Evaluation of *Aedes aegypti* Densonucleosis (AeDNV) Infection in Adult Mosquito Mortality

Emily M. Plake, Malena Doehling, Stacey Bray, Dr. Jon Carlson, and Dr. Erica Suchman

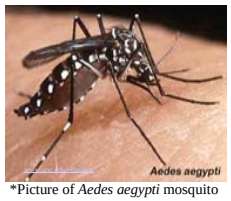
Department of Microbiology, Immunology, and Pathology, Colorado State University, 80523

Methods and Materials

1. *Mosquito Collection and Insectary Environment:*

A Rexville-D strain of *Aedes aegypti* mosquitoes, cultured since 1991, was obtained (C. Meredith, Arthropod Borne and Infectious Disease Laboratory, Colorado State University Fort Collins, CO) and used for all experiments. This room is on a 12 hour photoperiod and maintains a constant temperature of 80°F and 80% humidity by daily observation and monitoring. Larvae and Pupae were fed 50 ul of a mixture of mouse and fish food and water on an as need basis.

*Insectary and room containing it



*Picture of *Aedes aegypti* mosquito

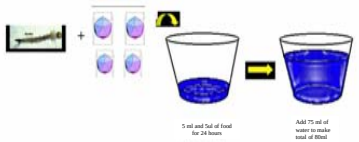
Picture provided by Jeremy P. Ledermann

2. *Virus Production:*

AeDNV was produced by transfecting cell cultures grown in L-15 medium supplemented with 10% fetal bovine serum and streptomycin-penicillin in 75 cm2 polystryene culture with infectious clones (Afanasiev, 1991). Transfection was done using the Qiagen Effectene® transfection protocol and cells were incubated at 28°C for 4 days following transfection. Post incubation, virus pellets were collected through multiple centrifugations. The pellets collected were then resuspended in 1ml of double deionized water per 30mls of original solution and filtered using a 0.45 µm syringe style filter (Uniflow®, Keene, NH) (Ledermann *et al*., 2004).

3. *Mosquito Infection Protocol:*

Mosquito eggs were hatched in approximately 500ml of tap water and 1 ml of Blood, Brain and Heart fusion. One hour post hatching 25 larvae were isolated and place into eight 100ml plastic cups (25 larvae per cup for a total of 200 larva) containing 5mls of tap water. Each cup is then infected with the appropriate volume of virus to get the desired dose ($1x10^{10}$ genome equivalents per ml) and fed 5 ul of food. The cups were allowed to incubate at 80°F and 80% humidity for 24 hours without food. After 24 hours 75 milliliters of water were added to the cup to total eighty milliliters. Dead adults were recorded and removed from existing populations. Removed adults were placed in 1.7 microfuge tubes for further analysis by Quantitative PCR.



Graphic provided by Malena Doehling

4. *Quantitative PCR:*

-Primer and Probe Design: Degenerate primers within the NS1 gene of densovirus genome were designed to detect viral genomes. The probe has a FAM fluorescent dye on the 5'end of the sequence and a TAMRA quencher at the 3' end of the sequence (fwd: CAT ACT ACA CAT TCG TCC TCC ACA A, probe: FAMCCA GGG CCA AGC AAG CGC C, rev: CTT GGT GAT TCT GGT TCT GAC TCT T) (Ledermann *et al*., 2004).

-Quantification of Viral Genomes: A plasmid containing the viral NS1 gene was used as a standard to establish a linear regression. The viral genome equivalence (geq/ul) in the samples were then calculated based on the fluorescence values obtained from these standards (Ledermann et al., 2004)

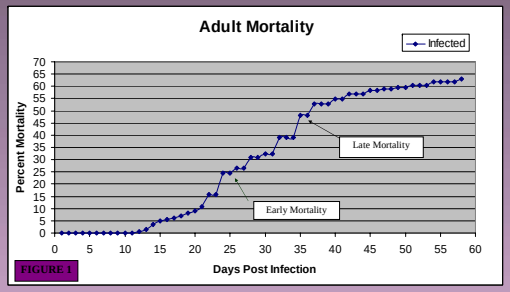
5. *Virus (DNA) Isolation*

Qiagen ® DNeasy protocol for insects is used to extract those biological samples to be analyzed for virus concentration by Quantitative PCR

Abstract

Aedes aegypti densonucleosis virus (AeDNV) has long been considered a likely agent in biologic control against arthropod-borne diseases. Extensive studies have been done to evaluate infection and pathogeneses in *Aedes aegypti* larvae and pupae, but only primary studies have been preformed in adults. The primary studies on adult mortality produced a two part mortality curve that showed high levels of early mortality, low levels of intermediate mortality and high levels of late mortality. It was hypothesized that the high levels of early mortality were due to high levels of virus infection, whereas the high levels late mortality were due to old age. In this study we analyzed the mosquitoes that died early and late in the mortality assay with quantitative PCR to determine virus titers in individual mosquitoes. We found that there is no significant difference between titer level of mosquitoes that died in the early part of the curve (Day 17-29), which had an average of 1.264×10^{11} genome equivalents per ml and those that died in the late part of the curve (Day 45-54), which had an average of 2.255×10^{11} genome equivalents per ml.

