EFFECT OF LA CROSSE VIRUS INFECTION ON OVERWINTERING OF AEDES TRISERIATUS

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Abstract. The effect of La Crosse (LAC) virus infection on Ae. triseriatus overwintering success was determined. Eggs from LAC virus transovarially infected (LAC TOT+) and uninfected (LAC TOT−) Ae. triseriatus colonies were induced into diapause, held in natural conditions, and returned to the laboratory at predetermined times for assay of diapause, mortality, and filial infection rates, and to examine viral transcription and replication during diapause. Embryos from the LAC TOT+ colony exhibited greater cumulative mortality (16.7%) than the LAC TOT− eggs (7.3%) throughout the overwintering periods. The increased mortality rate in LAC TOT+ eggs corresponded with a decrease in filial infection rates. Eggs from the LAC TOT+ colony terminated diapause more readily than the LAC TOT− colony. An RNA strand-specific reverse transcriptase–polymerase chain reaction technique was used to monitor viral transcription and replication in mosquito eggs during overwintering, and to compare viral replication in diapausing and nondiapausing embryos. Viral messenger and replicative form RNA were present in eggs in all sample periods, suggesting that some virus replication occurred during diapause.

La Crosse (LAC) virus is a member of the Bunyavirus genus in the family Bunyaviridae.1 This mosquito-borne virus is an important cause of pediatric arboviral encephalitis in the United States,2 and is biologically transmitted to susceptible vertebrate hosts by the mosquito Ae. triseriatus.3 Importantly, LAC virus is efficiently transovarially transmitted (TOT) by Ae. triseriatus and overwinters in diapausing eggs of the vector.4 Although altered feeding behavior in LAC virus–infected adult females has been noted,5 laboratory studies suggest that there is no major untoward effect associated with LAC virus infection on Ae. triseriatus progeny.6,7 However, there is little quantitative information concerning the impact of LAC virus infection on Ae. triseriatus embryos overwintering in natural conditions. Filial infection rates (FIRs) were determined in progeny of Ae. melaninom mosquitoes infected with California encephalitis virus: FIRs were somewhat lower in progeny held for two winters in natural conditions.8

In the northern part of their range, Ae. triseriatus mosquito eggs undergo facultative diapause as the mechanism for overwintering. There is both genetic and geographic variation in the response of Ae. triseriatus eggs to shortened photoperiods.9,10 Additionally, Ae. triseriatus mosquito eggs exhibit installment hatching, which can result in a percentage of eggs requiring repeated hatching stimuli to terminate diapause.11 The molecular mechanisms that induce and maintain diapause and condition installment hatching in mosquito eggs are not known.12,13 However, the reduction in metabolic activity that occurs during diapause may have an effect on virus replication in the diapausing eggs.14

Bunyaviruses have a unique replication strategy that could function in establishing persistent infections in mosquito eggs. These viruses require host cell mRNA 5′ caps plus adjacent oligonucleotides to prime their transcription.15,16 The restriction in host cell biosynthetic activities that occurs during diapause could indirectly result in a reduction of viral replication due to a limiting supply of mRNA caps or other macromolecules needed for replication.17 The reduction in host cell transcription during diapause could concomitantly reduce viral replication, thereby moderating deleterious effects to the embryo by preventing the depletion of host cell macromolecules and enhancing virus overwintering. We previously used a reverse transcriptase–polymerase chain reaction (RT-PCR) technique to demonstrate that LAC virus replicates continuously in metabolically active embryos of mosquitoes that ingest repeated blood meals to stimulate oogenesis; however, in mosquitoes that do not receive blood meals after their initial infection, LAC genomic RNA (vRNA) can be detected in the ovaries, but mRNA and virion-complementary RNA (vRNA) are greatly reduced or undetectable.18 An additional blood meal restimulates virus replication in the ovaries. In contrast, in both metabolically active (embryonating) and dormant (diapausing) LAC virus–infected embryos, the sequences of host-derived viral mRNA primers change over time, suggesting that viral transcription is continuous.19

In these studies, we tested the hypothesis that diapause functions to enhance both Ae. triseriatus embryo survival and LAC virus overwintering. Virus-infected and noninfected Ae. triseriatus eggs induced into diapause and placed in natural overwintering conditions were periodically returned to the laboratory and assayed for viability, diapause, and virus replication. Coregulation of vector biosynthesis and virus replication during diapause would result in mortality rates in LAC virus–infected eggs similar to those in noninfected eggs.

