PHYSIOLOGICAL AGEING AS IT IS RELATED TO GENE EXPRESSION IN THE LONE STAR TICK, *Amblyomma americanum*

A Thesis

by

AMANDA MARIE CATENA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2009

Major Subject: Entomology

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Approved by:

Co-Chairs of Committee,	Pete Teel
	Albert Mulenga
Committee Members,	Michael T. Longnecker
Head of Department,	Kevin Heinz

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ABSTRACT

Physiological Ageing as It Is Related to Gene Expression

in the Lone Star Tick, *Amblyomma americanum*. (May 2009)

Amanda Marie Catena, B.A., Indiana University

Co-Chairs of Advisory Committee: Dr. Pete Teel
Dr. Albert Mulenga

With advances in molecular technology, the study of human ageing has turned to DNA for answers as to how humans age. Due to the size of the human genome and the longevity of humans, organisms with smaller genomes and shorter lifespans have frequently been the center of research studies in ageing. Studies of *Drosophila melanogaster, Caenorhabditis elegans*, yeast, and mice have uncovered specific genes that up and down regulate with age and stress. Research has yet to produce, however, results from an organism known for its longevity. *Amblyomma americanum* is an excellent candidate for this, as it can survive for years unfed. Two groups of 75 unfed adult *A. americanum* were monitored in a control environment of 85% relative humidity and an experimental environment designed to induce physiological stress at 75% relative humidity. Five ticks were tested for transcript abundance of five candidate ageing genes initially and at the 25^{th}, 75^{th}, and 95^{th} percent mortality. These tests provided evidence that *Amblyomma americanum* undergoes changes in gene expression with age on a genetic level.

ACKNOWLEDGEMENTS

I would like to give special thanks to my committee co-chair, Dr. Teel, my committee co-chair, Dr. Mulenga, and my committee member, Dr. Longnecker. They provided the valuable input and guidance that made this project successful.

I also want to extend my gratitude to the United States Army Green to Gold Active Duty Option Program, which allowed me to pursue my degree while remaining in the course of my service.

A special thanks to my parents and friends for their encouragement and support during this process.

NOMENCLATURE

FC	Female Control Group
MC	Male Control Group
FE	Female Experimental Group
ME	Male Experimental Group
PCR	Polymerase Chain Reaction
NP	No PCR Product
Exp	Experimental Group

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1. INTRODUCTION

1.1 Introduction to Current Molecular Ageing Research

Researchers have studied other organisms to establish the molecular mechanisms of ageing to better understand human ageing. These model ageing organisms have been fruit flies (*Drosophila melanogaster),* nematodes *(Caenorhabditis elegans),* yeast (*Saccharomyces cerevisiae*) and mice (*Mus musculus*). This is owing to their relatively small genomes and brief life span that enable them to be exceptional subjects for molecular studies. Research has shown that ageing is regulated by specific genes and that a number of them can be silenced to increase longevity (Guarente et al. 2000). There is also strong evidence that many of the same ageing genes will express under physiological stress as well. In these scenarios physiological stress and chronological ageing have very similar physiological effects emphasizing the difference between chronological ageing and physiological ageing (Pletcher et al. 2005). Although these studies have been of great benefit to the study of senescence, some scientists argue organisms with unique ageing features and longer life spans should also be studied (Strehler 1986).

1.2 Justification of Research

Amblyomma americanum, the lone star tick, possesses features related to ageing that are distinctive. Possessing relatively long life spans for arthropods, they are also capable of surviving several years without feeding. Another important ageing feature in

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This thesis follows the style of the Journal of Medical Entomology.

ticks is their physiological state known as quiescence that is very similar to diapause, but unique in that it can be interrupted for host questing (Sonenshine 1994). The lone star tick provides an opportunity to study the molecular mechanisms of longevity, rather than that of ageing as in previous model ageing organisms. There are benefits to understanding ageing in ticks as well.

The lone star tick has long established its status as a medically important arthropod capable of vectoring numerous pathogens. Through their feeding activity they place humans at risk for *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Coxiella burneti*, *Francisella tularensis*, *Borrelia lonestari*, and there is also evidence for the transmission of *Rickettsia amblyommii* (Childs and Paddock 2003). Domestic animals, such as dogs, can be infected and act as reservoirs for *E. chaffeensis* and *E. ewingii* (Ewing et. al 1971).

Current knowledge on tick ageing as it relates to their population ecology is limited. Methods rely on measuring physiological changes in the unfed adult tick resulting from gradual nutrient depletion (Balashov 1961, Balashov 1967), muscle deterioration (Jaworski et al. 1983), changes in siderosomes in the midgut epithelium (Jaworski et al. 1983), and scutum/body weight ratios (Chaka et al. 2001). There have been research attempts to determine whether infection prevalence and intensity are related to tick age (Uspensky 2006). If a correlation is established, a need for an accurate measure of tick age is even more pressing. Physiological ageing as it relates to gene function in *A. americanum*, however, has not been undertaken.

Only a few animal genomes have been sequenced to the extent that a microarray analysis could be used to determine what genes up or down regulated with age or physiological stress. *Amblyomma americanum* is no exception, however quite a number a genes have been fully sequenced. By comparing gene name and function with those identified as candidate ageing genes in model ageing organisms there were a number a matches. The first match was ATP Synthase subunit c (Aljamali et al. 2009).

ATP synthase subunit c is a key component to an enzyme that moves proteins across membranes and helps create ATP within the cell (Rappas et al. 2004). Studies of *Drosophila melanogaster* have shown that both ageing and physiological stress causes the down regulation of genes involved with mitochondrial function including ATP synthase (Dubessay et al. 2007, Vermeulen et al. 2007). Hormone regulation in *Caenorhabditis elegans* enabled ATP synthase genes to stay active longer and increased lifespan in experimental subjects (Hansen et al. 2005). Increased expression in aged ticks could account for their longevity in the absence of a bloodmeal. There was also another match to the anti-oxidant gene peroxiredoxin that is strongly associated with ageing and stress in model ageing organisms.

A widely accepted theory of ageing is the free radical theory of ageing which involves the irrevocable accumulation of damage within cells, particularly those caused by oxidants (Harman 1956, Harman 1981). Cells react by producing anti-oxidants to degrade oxidants into inert compounds though chemical reactions (Ames et al. 1981). *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* showed an extended life span when induced mutations increased anti-oxidant activity (Guarente et

al. 2000). *Drosophila melanogaster* studies using microarray technology have shown an increase in transcript abundance with age for the gene peroxiredoxin (Lai et al. 2007). Abnormally high anti-oxidant activity in ticks may be linked to their longevity. Oxidative stress is not the only damage based theory of ageing that can be tested with gene matches.

The damaged DNA binding gene, also sequenced for the lone star tick, is a gene that has been in a number of ageing studies. The DNA Damage Theory is another ageing theory based on the accumulation of damage and mutations as a result of ageing (Gensler and Bernstein 1981, Browner et al. 2004). Multiple research studies have established that DNA mutations, alterations and chromosomal abnormalities increase with age in both mice (Martin et al. 1985, Dolle et al. 1997, Vijg 2000, Dolle and Vijg 2002) and also humans (Esposito et al., 1989, Lu et al. 2004). Correlations have been found between genes that control these DNA repair mechanisms and the rate of aging measured in some mammals (Hart and Setlow 1974, Grube and Burkle 1992, Cortopassi and Wang 1996). Unfortunately, whether DNA repair genes influence ageing is still controversial. For example, mice over-expressing a DNA repair gene called MGMT had a lower cancer incidence but did not age slower (Zhou et al. 2001). On the other hand, mice with extra copies of two DNA repair genes lived 16% longer than controls but it was not clear that aging was delayed (Matheu et al. 2007).Similarly, the mutation of the genes that repair damaged DNA causes shortened life span in *Saccharomyces cerevisiae* (Park et. al 1999). These genes have been vastly understudied in regard to expression with ageing and pose promising candidates for study in *A. americanum*.

A number of genes are commonly used as controls for RT-PCR experiments. These genes are considered ideal candidates because they are thought to be stable under a variety of conditions and are typically highly conserved throughout the animal kingdom. Of these control genes, both 16S (Black et al. 1994) and actin have been sequenced in the lone star tick and used in studies. However there has yet to be a study to see how stable these genes are under varying environmental conditions and whether expression changes with age. There is a need for a study to examine the true variability in transcript abundance of these genes in order to determine suitability for use in RT-PCR with field collected ticks.

There are now a total of five genes in *Amblyomma americanum* that are potentially age relevant and require further study. The ATP synthase gene, 16S gene, tick actin gene, peroxiredoxin gene, and damaged DNA binding gene are the names of the five candidate ageing genes in this experiment.