Results



* ***Figure 1***: Graph of Adult Mortality (percentage) vs. Day Post Infection. This graph is from the primary studies of adult mortality and shows the high levels of early and late mortality

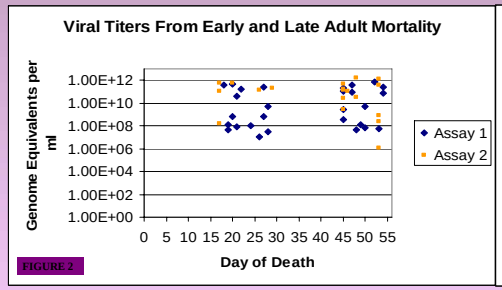


Figure 2: Viral genome equivalence per ml isolated from mosquitoes that died in the early and late parts of the mortality graph. Note that in both the early and late parts of the mortality graph there were individuals that contained viral titers anywhere from 1 x 10^6 genome equivalents per ml to 1 x 10^{12} genome equivalents per ml.

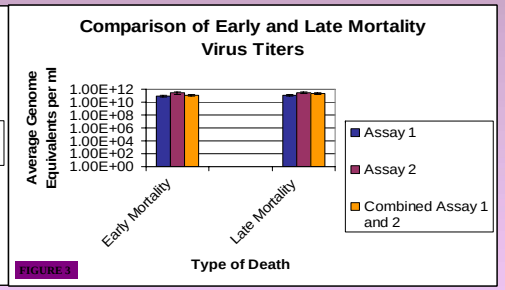


Figure 3: Average viral genome equivalents per ml for both early and late mortality groups. This graph allows for visual comparison between the two. Note that the average genome equivalents per ml isolated in individuals that died in the early part of the mortality graph was 1.264×10^{11}, whereas the average for those individuals that died in the late part was 2.255×10^{11}.

References

Afanasiev, B.N., E.E. Galyov, L.P. Butchatsky, and T.V. Kozlov. 1991. Nucleotide Sequence and Genomic Organization of ***Aedes Densonucleosis Virus.*** Virology. **185:** 323-36.

Buchatsky, L. P., M. A. Kuznetsova, N. N. Lebedinets, and G. A. Kononko. 1987. Development and basic properties of a virus preparation Viroden. Vopr. Virology S.U. **6:**729-733.

Jousset, F., E. Baquerizo, and M. Bergoin. 2000. A new densovirus isolated from the mosquito *Culex pipiens* (Diptera: culicidae). Virus Res. **67:**11-6.

Jousset, F.-X., C. Barreau, Y. Boublik, and M. Cornet. 1993. A Parvo-like virus persistently infecting a C6/36 clone of *Aedes albopictus* mosquito cell line and pathogenic for *Aedes aegypti larvae*. Virus Research. **29:**99-114.

Kittayapong, P., K. J. Baisley, and S. L. O'Neill. 1999. A Mosquito Densovirus Infecting *Aedes aegypti* and *Aedes albopictus* from Thailand. American Journal of Tropical Medicine and Hygeine. **61:**612-617.

Lebedeva, P. O., A. P. Zelenko, M. A. Kuznetsova, and A. P. Gudzgorban. 1972. Studies on the demonstration of a viral infection in larvae of *Aedes aegypti* mosquitoes. Microbiol. J.S.U. **34:**70-73.

Ledermann, J. P., E. Suchman, B. Black, and J. Carlson. 2004. Infection and Pathogenicity of Mosquito Densoviruses AeDNV, HeDNV, and APeDNV in *Aedes aegypti* Mosquitoes. Journal Of Economic Emtamology. **97(6):**1828-1835.

O'Neill, S. L., P. Kattayapong, H. R. Braig, T. G. Andreadis, J. P. Gonzalez, and R. B. Tesh. 1995. Insect densoviruses may be widespread in mosquito cell lines. Journal of General Virology. **76:**2067-2074.

Rwegoshora, R., K. Baisley, and P. Kittayapong. 2000. Seasonal and spatial variation in natural densovirus infection in *Anopheles minimus* S.L. in Thailand. Southeast Asian J Trop Med Public Health. **21:**3-9.

Introduction

- It has been shown that *Aedes aegypti* densovirus (AeDNV) cause morbidity and mortality in multiple genera of mosquitoes, making it a likely candidate for biologic control of arthropod borne infectious diseases (Ledermann *et al*., 2004). Due to the increase incidence of these diseases research is being done so that AeDNV can be used for this purpose in the future.