MATERIALS AND METHODS

Aedes triseriatus mosquito colonies. Aedes triseriatus mosquito eggs were collected in 1992 from breeding sites near La Crosse, Wisconsin, and used to start the Bluff colony. The LAC TOT− colony mosquitoes used in these studies were derived from the Bluff colony. To generate a colony of mosquitoes transovarially infected with LAC virus (the LAC TOT+ colony), female mosquitoes were orally infected by feeding on a blood meal containing LAC virus. Mosquitoes were assayed for LAC virus infection by removal of one leg, which was examined by immunofluorescence for LAC virus antigen.20 Virus-infected mosquitoes were selected, placed into a colony cage with male mosquitoes from the LAC TOT− colony, blood fed again, and eggs were
Mosquito colonies were maintained as previously described. Mortality of overwintering Aedes triseriatus mosquitoes. 

Mortality of overwintering Aedes triseriatus mosquitoes.

Field studies. Replicate trials were conducted to determine the effect of LAC virus infection on Ae. triseriatus overwintering in their natural environment at ambient temperatures (Table 1). Eggs from the LAC TOT+ and LAC TOT− colonies were used for the field diapause studies. The F3 generation was used in year 1 (1993–1994), and the F2 generation was used in year 2 (1994–1995). Eggs were collected over a one-month time period and allowed to embryonate as described above. Immediately after diapause was induced, diapause rates were calculated (Tables 2 and 3). Diapause rates were also calculated at 0 time for the year 2 studies (Table 3). In the late fall of 1993 and 1994, immediately following diapause induction, eggs were shipped on ice to Madison, Wisconsin and placed in a secured outdoor area in a used tire in an orientation that placed them out of direct sunlight and artificial light.

At five-week intervals (Table 1), randomly selected LAC TOT+ and TOT− OPs were shipped back in ice via overnight service to Colorado State University and processed immediately upon arrival. Hatching rates, FIRs, and egg egg collections to initiate the next generation. For each ensuing LAC TOT+ generation, female mosquitoes that were demonstrated by fluorescent antibody to have been infected transovarially were mated, blood fed, and the resultant eggs were collected. This selection resulted in FIRs in the LAC TOT+ colony of 62 and 67%, respectively, for the F3 and F2 generations used in these studies, and > 95% of the females transovarially transmitted virus to their progeny (McGaw MM, 1996, The Effect of La Crosse Virus Infection on the Mortality of Overwintering Aedes triseriatus Mosquitoes. Master’s Thesis, Colorado State University, Fort Collins, CO). Mosquito colonies were maintained as previously described.18

Mosquito egg collection. Wooden tongue depressors used as oviposition substrates (OPs), were placed in colony cages in a small container of water infused with oak leaves four days after a blood meal. The OPs were left in the cages for 3–5 days, then removed, air-dried briefly, and stored in plastic bags at 20–23°C and a daily photoperiod of 16 hr of light: 8 hr of dark (16L:8D). Eggs were allowed to embryonate for 10 days before starting the diapause experiments.

Induction of diapause. The diapause-inducing conditions previously described for Ae. triseriatus were used.19 Following embryonation, eggs were placed into an environmental chamber at 21°C, and subjected to a shortened day length (10L:14D) for two weeks. The start of diapause induction was designated 0 time. These conditions induced diapause in 100% of the Bluff colony Ae. triseriatus mosquitoes.