The overall goal of this study was to examine whether gene expression changes with ageing in *Amblyomma americanum*. To accomplish this goal the experiment:

1. Used the data to create a survivorship curve for both optimal and physiologically stressed conditions.
2. Determined whether transcript abundance of a select set of genes related to mitochondrial function, anti-oxidant activity, damaged DNA repair and RT-PCR controls changes with population age.
3. Compared transcript abundance of a select set of genes in a tick population under physiological stress.

The underlying hypothesis was that the six selected *Amblyomma americanum* genes will change expression under chronological ageing conditions and physiologically stressed conditions. Further, it was hypothesized that genetic response to ageing under physiological stress is different to genetic responses in a population under optimal environmental conditions.

2. MATERIALS AND METHODS

2.1. Tick Rearing and Test Population Establishment

The ticks that were used in this study are maintained in a colony at the Tick Research Laboratory, Department of Entomology, Texas A & M University, under Animal Use Protocol number 2008-203. Founding specimens were collected from cattle at the Texas AgriLife Sonora Experiment Station and Hill Ranch in Edwards and Sutton Counties in Texas. Ticks for this project fed on chickens as nymphs and were allowed to molt to adults. Two test populations of adult ticks, each containing a mortality indicator subset and a sample subset were established. These populations consisted of equal numbers of each gender and were all from a uniform age cohort with respect to timing nymph bloodmeal and molting.

2.2. Ageing the Ticks

Two glass cubicles were used to create the two environments differing only by saturation deficit. In each cubicle, a mortality indicator subset consisting of 75 unfed male and 75 unfed female adult *Amblyomma americanum* individually placed into 3-dram vials, with screw tops, and netting over top was established alongside a sample subset consisting of three mason jars containing between 4,000 and 5,000 unfed adult *Amblyomma americanum* from the same population the control and experimental groups were drawn. The control cubicle contained two pans of glycerol to maintain 85% relative humidity. The experimental cubicle contained one pan of glycerol and one pan of CaCl to maintain 75% relative humidity. The experiment began on Day 0 and each Monday,

Wednesday, and Friday the indicator subset was counted to assess mortality. At Day 0 and also when the male experimental group reaches the 25th, 50th, 75th, or 95th percentile mortality twenty samples were taken from the sample subsets in each cubicle in equal numbers of both male and female.

2.3. Gene Expression Determination Using Semi-Quantitative PCR

The remaining phases of the experiment were conducted under Institutional Biosafety Committee #2008052-Mulenga. Each specimen was cut in half using a razor on a glass microscope slide that had been soaked for one hour in a 10% bleach solution. The specimens were then placed into a 1.5µL Eppendorf tube containing 150µL of RNA*later*® Tissue Collection: RNA Stabilization Solution and frozen at -80°C. The samples were later thawed to room temperature, the RNA*later*® removed, and the sample rinsed with RNA grade water. The protocol for TRIZOL® Reagent produced by the Invitrogen Corporation was then completed (Chomczynski and Sacchi 1987). To ensure there was no DNA contamination the RNA samples were then treated with Promega DNAse according to the protocol Promega Corporation #TB220. In order to re-precipitate the RNA pellet and perform a second chloroform wash, to reduce protein contamination, the protocol for TRIZOL® Reagent by the Invitrogen Corporation was then completed again (Chomczynski and Sacchi 1987). Following completion, the RNA was quantified three times using a Nanodrop ND-1000, once using a spectrophotometer, and the median value taken. Using the Thermo Fisher Scientific, Inc. Verso™ cDNA Kit protocol the quantity necessary for each sample to equal 1µg was added to RNA grade water to total 11µL. This allowed the quantity of RNA to be equal in every sample. To

study the function of the genes, as they are related to age in *Amblyomma americanum*, each sample underwent polymerase chain reaction (PCR) with each candidate ageing gene primer set to total six reactions each. Using the sequences for the five known genes 16S (Black et al. 1994), ATP synthase c-subunit, damaged DNA binding gene, peroxiredoxin, and tick actin (Aljamali et al. 2009) primers were created. Table 1 outlines the selected genes and primers that were used.

Table 1. Forward and reverse primers to target specific genes in *Amblyomma americanum* for polymerase chain reaction (PCR) amplification

Gene/Locus Number	Forward Primer/Reverse Primer
ATP Synthase EZ000266	F - 5' – ATGTTCGCCCTCAAGCTCG – 3' R - 5' – CAGCAACAGGAAGGCCAT – 3'
Peroxiredoxin EZ000198	F - 5' – GGTGACTGAGACCAGGAAGG – 3' R - 5' – TGTCCTTTATTGATCGCAGGT – 3'
Damaged DNA Binding EZ000281	F - 5' – AGGCTTTATAGACGGTGACCTC – 3' R - 5' – TTTCCCCACCCCACATGTC – 3'
Tick Actin EZ000248	F - 5' – GGACAGCTACGTGGGCGACGAGG – 3' R - 5' – CGATTTCACGCTCAGCCGTGGTGG – 3'
16S ABLMTRGDB	F - 5' – GTGGTATTTTGACTATACAAAGG – 3' R - 5' – CCTAATCCAACATCGAGGTC – 3'

Each gene required its own specific annealing temperature and number of cycles to ensure yield could be seen, but saturation was not reached. This enables each sample to show its own expression quantity. Table 2 gives the specific annealing temperatures and number of cycles used for each gene.

Table 2. Annealing temperatures and polymerase chain reaction (PCR) thermo-cycler machine cycles used to amplify specific target genes of *Amblyomma americanum*

Gene/Locus Number	Annealing Temperature	PCR Cycles
ATP Synthase EZ000266	55°C	25 Cycles
Peroxiredoxin EZ000198	50°C	30 Cycles
Damaged DNA Binding EZ000281	50°C	40 Cycles
Tick Actin EZ000248	55°C	30 Cycles
16S ABLMTRGDB	50°C	25 Cycles

The PCR cycles all included an initial denaturation step at 94°C for 2 minutes, followed by the designated number of cycles of 94°C for 30s, 47-55°C for 30s, and 72°C for 1m, followed by 72°C for 5m. The results were loaded on a 2% agarose gel with a DNA ladder. The samples were grouped by gene and after being loaded they were separated using agarose gel electrophoresis for 20 minutes at 100 volts.

2.4. Analyzing and Quantifying the Data

The gel results were examined using Image J software that assigns a numeric value to the density of each band on the gel. In this manner the transcript abundance in each sample was compared to that of the others within the same gene. The resulting numbers were compared using an ANOVA analysis to assign a p-value to the comparison of treatment, gender, and time.

3. RESULTS

The experiment allowed for the comparison of changes to transcript abundance in six ageing genes in *Amblyomma americanum*. The comparison was between the physiologically stressed and unstressed, male and female, and whether there were significant changes over time.

3.1. Survivorship Curve

The survivorship curve shown in Figure 1 was plotted using the data from the mortality indicator subset in Appendix A. Every Monday, Wednesday, and Friday from Day 0 of the start of the experiment until Day 77 the number of ticks remaining alive in the vials were counted. When the experiment began the ticks had molted to the adult stage 142 days prior and this number reached 219 on Day 77 when the experiment ended.

Although all four groups showed a significant increase in mortality on Day 45 of the experiment, the male ticks showed greater mortality in relation to time than females under the same relative humidity. Due to the male experimental group having the most significant mortality in relation to time during the experiment, their mortality indicator subset was used to determine when samples were taken. After Day 0 the samples for the 25^{th} percentile mortality group were taken on Day 49, the 50^{th} on Day 56, the 75^{th} on Day 63, and the 95^{th} on Day 77 of the experiment.

