20**, 4648-4657.

Holcik, M., Lefebvre, C., Yeh, C. L., Chow, T. & Korneluk, R. G. (1999). A new internal ribosome entry site motif potentiates XIAP-mediated cytoprotection. *Nat Cell Biol* **1**, 190-192.

Holland, J. J., De La Torre, J. C. & Steinhauer, D. A. (1992). RNA virus populations as quasispecies. *Curr Top Microbiol Immunol* **176**, 1-21.

Hozak, R. R., Manji, G. A. & Friesen, P. D. (2000). The BIR motifs mediate dominant interference and oligomerization of inhibitor of apoptosis Op-IAP. *Mol Cell Biol* **20**, 1877-1885.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Huang, H. K., Joazeiro, C. A. P., Bonfoco, E., Kamada, S., Leverson, J. D. & Hunter, T. (2000). The inhibitor of apoptosis, cIAP2, functions as a ubiquitin-protein ligase and promotes in vitro monoubiquitination of caspases 3 and 7. *J Biol Chem* **275**, 26661-26664.

Hughes, M. T., Gonzalez, J. A., Reagan, K. L., Blair, C. D. & Beaty, B. J. (2006). Comparative potential of *Aedes triseriatus*, *Aedes albopictus*, and *Aedes aegypti* (Diptera: *Culicidae*) to transovarially transmit La Crosse virus. *J Med Entomol* **43**, 757-61.

Igaki, T., Yamamoto-Goto, Y., Tokushige, N., Kanda, H. & Miura, M. (2002). Down-regulation of DIAP1 triggers a novel *Drosophila* cell death pathway mediated by Dark and DRONC. *J Biol Chem* **277**, 23103-23106.

Jin, H. & Elliott, R. M. (1991). Expression of functional Bunyamwera virus-L protein by recombinant vaccinia viruses. *J Virol* **65**, 4182-4189.

Jin, H. & Elliott, R. M. (1993). Characterization of Bunyamwera virus-S RNA that is transcribed and replicated by the L-protein expressed from recombinant vaccinia virus. *J Virol* **67**, 1396-1404.

Jones, G., Jones, D., Zhou, L., Steller, H. & Chu, Y. X. (2000). Deterin, a new inhibitor of apoptosis from *Drosophila melanogaster*. *J Biol Chem* **275**, 22157-22165.

Jones, T. F., Craig, A. S., Nasci, R. S., Patterson, L. E. R., Erwin, P. C., Gerhardt, R. R., Ussery, X. T. & Schaffner, W. (1999). Newly recognized focus of La Crosse encephalitis in Tennessee. *Clin Infect Dis* **28**, 93-97.

Kamrud, K. I., Olson, K. E., Higgs, S., Carlson, J. O. & Beaty, B. J. (1998). Use of the Sindbis replicon system for expression of La Crosse virus envelope proteins in mosquito cells. *Arch Virol* **143**, 1365-1377.

Kappus, K. D., Monath, T. P., Kaminski, R. M. & Calisher, C. H. (1983). Reported encephalitis associated with California serogroup virus infections in United States, 1963 to 1981. In *California Serogroup Viruses*, pp. 31-41. Edited by C. H. Calisher & W. H. Thompson. New York: Liss.

Kappus, K. D. & Venard, C. E. (1967). Effects of photoperiod and temperature on induction of diapause in *Aedes triseriatus* (Say). *J Insect Physiol* **13**, 1007-&.

Keller, E. B. & Noon, W. A. (1985). Intron splicing: A conserved internal signal in introns of *Drosophila* pre-messenger RNAs. *Nucleic Acids Res* **13**, 4971-4981.

Kempf, B. J., Blair, C. D. & Beaty, B. J. (2006). Quantitative analysis of La Crosse virus transcription and replication in cell cultures and mosquitoes. *Am J Trop Med Hyg* **74**, 224-232.

Kimura, M. (1983). The Neutral Theory of Molecular Evolution. Cambridge: Cambridge University Press.

Kitron, U., Swanson, J., Crandell, M., Sullivan, P. J., Anderson, J., Garro, R., Haramis, L. D. & Grimstad, P. R. (1998). Introduction of *Aedes albopictus* into a La Crosse virus: Enzootic site in Illinois. *Emerg Infect Dis* **4**, 627-630.