- Densonucleosis is a single stranded DNA virus that belongs to the sub-family *Densovirinae* of the genus *Parvoviridea*. Densoviruses are known to infect and replicate in insects only (Buchatsky *et al*., 1987). The first mosquito densovirus (MDV) was discovered and isolated from a research colony of *Aedes aegypti* mosquitoes and therefore named *Aedes aegypti* densovirus (AeDNV) (Lebedeva *et al*., 1972). Other genera of mosquitoes including *Culex, Culiseta*, and other members of *Aedes* were found to be responsive to AeDNV (Buchatsky *et al*., 1987). Multiple other strains of MDV's have been isolated from natural and laboratory populations including *Haemogogus equines* densovirus (HeDNV), and *Aedes* Peruvian densovirus (APeDNV) (Kittayapong *et al*., 1999; Rwegoshora *et al*., 2000; Jousset *et al*., 2000; O'Neill *et al*., 1995; Jousset *et al*., 1993).

- Primary studies on adult mortality were performed by Malena Doehling and showed that the high morbidity and mortality rates that we saw in larvae and pupae remained true throughout the life of the mosquito. This was very promising information because it was found that most death in adults occurs prior to the time required for them to become infected and able to transmit human pathogens such as Dengue Fever. When mortality rates were plotted verses day post infection, the graph showed a two part mortality curve. The first part, which I have named early mortality, occurs between day 17 and day 29 and consists of high levels of mortality in adult mosquitoes. The second part of the curve, which I have named late mortality, occurs between day 45 and day 54 and also consists of high levels of mortality. Low levels of mortality occurred between days 30 and 40. It was hypothesized that the first part of the graph, early mortality, was due to high levels of infection. Whereas, the second part of the graph, late mortality, was due to old age and therefore, low levels of infection or virus titer. In this study we analyzed, using quantitative PCR, the mosquitoes that died between day 17 and 20, early mortality, and compared them to those that died between day 45 and 54, late mortality, to test this hypothesis.

Discussion

• Our hypothesis that early adult mosquito mortality is caused by high titers of virus, whereas late mortality is caused by old age is incorrect. In fact, high virus titers were found in individuals that died in both the early and late parts of the mortality curve.

• There is no correlation between the time of death in adult individuals and the virus titer of that individual. Therefore, there is no correlation between death and virus titer. This supports pervious data that showed high virus titers in adult mosquitoes that were infected with less virulent strains of densonucleosis viruses (Ledermann *et al*., 2004).

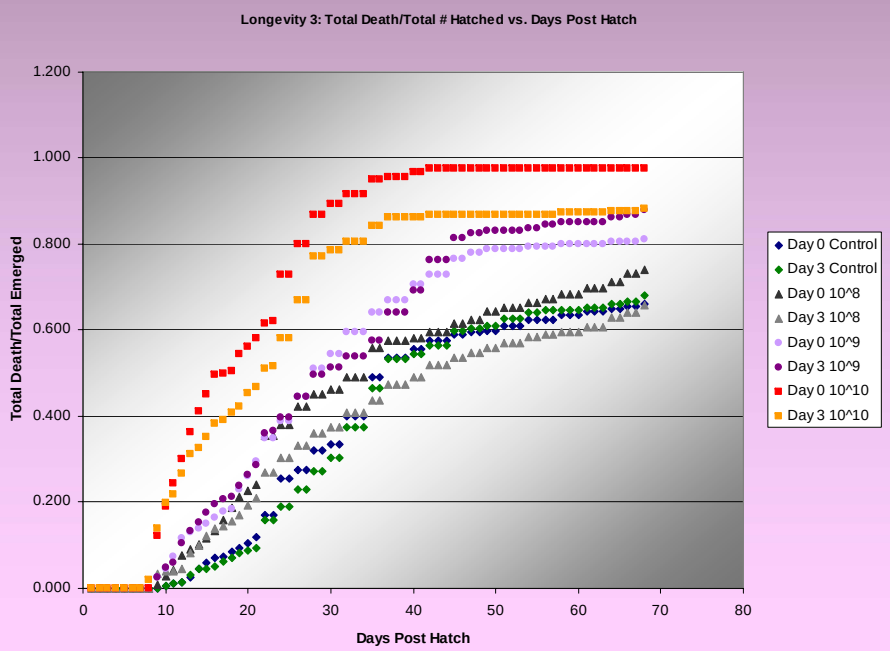
• No correlation between death and virus titer suggests that the viruses ability to cause mortality is dependent on something other than the amount of virus present in the individual. The viruses ability to cause mortality could be dependent on many other factors such as; the location of infection, the amount of time it takes for the virus to reach that location, host (mosquito) immunity to the virus, and environmental factors related to the viruses ability infect.

Acknowledgments

• I would like to thank the following people:

-Cindy Meredith for maintaining the Rexville-D strain of *Aedes aegypti* mosquitoes and providing the eggs used in this experiment.

-Joe Piper and Jesse Terry for their help in picking pupa and removing dead adults.


Longevity 3: Total Death/Total # Hatched vs. Days Post Hatch
Total Death/Total Emerged
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
10
20
30
40
50
60
70
80
Days Post Hatch
Day 0 Control
Day 3 Control
Day 0 10^8
Day 3 10^8
Day 0 10^9
Day 3 10^9
Day 0 10^10
Day 3 10^10