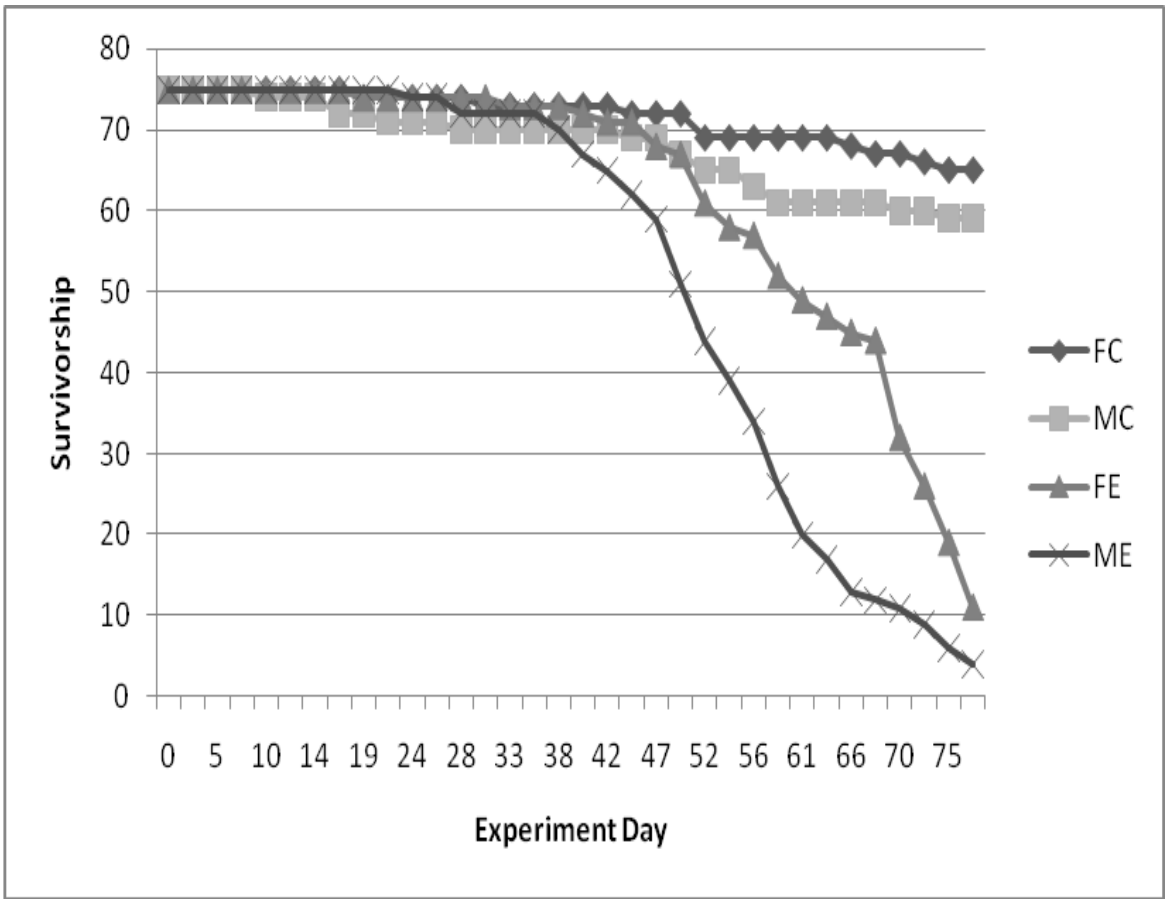

Fig. 1. Comparison of survivorship of *Amblyomma americanum* female control and male control maintained at 21°C, 85% relative humidity to female experimental and male experimental maintained at 21°C, 75% relative humidity consisting of 75 unfed adult individuals for each gender and treatment group

3.2. Semi-Quantitative PCR Results

Each sample was loaded into a well on an 2% agarose gel and gel electrophoresis was complete at 120V for twenty minutes. The results were then analyzed using Image J software. All four groups (FC,MC.FE,FE) has five samples analyzed except for 0% mortality. The experimental group was established after the start of the experiment so the initial collect on Day 0 consisted of onlycontrol specimens, as the experimental

group began that day. Due to this, the results for the experimental group at 0% are the results from the gender comparable control group.

3.2.1. ATP Synthase Expression Results

In order to analyze levels of mitochondrial activity in ageing ticksthe gene for ATP synthase subunit c was used. When the PCR results from Figure 2 were observed graphically, all four groups (FC, MC, FE, ME) showed changes in transcript abundance of the ATP synthase gene expression for each time interval in the study (Figure 3). The male stressed and unstressed groups appeared to show the most significant decrease in ATP synthase gene expression in relation to ageing according to the graphed data. The physiologically stressed males showing a greater decline between the start of the experiment and the 25^{th} percentile mortality. The female control and experimental groups both showed gradual decrease and the female experimental group showing increased expression after the 50^{th} percentile mortality to give it almost no change between the start of the experiment and Day 77 (95^{th} percentile mortality for the male experimental group). The male experimental also showed a slight increase in transcript abundance from the 25^{th} to 95^{th} mortality percentile.

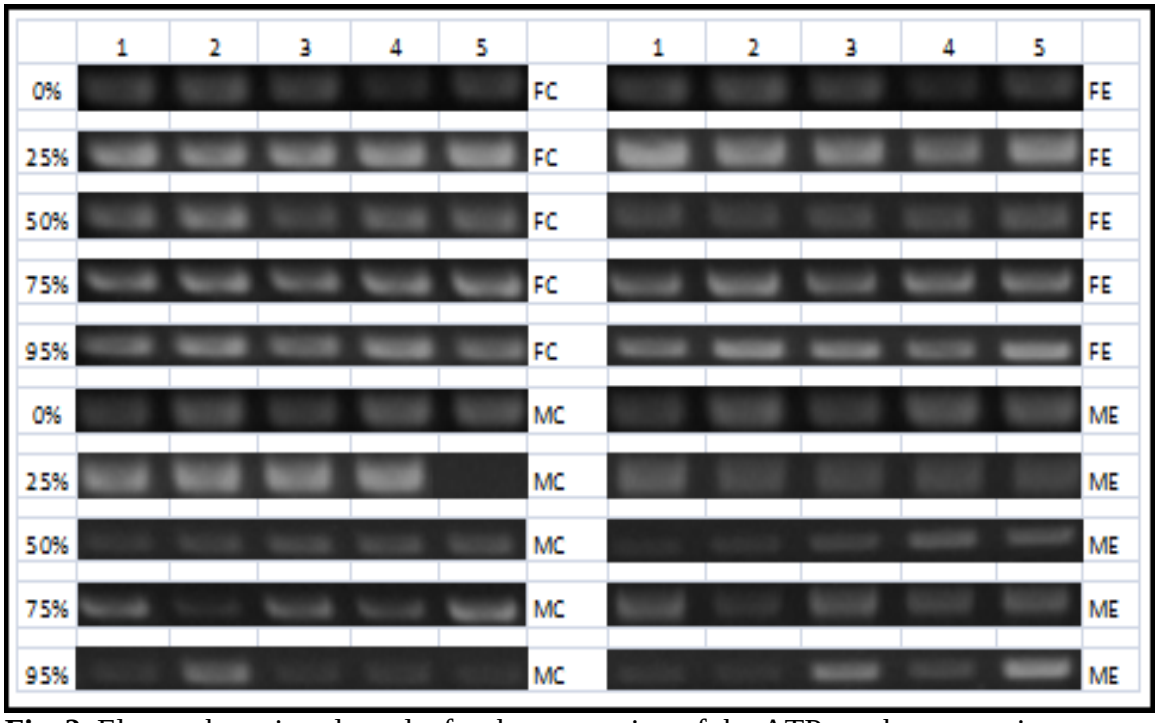

Fig. 2. Electrophoresis gel results for the expression of the ATP synthase gene in *Amblyomma americanum* subdivided by the mortality percentile to the right of the result, the sample number over the top of the result, and the group denoted as female control (FC), male control (MC), female experimental (FE), and male experimental (MC) to the left of the result