Klimas, R. A., Thompson, W. H., Calisher, C. H., Clark, G. G., Grimstad, P. R. & Bishop, D. H. L. (1981). Genotypic varieties of La Crosse virus isolated from different geographic regions of the continental United States and evidence for a naturally occurring intertypic recombinant La Crosse virus. *Am J Epidemiol* **114**, 112-131.

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Kokoza, V. A. & Raikhel, A. S. (1997). Ovarian- and somatic-specific transcripts of the mosquito clathrin heavy chain gene generated by alternative 5'-exon splicing and polyadenylation. *J Biol Chem* **272**, 1164-1170.

Komar, A. A. & Hatzoglou, M. (2005). Internal ribosome entry sites in cellular mRNAs: Mystery of their existence. *J Biol Chem* **280**, 23425-23428.

Kormelink, R., Dehaan, P., Peters, D. & Goldbach, R. (1992). Viral-RNA synthesis in tomato spotted wilt virus-infected *Nicotiana rustica* plants. *J Gen Virol* **73**, 687-693.

Kormelink, R., Storms, M., Van Lent, J., Peters, D. & Goldbach, R. (1994). Expression and subcellular location of the NSm protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. *Virology* **200**, 56-65.

Kornbluth, S. & White, K. (2005). Apoptosis in *Drosophila*: neither fish nor fowl (nor man, nor worm). *J Cell Sci* **118**, 1779-1787.

Kos, M., Denger, S., Reid, G. & Gannon, F. (2002). Upstream open reading frames regulate the translation of the multiple mRNA variants of the estrogen receptor alpha. *J Biol Chem* **277**, 37131-37138.

Ksiazek, T. G. & Yuill, T. M. (1977). Viremia and antibody response to La Crosse virus in sentinel gray squirrels (*Sciuris carolinensis*) and chipmunks (*Tamias striatus*). *Am J Trop Med Hyg* **26**, 815-21.

Lamb, R. A. & Kolakofsky, D. (2001). *Paramyxoviridae*: The viruses and their replication. In *Fields Virology*, 4th edn, pp. 1305-1340. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott, Williams, and Wilkins.

Lappin, D. F., Nakitare, G. W., Palfreyman, J. W. & Elliott, R. M. (1994). Localization of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not with NSm. *J Gen Virol* **75 (Pt 12)**, 3441-51.

Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J. & Hoffmann, J. A. (1995). A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defense. *Proc Natl Acad Sci USA* **92**, 9465-9469.

Li, Q., Li, H., Blitvich, B. J. & Zhang, J. (2007). The *Aedes albopictus* inhibitor of apoptosis 1 gene protects vertebrate cells from bluetongue virus-induced apoptosis. *Insect Mol Biol* **16**, 93-105.

Lim, L. P. & Burge, C. B. (2001). A computational analysis of sequence features involved in recognition of short introns. *Proc Natl Acad Sci USA* **98**, 11193-11198.

Lisitza, M. A., Defoliart, G. R., Yuill, T. M. & Karandinos, M. G. (1977). Prevalence rates of La Crosse virus (California encephalitis group) in larvae from overwintered eggs of *Aedes triseriatus*. *Mosq News* **37**, 745-750.

Liu, Z. H., Sun, C. H., Olejniczak, E. T., Meadows, R. P., Betz, S. F., Oost, T., Herrmann, J., Wu, J. C. & Fesik, S. W. (2000). Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* **408**, 1004-1008.

Lopez, N., Muller, R., Prehaud, C. & Bouloy, M. (1995). The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules. *J Virol* **69**, 3972-3979.