### Table 1

Environmental conditions during the field studies

<table>
<thead>
<tr>
<th>Week post diapause induction</th>
<th>Date processed</th>
<th>Average high in °C</th>
<th>Average low in °C</th>
<th>Precipitation in cm</th>
<th>Average monthly hours daylight</th>
</tr>
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<tr>
<td><strong>Year 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Dec 1, 1993</td>
<td>0.5</td>
<td>−13 to 9</td>
<td>−6.5</td>
<td>21 to 3</td>
</tr>
<tr>
<td>7</td>
<td>Jan 5, 1994</td>
<td>−7.5</td>
<td>−25 to 3</td>
<td>−18.4</td>
<td>−33 to −7</td>
</tr>
<tr>
<td>12</td>
<td>Feb 9, 1994</td>
<td>−3.0</td>
<td>−14 to 10</td>
<td>−15.0</td>
<td>−28 to 7</td>
</tr>
<tr>
<td>17</td>
<td>Mar 16, 1994</td>
<td>8.3</td>
<td>−1 to 20</td>
<td>−4.0</td>
<td>−10 to 3</td>
</tr>
<tr>
<td>22</td>
<td>Apr 20, 1994</td>
<td>15.6</td>
<td>2 to 29</td>
<td>2.5</td>
<td>−7 to 15</td>
</tr>
<tr>
<td>27</td>
<td>May 25, 1994</td>
<td>22.6</td>
<td>14 to 31</td>
<td>6.2</td>
<td>−1 to 16</td>
</tr>
<tr>
<td><strong>Year 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Dec 7, 1994</td>
<td>3.0</td>
<td>−7 to 13</td>
<td>−6.6</td>
<td>−18 to 0</td>
</tr>
<tr>
<td>7</td>
<td>Jan 11, 1995</td>
<td>−2.0</td>
<td>−14 to 5</td>
<td>−10.9</td>
<td>−22 to 1</td>
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<tr>
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<td>−9.7</td>
<td>−21 to −2</td>
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<tr>
<td>17</td>
<td>Mar 22, 1995</td>
<td>8.5</td>
<td>−7 to 24</td>
<td>−3.1</td>
<td>−19 to 9</td>
</tr>
<tr>
<td>22</td>
<td>Apr 26, 1995</td>
<td>12.3</td>
<td>−1 to 21</td>
<td>0.8</td>
<td>−10 to 7</td>
</tr>
<tr>
<td>27</td>
<td>May 31, 1995</td>
<td>20.9</td>
<td>13 to 29</td>
<td>7.5</td>
<td>1 to 13</td>
</tr>
</tbody>
</table>

Table 2

Year 1: effect of La Crosse (LAC) virus infection on overwintering of Aedes triseriatus

<table>
<thead>
<tr>
<th>Week†</th>
<th>LAC TOT− rates*</th>
<th>LAC TOT+ rates*</th>
</tr>
</thead>
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<tr>
<td></td>
<td>N1</td>
<td>Mortality Diapause</td>
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<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>357</td>
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<tr>
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</tr>
<tr>
<td>12</td>
<td>1,298</td>
<td>2.6</td>
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<td>5,381</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* TOT = transovarially transmitted; NA = not assayed.
† Number of weeks after the beginning of diapause induction.
†† Number of eggs per sample at a timepoint.
§ Total week 7 through week 27, when samples were in the field.

Table 3

Year 2: effect of LaCrosse (LAC) virus infection on overwintering of Aedes triseriatus

<table>
<thead>
<tr>
<th>Week†</th>
<th>LAC TOT− rates*</th>
<th>LAC TOT+ rates*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N1</td>
<td>Mortality Diapause</td>
</tr>
<tr>
<td>0</td>
<td>667</td>
<td>5.4</td>
</tr>
<tr>
<td>2</td>
<td>1,202</td>
<td>10.6</td>
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<tr>
<td>7</td>
<td>757</td>
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<td>12</td>
<td>794</td>
<td>23.3</td>
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<td>17</td>
<td>985</td>
<td>18.5</td>
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<tr>
<td>22</td>
<td>1,441</td>
<td>19.2</td>
</tr>
<tr>
<td>Total †</td>
<td>3,977</td>
<td>19.6</td>
</tr>
</tbody>
</table>
mortalities were determined at each time point. Rates expressed for time points are not cumulative rates. Molecular analyses were conducted on year 2 samples.

Environmental conditions in study sites. Weather information (temperature, precipitation, photoperiod) was obtained from the National Weather Service in Madison, Wisconsin on a monthly basis for the experimental year 1 and year 2 (Table 1).

Determination of diapause rates. To determine diapause rates, hatching was induced under optimal conditions.11 The OPs were submerged in hatching solution (1:100; Difco [Detroit, MI] brain heart infusion in deoxygenated, double-distilled water) in test tubes. After 24 hr, the number of larvae in each sample was determined. Each sample was subjected to multiple hatch attempts, with each hatch attempt separated by two weeks to allow the eggs to terminate diapause. Starting with the third hatch attempt, nitrogen was slowly bubbled through the hatching solution for 45 min immediately following immersion to promote hatching. During the first winter, each sample underwent four hatch attempts, while in the second winter, each sample underwent seven hatch attempts, although only the data from the four initial attempts are shown in Table 4. All samples were kept in nondiapause inducing conditions of 20–23°C, 80% relative humidity, and 16L:8D for the duration of the overwintering studies.