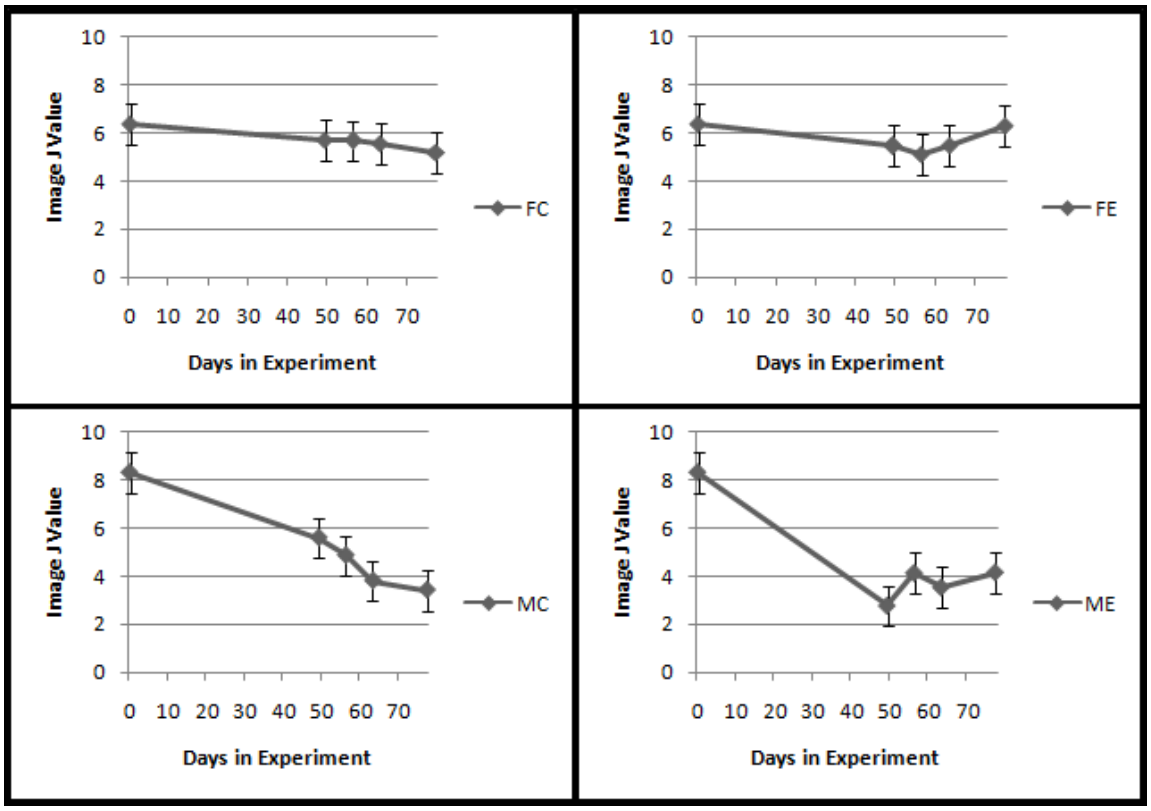

Fig. 3. Four graphs plotting electrophoresis gel band density as assessed by Image J software displaying the expression of *Amblyomma americaum* gene ATP synthase for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups from Day 0 to Day 77

3.2.2. 16S Expression Results

Levels of variance in expression for a widely utilized RT-PCR control was analyzed using the candidate ageing gene 16S. Stability with ageing gender, and physiological stress of any commonly used control would be of great importance to future research that used 16S as a control gene.

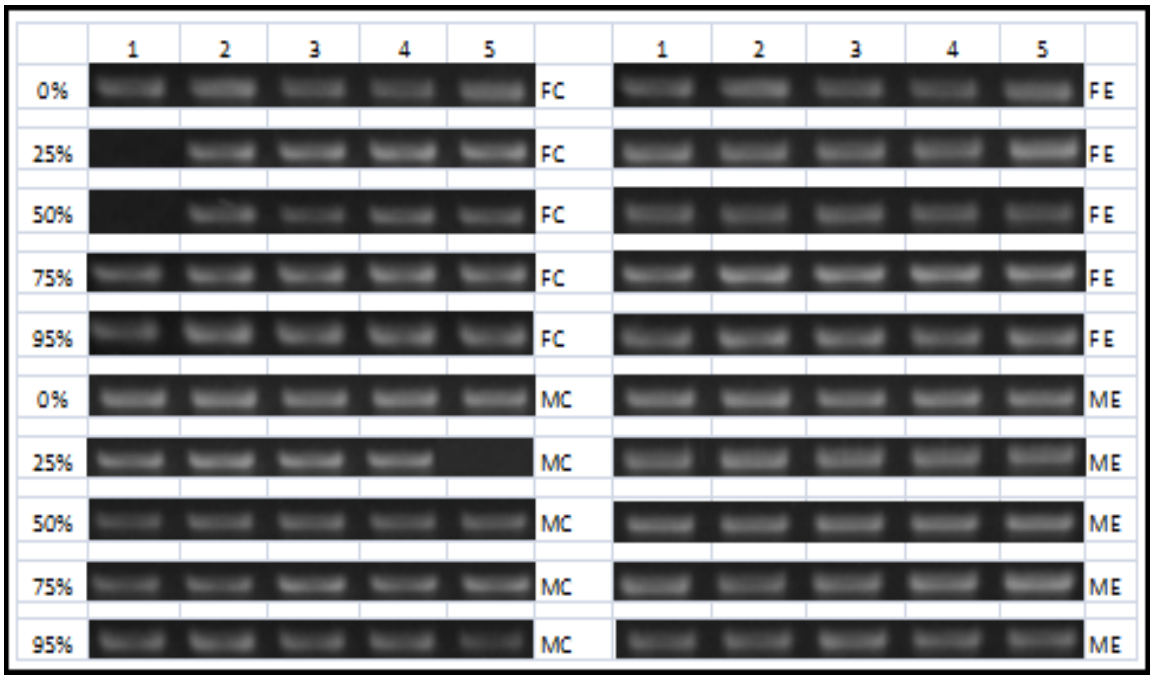

Fig. 4. Electrophoresis gel results for the expression of the 16S gene in Amblyomma americanum subdivided by the mortality percentile to the right of the result, the sample number over the top of the result, and the group denoted as female control (FC), male control (MC), female experimental (FE), and male experimental (MC) to the left of the result

The compiled PCR results displayed in Figure 4 indicate all four groups (FC, MC, FE, ME) appeared to show a change in transcript abundance of 16S gene during the experiment. From the graphs in Figure 5 (compiled from the Image J analysis of the results shown in Figure 4), the male control and experimental groups both showed significant decreases in 16S gene expression over time. Both male and female experimental groups showed a sudden spike in abundance around the 50th and 75th percentile mortality before beginning to decline again. The female control group showed a gradual decrease during the experiment.

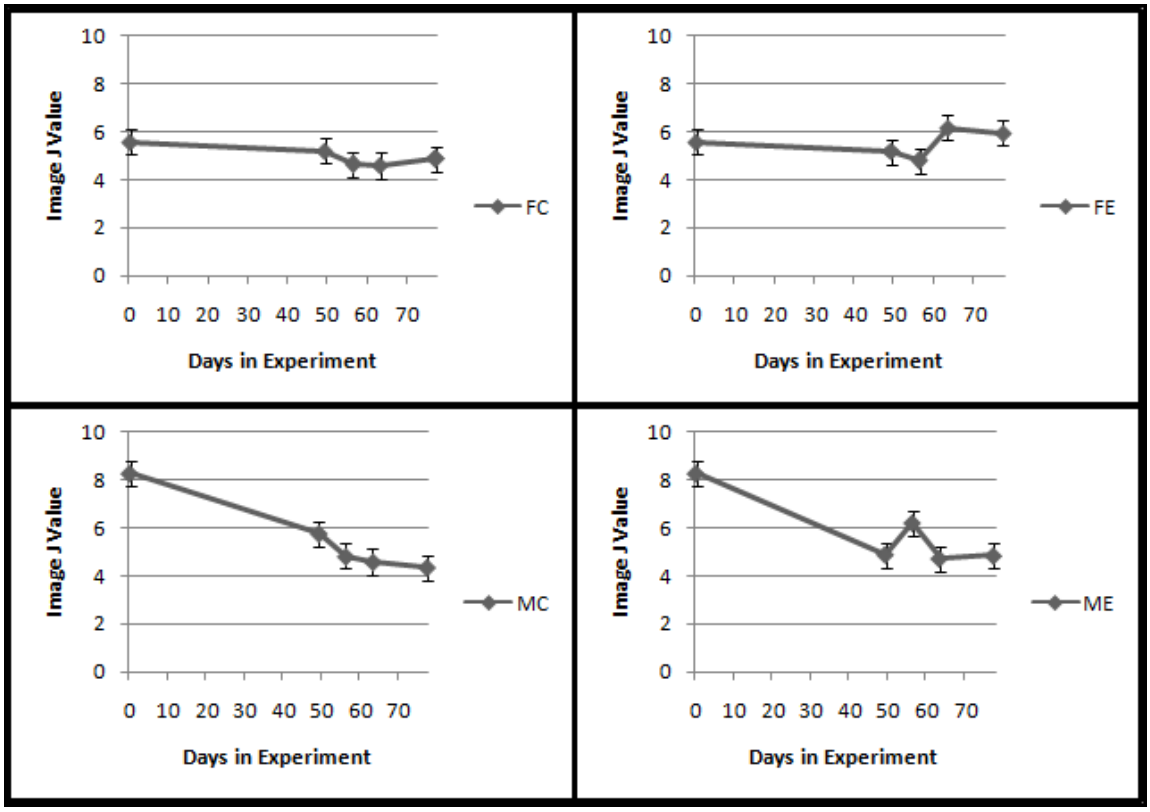

Fig. 5. Four graphs plotting electrophoresis gel band density as assessed by Image J software displaying the expression of *Amblyomma americaum* gene 16S for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups from Day 0 to Day 77

3.2.3. Tick Actin Expression Results

Tick actin has also been used as a control in RT-PCR studies. Like the 16S gene, the stability of this gene with age, between genders, and in a variety of environmental conditions is crucial to the accuracy of the studies it is used in.