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Ludwig, G. V., Christensen, B. M., Yuill, T. M. & Schultz, K. T. (1989). Enzyme processing of La Crosse virus glycoprotein G1: A bunyavirus-vector infection model. *Virology* **171**, 108-13.
Ludwig, G. V., Israel, B. A., Christensen, B. M., Yuill, T. M. & Schultz, K. T. (1991). Role of La Crosse virus glycoproteins in attachment of virus to host cells. *Virology* **181**, 564-71.
Lukashev, A. N. (2005). Evidence for recombination in Crimean-Congo hemorrhagic fever virus. *J Gen Virol* **86**, 2333-2338.
Lynd, A., Ranson, H., McCall, P. J., Randle, N. P., Black, W. C. t., Walker, E. D. & Donnelly, M. J. (2005). A simplified high-throughput method for pyrethroid knock-down resistance (kdr) detection in *Anopheles gambiae*. *Malar J* **4**, 16.
Mahmood, F., Crans, W. J. & Savur, N. S. (1997). Larval competition in *Aedes triseriatus* (Diptera: *Culicidae*): Effects of density on size, growth, sex ratio, and survival. *J Vector Ecol* **22**, 90-4.
Mantel, N. (1967). The detection of disease clustering and a generalized regression approach. *Cancer Res* **27**, 209-20.
Markham, N. R. & Zuker, M. (2005). DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res* **33**, W577-581.
Matthews, T. C. & Craig Jr., G. B. (1980). Genetic heterozygosity in natural populations of the tree-hole mosquito *Aedes triseriatus*. *Ann Entomol Soc Am* **73**, 739-43.
Matthews, T. C. & Munstermann, L. E. (1983). Genetic diversity and differentiation in northern populations of the tree-hole mosquito *Aedes hendersoni* (Diptera: *Culicidae*). *Ann Entomol Soc Am* **76**, 1005-1010.
McGaw, M. M., Chandler, L. J., Wasieloski, L. P., Blair, C. D. & Beaty, B. J. (1998). Effect of La Crosse virus infection on overwintering of *Aedes triseriatus*. *Am J Trop Med Hyg* **58**, 168-175.
McJunkin, J. E., Khan, R. R. & Tsai, T. F. (1998). California-La Crosse encephalitis. *Infect Dis Clin North Am* **12**, 83-93.
Meister, S., Kanzok, S. M., Zheng, X.-l., Luna, C., Li, T.-R., Hoa, N. T., Clayton, J. R., White, K. P., Kafatos, F. C., Christophides, G. K. & Zheng, L. (2005). Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* **102**, 11420-11425.
Meng, X., Khanuja, B. S. & Ip, Y. T. (1999). Toll receptor-mediated *Drosophila* immune response requires Dif, an NF-kappa B factor. *Genes Dev* **13**, 792-797.
Michel, T., Reichhart, J.-M., Hoffmann, J. A. & Royet, J. (2001). *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. *Nature* **414**, 756-759.
Miller, B. R., DeFoliart, G. R. & Yuill, T. M. (1977). Vertical transmission of La Crosse virus (California encephalitis group): transovarial and filial infection rates in *Aedes triseriatus* (Diptera: *Culicidae*). *J Med Entomol* **14**, 437-40.
Miller, B. R., DeFoliart, G. R. & Yuill, T. M. (1979). *Aedes triseriatus* and La Crosse virus: lack of infection in eggs of the first ovarian cycle following oral infection of females. *Am J Trop Med Hyg* **28**, 897-901.
Miller, L. K. (1999). An exegesis of IAPs: Salvation and surprises from BIR motifs. *Trends Cell Biol* **9**, 323-328.

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Mironov, A. A., Fickett, J. W. & Gelfand, M. S. (1999). Frequent alternative splicing of human genes. *Genome Res* **9**, 1288-1293.

Mitchell, S. E., Narang, S. K., Cockburn, A. F., Seawright, J. A. & Goldenthal, M. (1992). Mitochondrial and ribosomal DNA variation among members of the *Anopheles quadrimaculatus* (Diptera: *Culicidae*) species complex. *Genome* **35**, 939-50.

Moore, C. G. (1999). *Aedes albopictus* in the United States: Current status and prospects for further spread. *J Am Mosq Control Assoc* **15**, 221-227.

Muller, R., Poch, O., Delarue, M., Bishop, D. H. L. & Bouloy, M. (1994). Rift Valley fever virus L-segment: Correction of the sequence and possible functional role of newly identified regions conserved in RNA-dependent polymerases. *J Gen Virol* **75**, 1345-1352.

Muro, I., Hay, B. A. & Clem, R. J. (2002). The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J Biol Chem* **277**, 49644-49650.

Nakitare, G. W. & Elliott, R. M. (1993). Expression of the Bunyamwera virus M genome segment and intracellular localization of NSm. *Virology* **195**, 511-520.

Nasci, R. S. (1982). Differences in host choice between the sibling species of treehole mosquitoes *Aedes triseriatus* and *Aedes hendersoni*. *Am J Trop Med Hyg* **31**, 411-415.

Nei, M. (1987). Molecular evolutionary genetics. New York: Columbia University Press.

Nevins, T. A., Harder, Z. M., Korneluk, R. G. & Holcik, M. (2003). Distinct regulation of internal ribosome entry site-mediated translation following cellular stress is mediated by apoptotic fragments of eIF4G translation initiation factor family members eIF4GI and p97. *J Biol Chem* **278**, 3572-3579.