Determination of the FIRs. The larvae from LAC TOT+ samples were reared to third instar, and approximately six days after hatching were squashed onto microscope slides, fixed in acetone, and assayed by immunofluorescence for LAC virus antigen.20 The FIR was defined as the number of infected larvae divided by total number of larvae assayed.

Statistical analysis. Chi-square statistics and the associated probability values were computed using Epi-Info Version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Probability values less than 0.05 were considered significant.

Determination of LAC virus transcriptional activity. In the year 2 studies, one group of eggs at each time point was processed for molecular analyses. Total cellular RNA was extracted from the eggs for analyses of LAC virus RNA species and actin mRNA using a transcript-specific RT-PCR.16, 23 Nondiapause LAC TOT+ and TOT− controls were also used. These eggs were collected as above and were held in nondiapause inducing conditions of 20–23°C, 80% relative humidity, and 16L:8D for the duration of the overwintering studies.

Extraction of RNA from mosquito embryos. Eggs were scraped gently from OPs, transferred to a sterile microcentrifuge tube, and lysed by trituration in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sodium lauryl sarcosine, and 0.1 M 2-mercaptoethanol.17 Total RNA was extracted from eggs using the acid guanidinium thiocyanate-phenol-chloroform method.20 Each sample was analyzed for the presence of all three viral RNA species and actin mRNA.

La Crosse virus strand-specific RT-PCR. Primers were selected from published sequences, using Oligo 4.0 computer software (National Biosciences, Plymouth, MN) for specific reverse transcription of LAC virus small segment vRNA, mRNA, and vcRNA and PCR amplification of cDNA.8, 23, 25

Internal control for RT-PCR of mosquito RNA. An RT-PCR technique for detection of mosquito β-actin mRNA was developed as an internal control for RT reactions (Wasieloski LP. 1995, La Crosse Virus Gene Expression in Aedes triseriatus. Ph.D. Dissertation, Colorado State University, Fort Collins, CO).

RESULTS

Environmental conditions. Temperature, precipitation, and photoperiod data were obtained on a monthly basis for both years (Table 1). Clearly, the winter beginning in December 1993 (year 1) was much colder than the following year (year 2). The average low in January 1994 (year 1) was −18.4°C, 7.5°C colder than in January 1995 (year 2). Furthermore, temperatures as low as −33°C were recorded in January 1994. This temperature was 11°C below the coldest
temperature recorded in 1995. February 1994 was also colder than any of the months for the winter of 1995, both in terms of average low temperature and low temperature extreme. In contrast, the spring of 1994 was warmer than the spring of 1995. In April, the average high in 1994 exceeded that of 1995 by 3.3°C. Monthly figures for the precipitation and the average hours of daylight, a parameter critical for maintaining diapause in the overwintering eggs, are also included (Table 1).

**Year 1 overwintering study.** Diapausing eggs were shipped to Wisconsin on December 1, 1993, and returned at five-week intervals thereafter for analysis of diapause, mortality, and FIRs (Table 2).

**Diapause.** For the year 1 study, the diapause rate of both the LAC TOT+ and LAC TOT− samples was 100% from week 2 through week 17 postdiapause induction (PDI) (Table 2). At week 22 PDI, diapause rates were lower at 84.6% and 86.8% for the LAC TOT+ and the LAC TOT− samples, respectively. These rates suggest that the conditions inducing the eggs to respond to hatching stimuli occurred between the middle of March and the end of April. Apparently, the eggs began to terminate diapause in response to a daily photoperiod in excess of 13 hr (Table 1). The last set of samples for 1994 was returned from the field at week 27 PDI in the final week of May. These eggs exhibited greatly reduced diapause rates: 29.1% for the LAC TOT+ samples and 17.3% for the LAC TOT− samples.