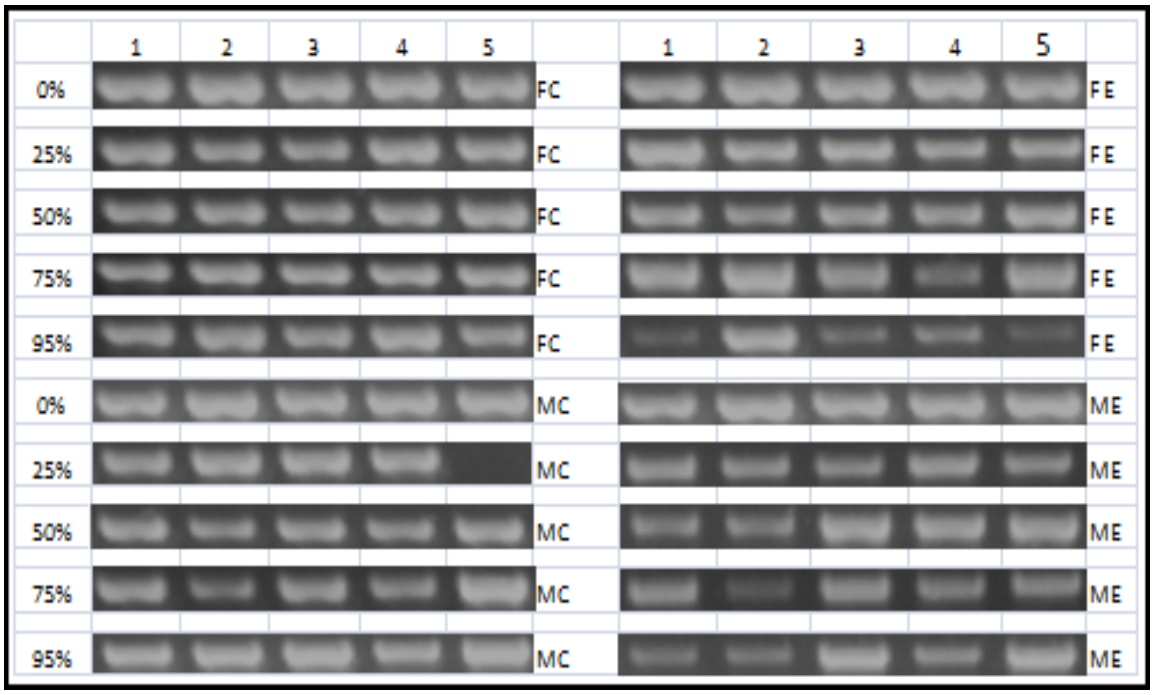

Fig. 6. Electrophoresis gel results for the expression of the tick actin gene in Amblyomma americanum subdivided by the mortality percentile to the right of the result, the sample number over the top of the result, and the group denoted as female control (FC), male control (MC), female experimental (FE), and male experimental (MC) to the left of the result

The PCR results for tick actin in Figure 6 show each of the four groups (FC, MC, FE, ME) demonstrating variance in transcript abundance throughout the study. When the PCR results were compiled graphically into Figure 7, the male and female control groups showed a gradual decline in tick actin expression with chronological ageing. The male experimental group showed a rapid decline upon being placed in the physiologically stressed environment and appeared to remain at those low levels for the duration of the study. The female experimental group differed from the other three groups by gradually upregulating tick actin during the study.

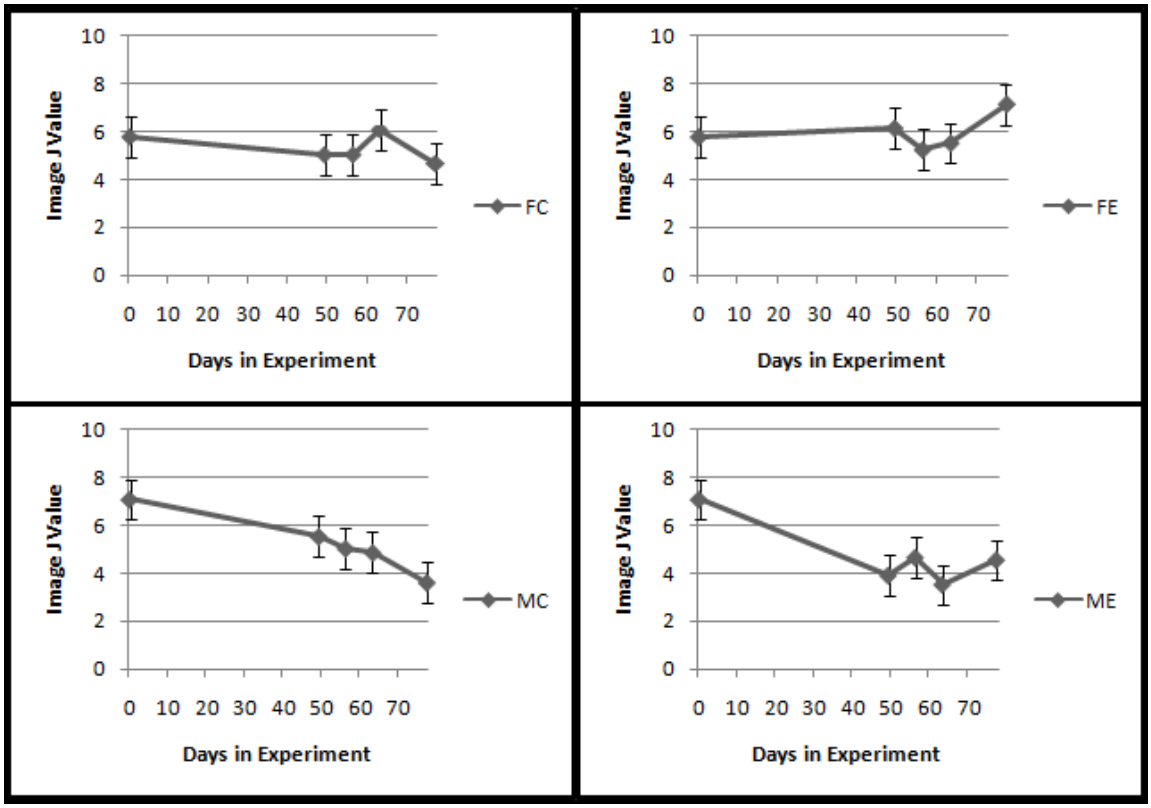

Fig. 7. Four graphs plotting electrophoresis gel band density as assessed by Image J software displaying the expression of *Amblyomma americaum* gene tick actin for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups from Day 0 to Day 77

3.2.4. Peroxiredoxin Expression Results

Multiple ageing studies indicate anti-oxidant expression changes with age, in particular the Peroxiredoxin gene. Lone stare tick anti-oxidant activity with age, gender, and environment was measured using the transcript abundance of the peroxiredoxin gene.

The PCR results in that are compiled in Figure 8 show a change in transcript abundance of peroxiredoxin gene during the experiment for all four groups (FC, MC, FE, ME). When these results were displayed graphically in Figure 9, the gradual down regulation of peroredoxin gene in both the male and female control groups was present. The female expermental showed an up regulation as mortality rose in the group. The male experimental group showed a rapid decline from the beginning of the study until the 25th mortality percentile when it began showing a gradual increase in expression of peroxiredoxin.

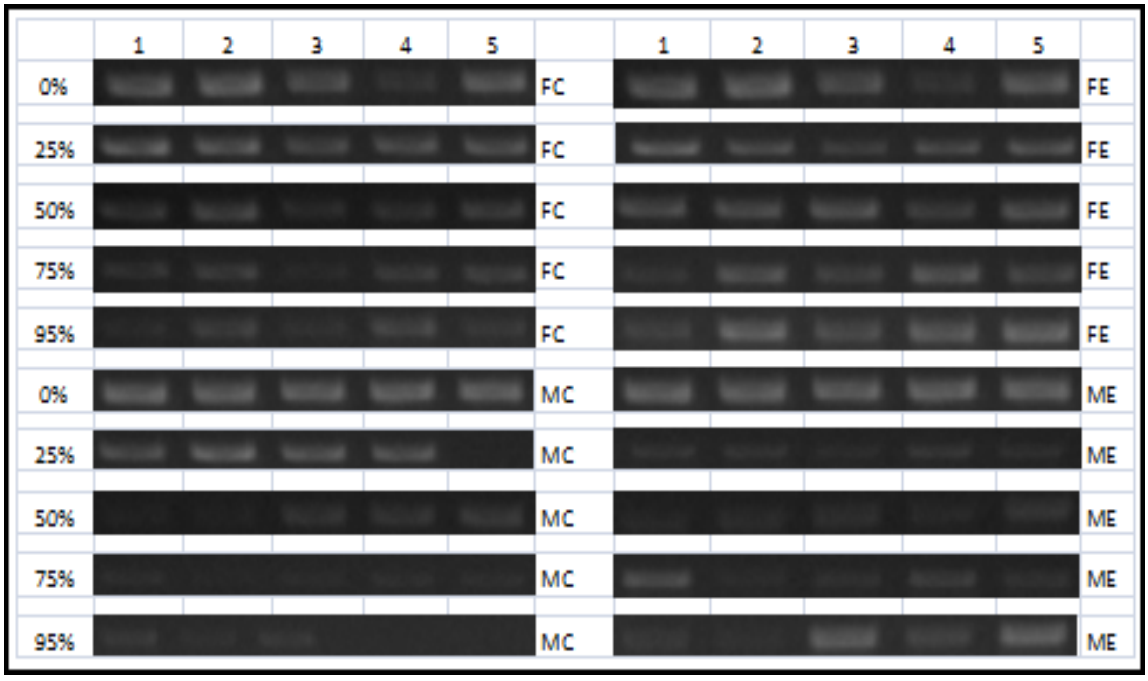

Fig. 8. Electrophoresis gel results for the expression of the peroxiredoxin gene in Amblyomma americanum subdivided by the mortality percentile to the right of the result, the sample number over the top of the result, and the group denoted as female control (FC), male control (MC), female experimental (FE), and male experimental (MC) to the left of the result