Nichol, S. T. (2001). Bunyaviruses. In *Fields Virology*, 4th edn, pp. 1603-1633. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott, Williams, and Wilkins.

Nunes, M. R. T., Travassos da Rosa, A. P. A., Weaver, S. C., Tesh, R. B. & Vasconcelos, P. F. C. (2005). Molecular epidemiology of group C viruses (*Bunyaviridae*, *Orthobunyavirus*) isolated in the Americas. *J Virol* **79**, 10561-10570.

Obijeski, J. F., Bishop, D. H. L., Murphy, F. A. & Palmer, E. L. (1976a). Structural proteins of La Crosse virus. *J Virol* **19**, 985-997.

Obijeski, J. F., Bishop, D. H. L., Palmer, E. L. & Murphy, F. A. (1976b). Segmented genome and nucleocapsid of La Crosse virus. *J Virol* **20**, 664-675.

Ohta, T. (1982a). Linkage disequilibrium due to random genetic drift in finite subdivided populations. *Proc Natl Acad Sci USA* **79**, 1940-1944.

Ohta, T. (1982b). Linkage disequilibrium with the island model. *Genetics* **101**, 139-155.

Olson, M. R., Holley, C. L., Yoo, S. J., Huh, J. R., Hay, B. A. & Kornbluth, S. (2003). Reaper is regulated by UP-mediated ubiquitination. *J Biol Chem* **278**, 4028-4034.

Palaga, T. & Osborne, B. (2002). The 3D's of apoptosis: death, degradation and DIAPs. *Nat Cell Biol* **4**, E149-E151.

Pantuwatana, S., Thompson, W. H., Watts, D. M., Yuill, T. M. & Hanson, R. P. (1974). Isolation of La Crosse virus from field collected *Aedes triseriatus* larvae. *Am J Trop Med Hyg* **23**, 246-250.

Pantuwatana, S., Watts, D. M., Thompson, W. H. & Hanson, R. P. (1972). Experimental infection of chipmunks and squirrels with La Crosse and trivittatus viruses and

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biological transmission of La Crosse virus by *Aedes triseriatus*. *Am J Trop Med Hyg* **21**, 476-&.

Patterson, J. L., Holloway, B. & Kolakofsky, D. (1984). La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *J Virol* **52**, 215-22.

Paulson, S. L. & Grimstad, P. R. (1989). Replication and dissemination of La Crosse virus in the competent vector *Aedes triseriatus* and the incompetent vector *Aedes hendersoni* and evidence for transovarial transmission by *Aedes hendersoni* (Diptera: *Culicidae*). *J Med Entomol* **26**, 602-9.

Paulson, S. L., Grimstad, P. R. & Craig, G. B., Jr. (1989). Midgut and salivary gland barriers to La Crosse virus dissemination in mosquitoes of the *Aedes triseriatus* group. *Med Vet Entomol* **3**, 113-23.

Pekosz, A. & Gonzalez-Scarano, F. (1996). The extracellular domain of La Crosse virus G1 forms oligomers and undergoes pH-dependent conformational changes. *Virology* **225**, 243-247.

Pekosz, A., Griot, C., Stillmock, K., Nathanson, N. & Gonzalez-Scarano, F. (1995). Protection from La Crosse virus encephalitis with recombinant glycoproteins: role of neutralizing anti-G1 antibodies. *J Virol* **69**, 3475-3481.

Plassmeyer, M. L., Soldan, S. S., Stachelek, K. M., Martin-Garcia, J. & Gonzalez-Scarano, F. (2005). California serogroup Gc (G1) glycoprotein is the principal determinant of pH-dependent cell fusion and entry. *Virology* **338**.

Pringle, C. R., Lees, J. F., Clark, W. & Elliott, R. M. (1984). Genome subunit reassortment among bunyaviruses analyzed by dot hybridization using molecularly cloned complementary-DNA probes. *Virology* **135**, 244-256.

Quinn, L. M., Dorstyn, L., Mills, K., Colussi, P. A., Chen, P., Coombe, M., Abrams, J., Kumar, S. & Richardson, H. (2000). An essential role for the caspase Dronc in developmentally programmed cell death in *Drosophila*. *J Biol Chem* **275**, 40416-40424.

Reed, J. C. (2000). Mechanisms of apoptosis. *Am J Pathol* **157**, 1415-1430.