**Egg mortality.** For the year 1 study, the mortality rates of eggs at two weeks PDI were 9.8% and 12.2% for the LAC TOT+ and LAC TOT− eggs, respectively (Table 2 and Figure 1). Mortality rates were significantly lower at week seven PDI at 7.1% in the LAC TOT+ sample and 2.6% in the LAC TOT− sample (χ² = 3.10, P = 0.078; χ² = 47.27, P < 0.001, respectively). The mortality rate in the LAC TOT+ eggs was 2.6% at week 12, and was higher in each of the ensuing sample periods. The percent of nonviable eggs in the LAC TOT− sample remained essentially unchanged at 3.3% at week 12 PDI, was slightly higher for weeks 17 and 22 PDI, and was 19.2% at week 27 PDI. Mortality rates did not differ significantly between the LAC TOT+ and LAC TOT− eggs for weeks 12 and 17 PDI (χ² = 1.14, P = 0.286; χ² = 0.06, P = 0.802, respectively).

At week 22 PDI, egg mortality rates in the LAC TOT+ sample were significantly higher than at week 17 PDI, measuring 23.3% at week 22 and 8.1% at week 17 (χ² = 51.84, P < 0.001) (Table 2). The average high temperature also increased from 8.3°C to 15.6°C during this time (Table 1). The percent of nonviable eggs remained essentially unchanged in the LAC TOT− samples at 6.6%. Egg mortality was significantly greater in the LAC TOT+ sample than in
the LAC TOT− samples at week 22 PDI ($\chi^2 = 115.88, P < 0.001$).

At week 27 PDI, mortality rates were significantly greater than in the prior time point in both the LAC TOT+ samples and the LAC TOT− samples at 43.6% ($\chi^2 = 62.87, P < 0.001$) and 19.2% ($\chi^2 = 94.19, P < 0.001$), respectively. Therefore, only 56.4% of the LAC TOT+ eggs survived the winter, while 80.8% of the LAC TOT− eggs survived. The 24.3% difference was significant ($\chi^2 = 134.83, P < 0.001$). Thus, mortality was substantially greater in the LAC TOT+ eggs than the LAC TOT− eggs at the end of the overwintering period.

Filial infection rates. For the year 1 study, the t = 0 FIR was 61.9%, and at two weeks PDI, it was 63.4% (Table 2). The FIR was 57.4% at week 7, 46.0% at week 17, and was slightly higher at the final two time points. The difference in the FIR from 63.4% at week 2 to 53.4% at week 27 was significant ($\chi^2 = 10.16, P = 0.001$). The reason for the low FIR at week 17 PDI is unknown.

Year 2 overwintering study. Eggs for the year 2 study were shipped to Wisconsin at a slightly later date, December 6, 1994, and returned at five-week intervals. During this second winter, there were also significant differences between the mortality rates of LAC TOT+ and LAC TOT− samples and a significant change in the FIRs through time (Table 3).

Diapause. For the year 2 study, at t = 0, the diapause rate for both the LAC TOT+ and LAC TOT− eggs was less than 1% (Table 3). Following induction, the diapause rate from week 2 until week 12 PDI was 100% for both groups. Unfortunately, for unknown reasons, eggs from the week 17 PDI samples did not hatch. They were checked for viability using the bleaching technique, but did not appear viable. Samples received in late April at week 22 PDI were beginning to emerge from diapause; the diapause rates for the LAC TOT+ and the LAC TOT− samples were 67.2% and 65.1%, respectively. Diapause rates from the same time point PDI in year 1 were approximately 20% higher. At week 27 PDI, diapause rates were 3.1% in the LAC TOT+ sample and 11.4% in the LAC TOT− sample. These rates were also considerably lower than the diapause rates of 29.1% and 17.3%, respectively, for this time point in the year 1 study (Table 2).

Egg mortality. At t = 0, the percentages of nonviable eggs were 5.4% and 4.5% in the LAC TOT+ and the LAC TOT− samples, respectively (Table 3 and Figure 1). At week 2 PDI, the egg mortality rate was 10.6% in the LAC TOT+ samples and 3.9% in the LAC TOT− samples. The 6.7% difference was significant ($\chi^2 = 25.70, P < 0.001$), although the change since t = 0 was significant only for the LAC TOT+ sample (LAC TOT+ $\chi^2 = 14.39, P < 0.001$). At seven weeks PDI, the egg mortality rate was 18.0% in the LAC TOT+ samples and 7.2% in the LAC TOT− samples. The 10.8% difference between the samples was statistically significant ($\chi^2 = 52.64, P < 0.001$), as was the change in the egg mortality rates between the week two and week seven PDI LAC TOT+ samples ($\chi^2 = 21.88, P < 0.001$). These samples were returned from the field on January 11, after they had been exposed to the coldest temperatures of the season (−22°C) (Table 1). This temperature was 11°C warmer than the coldest temperatures in 1994 (year 1), when daily lows exceeded −22°C on 19 occasions.