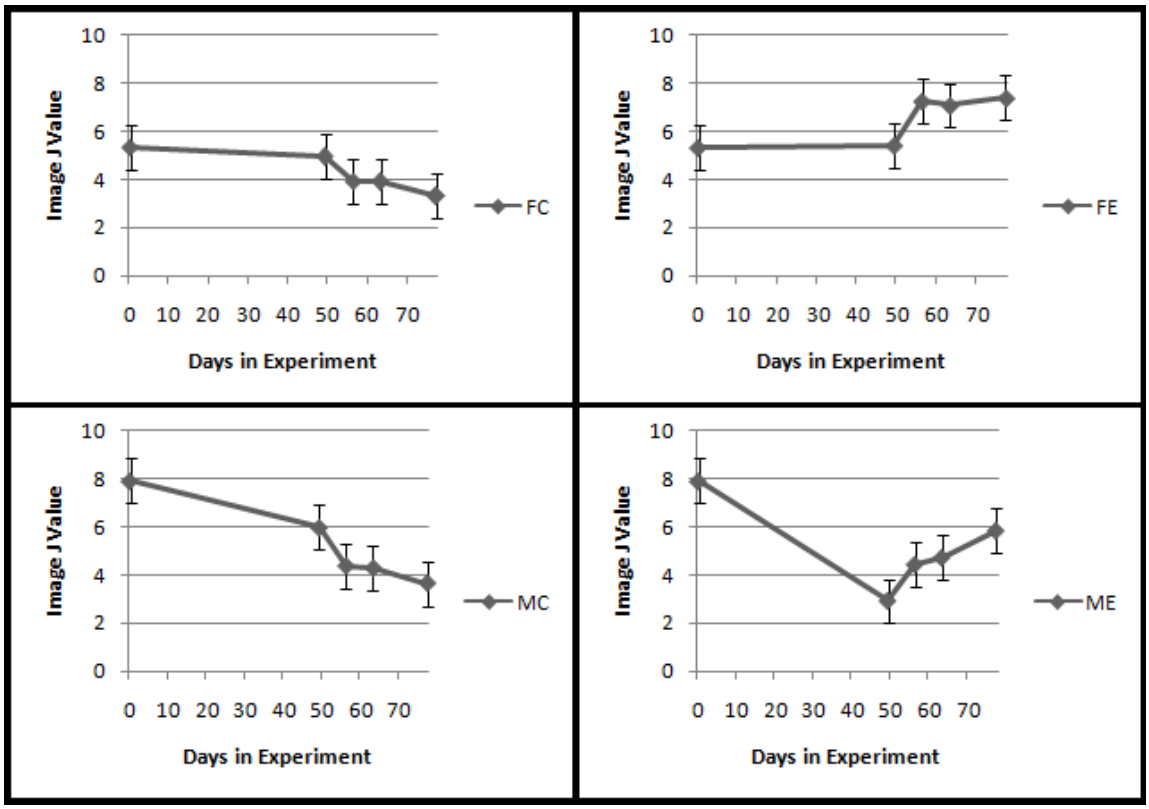

Fig. 9. Four graphs plotting electrophoresis gel band density as assessed by Image J software displaying the expression of *Amblyomma americaum* gene peroxiredoxin for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups from Day 0 to Day 77

3.2.5. Damaged DNA Binding Expression Results

The accumulation of mutated and damaged DNA is one theory of ageing currently accepted. In order to measure the cellular reaction to DNA damage with age, gender, and physiological stress damaged DNA binding gene was used as a candidate ageing gene for this experiment.

The PCR results displayed in Figure 10 show changes in transcript abundance for all four groups (FC, FE, MC, ME) of the damaged DNA binding gene during the experiment. Figure 11 graphically displays the PCR results showing the female control group began and ended the study with nearly the same mean value, but experienced both up and down regulation throughout the study. The male control group, on the other hand, showed a steady down regulation during the study. The female experimental group showed significant up regulation while the male experimental show marked down regulation up until the 25th mortality percentile where it remained at a lowered expression.

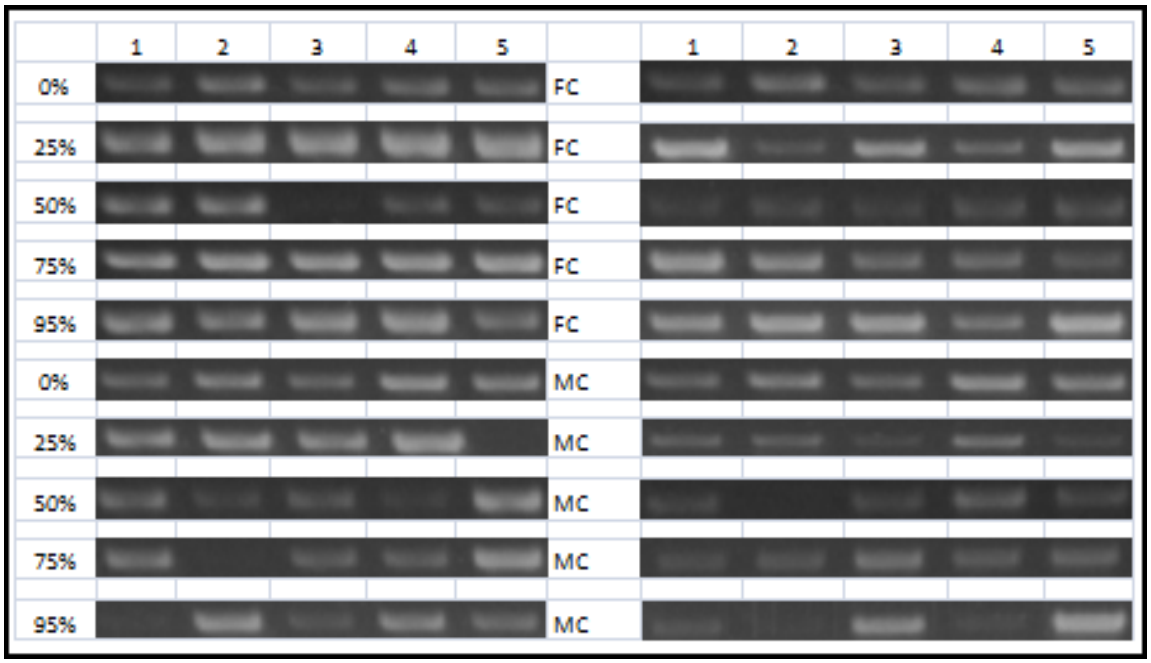

Fig. 10. Electrophoresis gel results for the expression of the damaged DNA binding gene in Amblyomma americanum subdivided by the mortality percentile to the right of the result, the sample number over the top of the result, and the group denoted as female control (FC), male control (MC), female experimental (FE), and male experimental (MC) to the left of the result