Roberts, A., Rossier, C., Kolakofsky, D., Nathanson, N. & Gonzalez-Scarano, F. (1995). Completion of the La crosse virus genome sequence and genetic comparisons of the L proteins of the *Bunyaviridae*. *Virology* **206**, 742-745.

Rosenberg, M. S. (2000). Passage: Pattern Analysis, Spatial Statistics, and Geographic Exegesis, 1.0 edn. Tempe, AZ: Department of Biology, Arizona State University.

Roy, N., Deveraux, Q. L., Takahashi, R., Salvesen, G. S. & Reed, J. C. (1997). The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* **16**, 6914-6925.

Rozas, J., Sanchez-DelBarrio, J. C., Messeguer, X. & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496-2497.

Rozhon, E. J., Gensemer, P., Shope, R. E. & Bishop, D. H. L. (1981). Attenuation of virulence of a bunyavirus involving an L RNA defect and isolation of LAC-SSH-LAC and LAC-SSH-SSH reassortants. *Virology* **111**, 125-138.

Rust, R. S., Thompson, W. H., Matthews, C. G., Beaty, B. J. & Chun, R. W. (1999). La Crosse and other forms of California encephalitis. *J Child Neurol* **14**, 1-14.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Rutledge, L. C., Ward, R. A. & Gould, D. J. (1964). Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. *Mosquito News* **24**, 407-419.

Ruusala, A., Persson, R., Schmaljohn, C. S. & Pettersson, R. F. (1992). Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. *Virology* **186**, 53-64.

Sall, A. A., Zanotto, P. M. d. A., Sene, O. K., Zeller, H. G., Digoutte, J. P., Thiongane, Y. & Bouloy, M. (1999). Genetic reassortment of Rift Valley fever virus in nature. *J Virol* **73**, 8196-8200.

Salvesen, G. S. & Duckett, C. S. (2002). IAP proteins: Blocking the road to death's door. *Nat Rev Mol Cell Biol* **3**, 401-410.

Saul, S. H., Sinsko, M. J., Grimstad, P. R. & Craig, G. B. (1978). Population genetics of mosquito *Aedes triseriatus*: Genetic-ecological correlation at an esterase locus. *Am Nat* **112**, 333-339.

Schmaljohn, C. S. & Hooper, J. W. (2001). *Bunyaviridae*: The viruses and their replication. In *Fields Virology*, 4th edn, pp. 1581-1602. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott, Williams, and Wilkins.

Scholl, P. J. & DeFoliart, G. R. (1977). *Aedes triseriatus* and *Aedes hendersoni* (Diptera: *Culicidae*): Vertical and temporal distribution as measured by oviposition. *Environ Entomol* **6**, 355-358.

Scholl, P. J., DeFoliart, G. R. & Nemenyi, P. B. (1979). Vertical distribution of biting activity by *Aedes triseriatus* (Diptera: *Culicidae*). *Ann Entomol Soc Am* **72**, 537-539.

Schopen, S., Labuda, M. & Beaty, B. (1991). Vertical and venereal transmission of California group viruses by *Aedes triseriatus* and *Culiseta inornata* mosquitoes. *Acta Virol* **35**, 373-82.

Schwerk, C. & Schulze-Osthoff, K. (2005). Regulation of apoptosis by alternative pre-mRNA splicing. *Mol Cell* **19**, 1-13.

Shalev, A., Blair, P. J., Hoffmann, S. C., Hirshberg, B., Peculis, B. A. & Harlan, D. M. (2002). A proinsulin gene splice variant with increased translation efficiency is expressed in human pancreatic islets. *Endocrinology* **143**, 2541-2547.

Shi, X., Kohl, A., Leonard, V. H. J., Li, P., McLees, A. & Elliott, R. M. (2006). Requirement of the N-terminal region of orthobunyavirus nonstructural protein NSm for virus assembly and morphogenesis. *J Virol* **80**, 8089-8099.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* **9**, 459-70.

Shi, Y. (2004). Caspase activation, inhibition, and reactivation: A mechanistic view. *Protein Sci* **13**, 1979-87.

Shin, S. W., Kokoza, V., Ahmed, A. & Raikhel, A. S. (2002). Characterization of three alternatively spliced isoforms of the Rel/NF-kappa B transcription factor Relish from the mosquito *Aedes aegypti*. *Proc Natl Acad Sci USA* **99**, 9978-9983.