The egg mortality rates in the week 12 PDI LAC TOT+ and LAC TOT− field samples were 23.3% and 5.2%, respectively ($\chi^2 = 134.91, P < 0.001$). At week 22 PDI, the egg mortality rate was 18.5% for the LAC TOT+ sample and 6.4% for the LAC TOT− sample ($\chi^2 = 79.75, P < 0.001$). At week 27 PDI, the egg mortality rates were 19.2% and 7.3%, respectively, for the LAC TOT+ and LAC TOT− eggs. Thus, as in year 1, the mortality rate in the LAC TOT+ eggs was significantly greater than in the LAC TOT− eggs ($\chi^2 = 106.16, P < 0.001$).

Overall, mortality rates were greater in year 1 (1994) than in year 2 (1995). In year 1, 56.4% of the LAC TOT+ eggs and 80.8% of the LAC TOT− eggs successfully overwintered. In year 2, 80.8% of the LAC TOT+ eggs and 92.7% of the LAC TOT− eggs successfully overwintered. Much of the mortality in year 1 occurred in later months when diapause had been terminated.

Filial infection rates. In the year 2 study, the beginning FIR was 66.5%, approximately 5% higher than the FIR at t = 0 in the previous winter (Tables 2 and 3). The FIR remained relatively constant from week 2 to week 22, and was lower at week 27 PDI (Table 3). The FIRs were 70.4% at week 2 and 64.9% at week 27, the final timepoint for the year. The 5.5% change was significant ($\chi^2 = 6.09, P = 0.041$).

Effect of LAC virus infection on hatching. The percent of eggs hatching at each attempt for a given LAC TOT+ sample was determined and compared with the percent hatching for the corresponding LAC TOT− sample at the same time point (Table 4). Only hatches 1–4 are shown for year 2. In sample periods 7–17 weeks PDI, when eggs were still in diapause upon receipt from the field, the percentage of eggs hatching after diapause was terminated was significantly greater in the LAC TOT+ sample. Therefore, the LAC TOT+ samples emerged from diapause more quickly than the LAC TOT− samples. In the final two time points (weeks 22 and 27), when the embryos were emerging from diapause in the field, the differences in hatching rates between the two groups were smaller and more variable (Table 4).

La Crosse virus transcription during diapause. Persistence of LAC virus was monitored by RT-PCR analysis of RNA extracted from eggs for the year 2 field studies. Virion RNA, mRNA, and vcRNA were all detected at all time points in both diapassing and nondiapassing (control) LAC TOT+ eggs (Figure 2). No apparent difference between the field diapassing and control nondiapassing groups could be seen. No PCR product was seen in the reactions using RNA from uninfected eggs. Actin mRNA was detectable by RT-PCR at all time points in the diapassing eggs.

DISCUSSION

La Crosse virus infection did adversely affect overwintering in Ae. triseriatus. In both years, the LAC TOT+ eggs experienced higher mortality rates during overwintering than the LAC TOT− eggs (Tables 2 and 3 and Figure 1). Similar results were obtained under carefully controlled conditions in the laboratory (McGaw MM, 1996, The Effect of La Crosse Virus Infection on the Mortality of Overwintering Aedes triseriatus Mosquitoes. Master's Thesis, Colorado
FIGURE 2. Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of La Crosse (LAC) virus gene expression in field diapausing and nondiapausing (laboratory control) *Aedes triseriatus* eggs. Total cellular RNA extracted from eggs (0.5 µg per reaction) or BHK-21 cells (0.3 µg per reaction) was subjected to RT-PCR using LAC virus–specific primers. Eggs from March were in full diapause (100%); eggs from April were in partial diapause (65%). V\(^+\) = LAC virus infected; V\(^-\) = uninfected; m = virion complementary (vc) and mRNA; v = virion RNA; vc = vcRNA only. Values on the left are in basepairs.