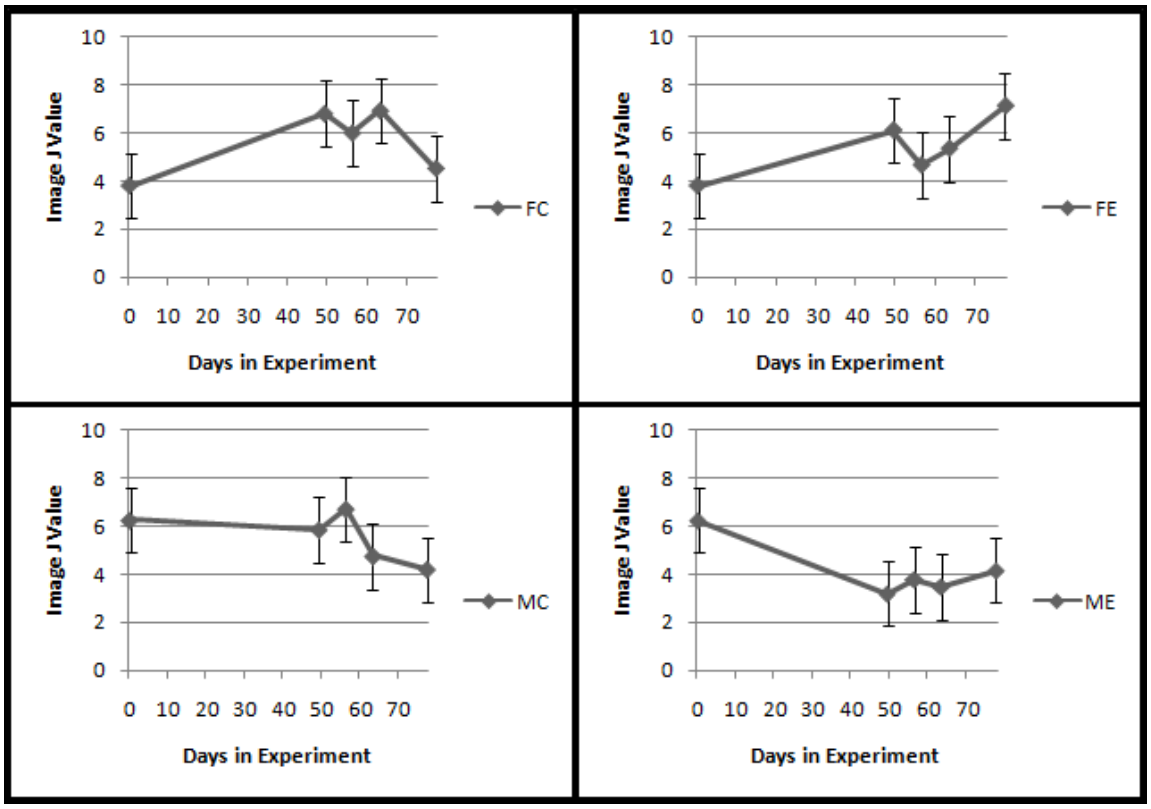

Fig. 11. Four graphs plotting electrophoresis gel band density as assessed by Image J software displaying the expression of *Amblyomma americaum* gene 16S for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups from Day 0 to Day 77

3.3. Statistical Analysis

The statistical analysis of the experiment was focused on the variance in transcript abundance between the samples using an ANOVA test (analysis of variance). These tests establish whether there is a statistically significant change in transcript abundance between the ungrouped and samples grouped by gender and treatment (FC, MC, FE, ME) with a comparison for time, gender, or physiological stress. Using 95% confidence a statistically significant change could indicate that either time, gender, or physiological stress influence ageing.

The first set of ANOVA tests were for variance in candidate ageing gene expression between the ungrouped samples to examine whether time influenced ageing. When all the ungrouped samples were compared the ATP synthase gene, 16S gene, tick actin gene, and peroxiredoxin gene had p-values of < 0.0001. The damaged DNA binding gene had a p-value of 0.9789.

The second set of ANOVA tests was for variance related to gender to determine whether male or female ticks experience a difference in gene expression with age. When the ungrouped samples were compared the ATP synthase gene (p-value < 0.0001), 16S gene (p-value 0.0010), and tick actin gene (p-value 0.0010) were statistically significant for a difference for gender. The peroxiredoxin gene (p-value 0.3900) and damaged DNA binding gene (p-value 0.1020) were not. In tables 11, 13, 15, 17, and 19 in Appendix B the samples were grouped (FC, MC, FE, ME) and compared within their respective mortality percentile and age bracket to have gender be the only point of difference between the samples. All of the candidate ageing genes showed significant variance for at least one group except the damaged DNA binding gene which showed no statistically significant change for any group.

The third set of ANOVA tests were to determine whether the control or experimental treatment caused greater variance in expression. When the ungrouped samples were compared the 16S gene (p-value 0.0016), peroxiredoxin gene (p-value < 0.0001), and damaged DNA binding gene (p-value 0.0355) showed statistically significant variance between the two treatments. The ATP synthase gene (p-value 0.1395) and tick actin gene (p-value 0.7038) did not. In Appendix B, tables 10, 12, 14,

16, and18 group the samples (FC, MC, FE, ME) and place them within subgroups according to gender and mortality percentile. In this way the only difference between each group was whether it was in the control or experimental treatment. All of the variance comparisons yielded at least one statistically significant group except the damaged DNA binding.

4. DISCUSSION AND CONCLUSIONS

The objectives of the experiment were to determine whether the transcript abundance of five candidate ageing genes in *Amblyomma americanum* changed with age. Further, the experiment was set to determine whether gender or treatment caused greater or less change in expression. The data from these objectives has many applications to the field of both ageing and tick research.

4.1 Potential Applications

The environmental conditions and methods of lowering the relative humidity in the chambers provide insight for tick rearing facilities to use on their own colonies. The rate of mortality for *Amblyomma americanum* under two separate treatments will give survivorship expectations.

There is also great importance in the field of medical entomology. Arthropod vectors have shown resistance to conventional control measures. Once ageing mechanisms in ticks can be fully understood, there is potential for new control methods. Ageing mechanisms are unique to disease vectors could be exploited in the form of pesticides and vaccines to limit tick population size.

Developing methods for accurate ageing of field collected ticks will enable acarologists to establish age distribution in tick populations. If pathogen infection prevalence and intensity become linked to tick age through research efforts then accurate ageing methodology will be desperately needed.

Ageing data from ticks does have applications to human ageing. Ageing and death appear to be universal in the animal kingdom, but not nearly enough information is known at present. If the chemical stimuli could be uncovered that controlled the mechanisms responsible for tick longevity those could be used to increase human longevity. These same chemicals could even facilitate human hibernation comparable to quiescence.

4.2 Limitations of the Experiment

The struggle to replicate field conditions in the laboratory for authenticity sake is often an issue in laboratory studies. Ultimately the information will be helpful to use with field collected ticks, but the lack of a stable field environment will present stark differences. Ticks have varying stimuli in the field such as temperature, humidity, host presence, etc. These events are variables that may influence gene expression at any age, but must be controlled in an experiment to minimize confounding factors to the study.

Both RT-PCR work and semi-quantitative PCR work involves the use of a control gene for comparison. This experiment however was unable to use a control because of the instability of common control genes in ticks. The lack of a control leaves no standard for comparison. All samples must be compared to one another so intensity then becomes relative within the single gene. This unfortunately was unavoidable without resorting to costly measures such as microarray analysis to map the changes in ageing genes over time in ticks.

The 25^{th}, 50^{th}, and 75^{th} percentile mortality samples were taken only a week, or at most two, apart making this only a short term study. The frequent opening of the

chambers during this time to collect samples may have caused some of the variability seen in the graphs for this time. Even though error was accounted for it can't be fully known whether the environmental changes added stress.

4.3 Future Experiments

This study could be repeated in the field using weather station data to correspond with the responses in the RNA samples. This could give insight as to weather ageing field collected ticks in this manner would be accurate or not. If it proves accurate it would provide a new method of tick population ageing in the field.

The issue of whether quiescence is influencing ageing should also be addressed. Tick activity could be monitored to draw correlations between inactivity and gene expression. It could show whether activity decreases with age. Ticks could be grouped by types of stimuli they will continuously be exposed to in order to measure energy metabolism after questing versus quiescent ticks.

It should be established whether age and ageing gene expression influence infection prevalence in tick populations. The 0 and 95^{th} percentile mortality could be screened to determine whether the infection rate is greater in older ticks. Expression can be examined in the samples to see how anti-biotic genes express with age to establish whether they influence the infection.

More arthropod vectors should be studied to discover unique ageing mechanisms to use for control. Urban entomologists and agricultural entomologists face major issues with pesticide resistance and creating a new type of control measure would be extremely

beneficial to pest management programs. Silencing different genes with different pesticides used in rotation would lower resistance and increase effectiveness.