Shope, R. E. & Causey, O. R. (1962). Further studies on the serological relationships of group C arthropod-borne viruses and the application of these relationships to rapid identification of types. *Am J Trop Med Hyg* **11**, 886-887.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Shroyer, D. A. & Craig, G. B. (1980). Egg hatchability and diapause in *Aedes triseriatus* (Diptera: *Culicidae*): Temperature-induced and photoperiod-induced latencies. *Ann Entomol Soc Am* **73**, 39-43.

Shroyer, D. A. & Craig, G. B. (1983). Egg diapause in *Aedes triseriatus* (Diptera: *Culicidae*): Geographic variation in photoperiodic response and factors influencing diapause termination. *J Med Entomol* **20**, 601-607.

Sibold, C., Meisel, H., Kruger, D. H., Labuda, M., Lysy, J., Kozuch, O., Pejcoch, M., Vaheri, A. & Plyusnin, A. (1999). Recombination in Tula hantavirus evolution: Analysis of genetic lineages from slovakia. *J Virol* **73**, 667-675.

Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D. & Maniatis, T. (2000). A Drosophila I-kappa B kinase complex required for Relish cleavage and antibacterial immunity. *Genes Dev* **14**, 2461-2471.

Sims, S. R. (1982). Larval diapause in the Eastern tree-hole mosquito, *Aedes triseriatus*: Latitudinal variation in induction and intensity. *Ann Entomol Soc Am* **75**, 195-200.

Sinsko, M. J. & Craig Jr., G. B. (1979). Dynamics of an isolated population of *Aedes triseriatus* (Diptera: *Culicidae*) 1. Population Size. *J Med Entomol* **15**, 89-98.

Sinsko, M. J. & Grimstad, P. R. (1977). Habitat separation by differential vertical oviposition of two treehole *Aedes* (Diptera: *Culicidae*) in Indiana. *Environ Entomol* **6**, 485-487.

Slatkin, M. (1993). Isolation by distance in equilibrium and nonequilibrium populations. *Evolution* **47**, 264-279.

Smith, J. F. & Pifat, D. Y. (1982). Morphogenesis of sandfly fever viruses (*Bunyaviridae* family). *Virology* **121**, 61-81.

Smithburn, K. C., Haddow, A. J. & Mahaffy, A. F. (1946). A neurotropic virus isolated from *Aedes* mosquitoes caught in the Semliki Forest. *Am J Trop Med Hyg* **26**, 189-208.

Soellick, T.-R., Uhrig, J. F., Bucher, G. L., Kellmann, J.-W. & Schreier, P. H. (2000). The movement protein NSm of tomato spotted wilt tospovirus (TSWV): RNA binding, interaction with the TSWV N protein, and identification of interacting plant proteins. *Proc Natl Acad Sci USA* **97**, 2373-2378.

Soldan, S. S., Plassmeyer, M. L., Matukonis, M. K. & Gonzalez-Scarano, F. (2005). La Crosse virus nonstructural protein NSs counteracts the effects of short interfering RNA. *J Virol* **79**, 234-244.

Sprenger, D. & Wuithiranyagool, T. (1986). The discovery and distribution of *Aedes albopictus* in Harris County, Texas. *J Am Mosq Control Assoc* **2**, 217-219.

Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y. S., Zhang, Z. Y., Toiber, D., Thanaraj, T. A. & Soreq, H. (2005). Function of alternative splicing. *Gene* **344**, 1-20.

Storms, M. M. H., Kormelink, R., Peters, D., Van Lent, J. W. M. & Goldbach, R. W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. *Virology* **214**, 485-493.

Sullivan, M. F., Gould, D. J. & Maneecha.S (1971). Observations on host range and feeding preferences of *Aedes albopictus* (Skuse). *J Med Entomol* **8**, 713-&.

Sundin, D. R. & Beaty, B. J. (1988). Interference to oral superinfection of *Aedes triseriatus* infected with La Crosse virus. *Am J Trop Med Hyg* **38**, 428-32.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Sundin, D. R., Beaty, B. J., Nathanson, N. & Gonzalez-Scarano, F. (1987). A G1 glycoprotein epitope of La Crosse virus: a determinant of infection of Aedes triseriatus. *Science* **235**, 591-3.

Talmon, Y., Prasad, B. V. V., Clerx, J. P. M., Wang, G. J., Chiu, W. & Hewlett, M. J. (1987). Electron microscopy of vitrified-hydrated La Crosse virus. *J Virol* **61**, 2319-2321.