State University, Fort Collins, CO). In the final two time points of each year, when the eggs were emerging from diapause under natural conditions, LAC TOT\(^+\) egg mortality was nearly three times the mortality of the LAC TOT\(^-\) samples. Furthermore, the additional mortality experienced by the LAC TOT\(^+\) embryos reduced the filial infection rate by 6–10% at the end of the overwintering period (Tables 2 and 3). Based on an average of the final two time points of both years, approximately 74% of LAC TOT\(^+\) eggs and 90% of LAC TOT\(^-\) eggs could be expected to survive the winter. If it is assumed that the 16% excess mortality in the LAC TOT\(^+\) population was due solely to LAC infection, then the FIR at the end of overwintering should be roughly 84% of its original value. Using an average FIR of 67% from week two PDI, the average FIR at the end of overwintering would be expected to be roughly 56%. The average of the observed FIR for the week 27 time point for both years was 59%. These results suggest that LAC virus infection was the major factor in the increased mortality experienced by this population, and these mortality differences were not simply attributable to genetic differences between the LAC TOT\(^+\) and LAC TOT\(^-\) populations. A second, less plausible, explanation is that the virus was somehow being cleared in the infected, LAC TOT\(^+\) mosquitoes and that selection of this line had also produced embryos less well-suited to overwintering. This seems unlikely since we were able to detect all forms of the viral RNA throughout the winter.

It is possible that the results may be complicated by potential genetic differences between the two groups. Female mosquitoes in the LAC TOT\(^+\) colony were selected for TOT. The LAC TOT\(^-\) and TOT\(^+\) colonies were separated by two generations in the first winter’s experiment and six generations in the second winter’s experiment. Genetic differences between the colonies could have resulted in a phenotype less adapted to survive natural conditions and more sensitive to environmental stimuli for hatching. However, the FIRs argue against this possibility (Tables 3 and 4). If the increase in the mortality rates were due to genetic or environmental factors, the FIR would have either remained constant or randomly fluctuated over the course of each winter. The steady decreases of FIRs with time seem to be the result of a higher mortality rate in infected embryos. In addition, the male mosquitoes used for mating both the LAC\(^+\) and LAC\(^-\) females were from the LAC TOT\(^-\) Bluff colony, which would reduce the genetic variability between the colonies (McGaw MM, 1996, *The Effect of La Crosse Virus Infection on the Mortality of Overwintering Aedes triseriatus Mosquitoes*. Master’s Thesis, Colorado State University, Fort
Collins, CO). Finally, the LAC TOT+ and TOT− colonies responded similarly in both experimental years. Since different generations of mosquitoes were used, this also suggests that virus infection status is the major determinant of the increased mortality and early hatching response.

The increased mortality in the LAC TOT+ overwintering Aedes triseriatus eggs clearly demonstrates an arbovirus-associated untoward effect on a natural mosquito vector. Although LAC virus infection may induce alterations in the blood feeding behavior of Aedes triseriatus, transovarially infected Aedes triseriatus females maintained their full reproductive capacity. In addition, no adverse effects were detected with regard to the duration of larval period, sex ratio, hatching success, time to ovarian maturation, fecundity, or adult survival through the second oviposition as a result of LAC virus infection. These studies were conducted in laboratory conditions and for relatively short periods of time. In contrast, our studies were conducted over more than six months and in natural conditions. Differential mortality was not seen in mosquitoes assayed in the early time points of each winter. The increased mortality in the LAC TOT+ eggs was most apparent after emergence from diapause, when the embryo would presumably be under the greatest stress from virus replication. It is noteworthy that similar LAC virus–infected egg mortality was observed in long-term laboratory studies (McGaw MM, 1996, The Effect of La Crosse Virus Infection on the Mortality of Overwintering Aedes triseriatus Mosquitoes. Master’s Thesis, Colorado State University, Fort Collins, CO).

Extreme cold temperatures appeared to have little effect on the mortality of the overwintering eggs. The winter of 1994 (year 1) was unusually cold, while 1995 (year 2) was relatively mild (Table 1). In year 1, the second set of field samples was returned in the second week of February, following nine nights when temperatures fell to −33°C. Egg mortality in February was actually lower in both the LAC TOT+ and LAC TOT− samples relative to the January time point (Table 2). In contrast, year 2 mortality rates were higher in February than in January, even though the daily low was never less than −22°C. The mortality rates increased dramatically in the spring of 1994, when temperatures were warmer. In contrast, in 1995, the spring was cooler than that in 1994, and egg mortality remained relatively constant from one time point to the next. These results suggest that in warm winters fewer mosquitoes, especially infected ones, overwinter successfully than in cold winters.