Tesh, R. B. & Beaty, B. J. (1983). Localization of California serogroup viruses in mosquitoes. *Prog Clin Biol Res* **123**, 67-75.

Tesh, R. B. & Gubler, D. J. (1975). Laboratory studies of transovarial transmission of La Crosse and other arboviruses by *Aedes albopictus* and *Culex fatigans*. *Am J Trop Med Hyg* **24**, 876-880.

Tesh, R. B. & Shroyer, D. A. (1980). The mechanism of arbovirus transovarial transmission in mosquitoes: San Angelo virus in *Aedes albopictus*. *Am J Trop Med Hyg* **29**, 1394-1404.

Thompson, W. H., Anslow, R. O., Hanson, R. P. & DeFoliart, G. R. (1972). La Crosse virus isolations from mosquitoes in Wisconsin, 1964-68. *Am J Trop Med Hyg* **21**, 90-6.

Thompson, W. H. & Beaty, B. J. (1977). Venereal transmission of La Crosse (California encephalitis) arbovirus in *Aedes triseriatus* mosquitoes. *Science* **196**, 530-1.

Thompson, W. H. & Beaty, B. J. (1978). Venereal transmission of La Crosse virus from male to female *Aedes triseriatus*. *Am J Trop Med Hyg* **27**, 187-96.

Thompson, W. H., Kalfayan, B. & Anslow, R. O. (1965). Isolation of California encephalitis group virus from a fatal human illness. *Am J Epidemiol* **81**, 245-253.

Truman, J. W. & Craig, G. B. (1968). Hybridization between *Aedes hendersoni* and *Aedes triseriatus*. *Ann Entomol Soc Am* **61**, 1020-&.

Turell, M. J., Hardy, J. L. & Reeves, W. C. (1982). Stabilized infection of California encephalitis virus in *Aedes dorsalis*, and its implications for viral maintenance in nature. *Am J Trop Med Hyg* **31**, 1252-1259.

Turell, M. J., Saluzzo, J.-F., Tammariello, R. F. & Smith, J. F. (1990). Generation and transmission of Rift Valley fever viral reassortants by the mosquito *Culex pipiens*. *J Gen Virol* **71**, 2307-2312.

Twomey, C. & McCarthy, J. V. (2005). Pathways of apoptosis and importance in development. *J Cell Mol Med* **9**, 345-359.

Utz, J. T., Apperson, C. S., Maccormack, J. N., Salyers, M., Dietz, E. J. & McPherson, J. T. (2003). Economic and social impacts of La Crosse encephalitis in western North Carolina. *Am J Trop Med Hyg* **69**, 509-518.

Vaux, D. L. & Silke, J. (2003). Mammalian mitochondrial IAP binding proteins. *Biochem Biophys Res Commun* **304**, 499-504.

Verhagen, A. M., Kratina, T. K., Hawkins, C. J., Silke, J., Ekert, P. G. & Vaux, D. L. (2007). Identification of mammalian mitochondrial proteins that interact with IAPs via N-terminal IAP binding motifs. *Cell Death Differ* **14**, 348-57.

Vernooy, S. Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M. R. & Hay, B. A. (2002). *Drosophila* Bruce can potently suppress Rpr- and Grim-dependent but not hid-dependent cell death. *Curr Biol* **12**, 1164-1168.

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Vernooy, S. Y., Copeland, J., Ghaboosi, N., Griffin, E. E., Yoo, S. J. & Hay, B. A. (2000). Cell death regulation in *Drosophila*: Conservation of mechanism and unique insights. *J Cell Biol* **150**, 69F-76.

Vucic, D., Kaiser, W. J., Harvey, A. J. & Miller, L. K. (1997). Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc Natl Acad Sci USA* **94**, 10183-10188.

Vucic, D., Kaiser, W. J. & Miller, L. K. (1998). Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. *Mol Cell Biol* **18**, 3300-3309.

Wang, G. R., Guo, X. X. & Floros, J. (2005). Differences in the translation efficiency and mRNA stability mediated by 5'-UTR splice variants of human SP-A1 and SP-A2 genes. *Am J Physiol Lung Cell Mol Physiol* **289**, L497-L508.

Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. J. & Hay, B. A. (1999). The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**, 453-463.

Warnakulasuriyarachchi, D., Cerquozzi, S., Cheung, H. H. & Holcik, M. (2004). Translational induction of the inhibitor of apoptosis protein HIAP2 during endoplasmic reticulum stress attenuates cell death and is mediated via an inducible internal ribosome entry site element. *J Biol Chem* **279**, 17148-17157.