At week 22 PDI, embryos were beginning to emerge from diapause for both years. Interestingly, diapause rates for eggs from the same time PDI in year 1 were approximately 20% greater. The year 2 samples were returned from the field on April 26, six days later than the shipment from the prior year. The daily photoperiod on April 20 in Madison, Wisconsin is 13 hr 30 min, while on April 26 it is 19 min longer at 13 hr 49 min. While the differences in photoperiod due to the six additional days in year 2 may have resulted in lower diapause rates, it is more likely that ambient temperatures at earlier periods may have conditioned the differences. Since the spring in year 1 was warmer than in year 2, it is unlikely that ambient temperatures at the time of diapause termination affected diapause. In general, the spring of 1995 was cooler than the prior spring. Furthermore, the temperature in the week prior to week 22 PDI shipping was colder in year 2 than in year 1 (Table 1). The much warmer winter experienced by the embryos in year 2 may have induced a reduced diapause response. At week 27 PDI, year 2 diapause rates were 3.1% in the LAC TOT+ sample and 11.4% in the LAC TOT− sample. These rates were also considerably lower than the diapause rates of 29.1% and 17.3%, respectively, for this timepoint in the year 1 study (Table 2). The marginal diapause rates exhibited at the end of overwintering were probably due to installment hatching rather than a true diapause in the embryo. The mechanisms triggering installment hatching are poorly understood, but the phenomenon has great significance on the maintenance of LAC virus by enabling a small percentage of embryos to survive multiple seasons before hatching. Development and use of molecular (instead of descriptive) markers of diapause and installment hatching would be extremely useful for elucidation of environmental factors conditioning these important vector phenotypes.

La Crosse virus infection apparently altered the hatching response of diapausing eggs; eggs from the LAC TOT+ colony emerged from diapause more readily than those from the LAC TOT− colony (Table 4). This may have been due to viral transcription scavenging caps from cellular messages necessary for diapause maintenance, resulting in LAC virus–infected eggs hatching earlier than uninfected eggs. This could have epidemiologic significance. If the LAC+ eggs break diapause and emerge sooner than their uninfected counterparts, infected larvae would have a temporal advantage in initiating the seasonal amplification cycle.

Coregulation of host and viral gene expression during diapause is an attractive hypothesis to describe the molecular mechanisms that promote efficient LAC virus overwintering in Aedes triseriatus. In these studies, the housekeeping gene selected to monitor host transcription, actin, was detectable in relatively constant amount in every sample tested, suggesting ongoing transcription. In addition, LAC virus mRNA and vcRNA, along with virion RNA, were also demonstrated throughout diapause (Figure 2). Viral transcription could continue throughout diapause if certain host mRNAs were continuously synthesized or if a stable pool of host mRNAs synthesized during embryogenesis was available for cap scavenging. Our methods did not allow us to determine if host mRNA synthesis was occurring continuously. La Crosse virus mRNA and vcRNA become undetectable in persistently infected mosquito midguts and metabolically inactive ovaries, but these results may not be applicable to embryos and to the complicating factors associated with overwintering in a natural environment. In this regard, the sequences of host-derived LAC virus mRNA primers changed over time in both embryonating and diapausing Aedes triseriatus embryos. Thus, we believe that limited host transcription and viral replication occur during overwintering and diapause. Further studies will be necessary to determine the molecular mechanisms that permit Aedes triseriatus overwintering and that modulate the deleterious effects of virus replication on mosquito embryos.

It is important to note that despite the adverse effect of LAC virus infection, most embryos successfully overwintered. Thus, TOT and overwintering in diapausing Aedes triseriatus embryos provides an extremely successful survival
and amplification mechanism for the virus. Understanding the molecular determinants of this remarkable host-parasite relationship should provide considerable insight into vector pathogen interactions and into the determinants of arbovirus cycle integrity in nature.

Acknowledgments: We acknowledge the expert technical assistance of Cynthia Oray and Lyric Beaty.

Financial support: This work was supported by Public Health Service Grant AI-32543 from the National Institutes of Health.

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