Wasmoen, T. L., Kakach, L. T. & Collett, M. S. (1988). Rift Valley fever virus M segment: Cellular localization of M segment-encoded proteins. *Virology* **166**, 275-80.

Watson, F. L., Puttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., Rebel, V. I. & Schmucker, D. (2005). Extensive diversity of Ig-superfamily proteins in the immune system of insects. *Science* **309**, 1874-1878.

Watts, D. M., Grimstad, P. R., DeFoliart, G. R. & Yuill, T. M. (1975a). *Aedes hendersoni*: Failure of laboratory-infected mosquitoes to transmit La Crosse virus (California encephalitis Group). *J Med Entomol* **12**, 451-453.

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

Watts, D. M., Pantuwatana, S., Yuill, T. M., DeFoliart, G. R., Thompson, W. H. & Hanson, R. P. (1975b). Transovarial transmission of La Crosse virus in *Aedes triseriatus*. *Ann N Y Acad Sci* **266**, 135-143.

Watts, D. M., Thompson, W. H., Yuill, T. M., DeFoliart, G. R. & Hanson, R. P. (1974). Overwintering of La Crosse virus in *Aedes triseriatus*. *Am J Trop Med Hyg* **23**, 694-700.

Watts, D. M., Wright, R. E., Hanson, R. P., DeFoliart, G. R. & Morris, C. D. (1972). Transmission of La Crosse virus (California encephalitis group) by the mosquito *Aedes triseriatus*. *J Med Entomol* **9**, 125-&.

Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. & Elliott, R. M. (2002). Bunyamwera bunyavirus nonstructural protein NSs counteracts the induction of Alpha/Beta interferon. *J Virol* **76**, 7949-7955.

Weir, B. S. & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370.

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Winstanley, P., Ward, S., Snow, R. & Breckenridge, A. (2004). Therapy of *falciparum* Malaria in sub-Saharan Africa: From molecule to policy. *Clin Microbiol Rev* **17**, 612-637.

Woodring, J., Chandler, L. J., Oray, C. T., McGaw, M. M., Blair, C. D. & Beaty, B. J. (1998). Short report: Diapause, transovarial transmission, and filial infection rates in geographic strains of La Crosse virus-infected *Aedes triseriatus*. *Am J Trop Med Hyg* **58**, 587-8.

Wright, J. E., Kappus, K. D. & Venard, C. E. (1966). Swarming and mating behavior in laboratory colonies of *Aedes triseriatus* (Diptera: *Culicidae*). *Ann Entomol Soc Am* **59**, 1110-&.

Wright, J. E. & Venard, C. E. (1967). Influence of a blood meal on copulation in *Aedes triseriatus* (Diptera: *Culicidae*). *Ann Entomol Soc Am* **60**, 861-&.

Wright, S. (1965). The interpretation of population structure by F-Statistics with special regard to systems of mating. *Evolution* **19**, 395-420.

Wu, G., Chai, J. J., Suber, T. L., Wu, J. W., Du, C. Y., Wang, X. D. & Shi, Y. G. (2000). Structural basis of IAP recognition by Smac/DIABLO. *Nature* **408**, 1008-1012.

Wu, J. Y., Tang, H. & Havlioglu, N. (2003). Alternative pre-mRNA splicing and regulation of programmed cell death. *Prog Mol Subcell Biol* **31**, 153-85.

Yanase, T., Kato, T., Yamakawa, M., Takayoshi, K., Nakamura, K., Kokuba, T. & Tsuda, T. (2006). Genetic characterization of Batai virus indicates a genomic reassortment between orthobunyaviruses in nature. *Arch Virol*.

Yang, Y., Fang, S. Y., Jensen, J. P., Weissman, A. M. & Ashwell, J. D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* **288**, 874-877.

Zar, J. H. (1999). Data Transformations. In *Biostatistical Analysis*, pp. 273-281. Edited by S. L. Snavely & T. Ryu. New Jersey: Prentice-Hall.

Zhou, L., Jiang, G. H., Chan, G., Santos, C. P., Severson, D. W. & Xiao, L. (2005). Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes. *EMBO Rep* **6**, 769-774.

Zimmermann, K. C., Bonzon, C. & Green, D. R. (2001). The machinery of programmed cell death. *Pharmacol Ther* **92**, 57-70.

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