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APPENDIX A

EXPERIMENTAL DATA TABLES

Table A.1. Mortality of *Amblyomma americanum* female control and male control maintained at 21°C, 85% relative humidity to female experimental and male experimental maintained at 21°C, 75% relative humidity consisting of 75 unfed adult individuals for each gender and treatment group

Experiment Day	**0**	**3**	**5**	**7**	**10**	**12**	**14**	**17**	**19**	**21**	**24**	**26**	**28**	**31**	**33**	**35**	**38**
Female Control	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Total Female Control	0	0	0	0	0	0	0	0	1	1	1	1	1	2	2	2	2
Male Control	0	0	0	0	1	0	0	2	0	1	0	0	1	0	0	0	0
Total Male Control	0	0	0	0	1	1	1	3	3	4	4	4	5	5	5	5	5
Female Experimental	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0
Total Female Exp	0	0	0	0	0	0	0	0	1	1	1	1	1	1	2	2	2
Male Experimental	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	2
Total Male Exp	0	0	0	0	0	0	0	0	0	0	1	1	3	3	3	3	5
Experiment Day	**40**	**42**	**45**	**47**	**49**	**52**	**54**	**56**	**59**	**61**	**63**	**66**	**68**	**70**	**73**	**75**	**77**
Female Control	0	0	1	0	0	3	0	0	0	0	0	1	1	0	1	1	0
Total Female Control	2	2	3	3	3	6	6	6	6	6	6	7	8	8	9	10	10
Male Control	0	0	1	0	2	2	0	2	2	0	0	0	0	1	0	1	0
Total Male Control	5	5	6	6	8	10	10	12	14	14	14	14	14	15	15	16	16
Female Experimental	1	1	0	3	1	6	3	1	5	3	2	2	6	7	6	7	8
Total Female Exp	3	4	4	7	8	14	17	18	23	26	28	30	36	43	49	56	64
Male Experimental	3	2	3	3	6	7	5	5	8	6	3	4	1	1	2	3	2
Total Male Exp	8	10	13	16	24	31	36	41	49	55	58	62	63	64	66	69	71

Table A.2. Electrophoresis gel band density expressed as a numeric value as assessed by Image J software displaying the expression of *Amblyomma americaum* gene ATP synthase, 16S, tick actin, peroxiredoxin, and damaged DNA binding for the female control (FC), male control (MC), female experimental (FE), and male experimental (ME) groups at the 0, 25th, 50th, 75th, and 95th mortality percentiles

Group	Sex	Mortality	Experiment Day	Tick #	Tick Actin	16S	ATP Synthase	Peroxiredoxin	Damaged DNA Binding
Control	F	0%	0	1	4.66	4.46	6.322	4.742	1.943
Control	F	0%	0	2	6.02	6.25	7.868	6.348	4.194
Control	F	0%	0	3	5.9	5.11	6.656	5.461	3.549
Control	F	0%	0	4	6.28	5.29	4.544	3.335	4.626
Control	F	0%	0	5	6.01	6.83	6.442	6.801	4.701
Control	M	0%	0	6	6.37	8.13	6.605	7.774	4.96
Control	M	0%	0	7	7.48	8.75	9.364	7.694	6.431
Control	M	0%	0	8	6.96	7.97	6.911	7.664	5.117
Control	M	0%	0	9	7.26	8.5	9.481	8.21	8.051
Control	M	0%	0	10	7.35	8.08	9.202	8.273	6.728
Control	F	25%	49	11	4.47	N/A	5.132	4.954	3.179
Control	F	25%	49	12	4.19	4.38	5.173	5.234	5.143
Control	F	25%	49	13	4.26	5.18	5.543	4.124	5.62
Control	F	25%	49	14	6.29	5.71	6.133	4.954	6.62
Control	F	25%	49	15	5.97	5.6	6.584	5.482	6.759
Control	M	25%	49	16	4.72	5.36	5.146	4.99	5.034
Control	M	25%	49	17	5.9	6.1	5.683	7.072	5.873
Control	M	25%	49	18	5.85	5.92	5.707	5.95	5.455
Control	M	25%	49	19	5.72	5.72	5.87	5.894	7.073
Control	M	25%	49	20	N/A	N/A	N/A	N/A	N/A
Exp	F	25%	49	21	7.42	5.18	6.645	6.68	9.027
Exp	F	25%	49	22	6.04	4.82	5.647	5.314	3.303
Exp	F	25%	49	23	6.47	4.92	5.171	4.25	6.76
Exp	F	25%	49	24	5.27	4.93	4.291	5.105	3.94
Exp	F	25%	49	25	5.48	6.07	5.585	5.783	7.508
Exp	M	25%	49	26	4.55	4.84	3.502	3.38	3.577
Exp	M	25%	49	27	3.77	5.21	2.869	3.096	3.694
Exp	M	25%	49	28	3.49	4.98	2.525	2.587	2.445
Exp	M	25%	49	29	4.52	4.95	2.611	3.097	4.008
Exp	M	25%	49	30	3.19	4.4	2.381	2.447	2.316

Table A.2. Continued

Group	Sex	Mortality	Experiment Day	Tick #	Tick Actin	16S	ATP Synthase	Peroxiredoxin	Damaged DNA Binding
Control	F	50%	56	31	3.83	N/A	4.721	2.763	6.337
Control	F	50%	56	32	4.75	4.86	6.85	4.941	8.15
Control	F	50%	56	33	4.51	3.78	4.474	3.086	N/A
Control	F	50%	56	34	5.56	5.1	6.147	3.812	4.59
Control	F	50%	56	35	6.57	4.87	6.203	5.03	4.983
Control	M	50%	56	36	5.33	4.07	4.01	3.143	7.006
Control	M	50%	56	37	4.12	4.98	4.739	3.126	4.819
Control	M	50%	56	38	5.22	5.23	5.151	4.874	6.076
Control	M	50%	56	39	4.62	4.82	5.171	5.189	4.297
Control	M	50%	56	40	5.99	5.01	5.249	5.538	11.437
Exp	F	50%	56	41	5.43	4.92	5.15	7.231	4.769
Exp	F	50%	56	42	4.37	4.61	5.388	7.457	4.738
Exp	F	50%	56	43	5.22	5.22	4.73	7.975	4.204
Exp	F	50%	56	44	5.06	4.66	4.888	5.876	4.84
Exp	F	50%	56	45	6.14	4.49	5.368	7.763	4.75
Exp	M	50%	56	46	3.55	5.98	3.193	4.099	3.654
Exp	M	50%	56	47	3.47	5.94	3.727	4.557	N/A
Exp	M	50%	56	48	5.9	6.42	4.173	4.676	3.309
Exp	M	50%	56	49	5.17	6.34	5.043	3.911	5.066
Exp	M	50%	56	50	5.22	6.36	4.526	4.953	3.029
Control	F	75%	63	51	4.56	3.25	4.354	2.42	4.963
Control	F	75%	63	52	6.19	4.29	5.645	4.071	7.042
Control	F	75%	63	53	6.26	4.86	4.928	2.787	6.907
Control	F	75%	63	54	6.37	5.53	6.048	4.667	7.543
Control	F	75%	63	55	7.03	5.02	6.823	5.562	8.299
Control	M	75%	63	56	4.93	3.66	4.24	3.833	4.472
Control	M	75%	63	57	3.1	4.04	1.957	3.822	N/A
Control	M	75%	63	58	5.01	5.18	3.938	4.198	3.455
Control	M	75%	63	59	4.14	4.59	3.184	4.613	3.736
Control	M	75%	63	60	7.2	5.13	5.703	4.969	7.357
Exp	F	75%	63	61	6.08	5.41	5.023	5.589	8.063
Exp	F	75%	63	62	6.87	6.67	6.341	8.409	6.409
Exp	F	75%	63	63	5.25	6.27	4.709	6.273	4.477
Exp	F	75%	63	64	3.25	6.45	5.711	8.554	4.429
Exp	F	75%	63	65	6.17	6.12	5.628	6.575	3.368

Table A.2. Continued

Group	Sex	Mortality	Experiment Day	Tick #	Tick Actin	16S	ATP Synthase	Peroxiredoxin	Damaged DNA Binding
Exp	M	75%	63	66	4.13	4.87	3.777	7.094	2.923
Exp	M	75%	63	67	2.33	3.9	2.948	3.958	3.29
Exp	M	75%	63	68	4.42	4.36	3.759	4.123	4.354
Exp	M	75%	63	69	3.36	4.92	3.402	4.796	3.257
Exp	M	75%	63	70	3.36	5.47	3.82	3.685	3.525
Control	F	95%	77	71	3.54	3.27	3.83	1.183	3.742
Control	F	95%	77	72	4.86	5.51	5.738	3.503	3.57
Control	F	95%	77	73	4.4	4.96	4.897	2.796	5.429
Control	F	95%	77	74	5.87	5.32	6.284	5.134	6.16
Control	F	95%	77	75	4.74	5.25	5.141	3.88	3.729
Control	M	95%	77	76	2.38	4.3	2.823	3.583	2.432
Control	M	95%	77	77	6.07	5.1	4.841	4.03	5.854
Control	M	95%	77	78	3.15	4.42	3.062	3.35	3.362
Control	M	95%	77	79	3.72	4.57	3.33	N/A	5.019
Control	M	95%	77	80	2.86	3.41	3.033	N/A	4.297
Exp	F	95%	77	81	6.51	5.46	5.831	5.553	6.567
Exp	F	95%	77	82	7.26	6.35	7.004	8.403	8.104
Exp	F	95%	77	83	7.76	6.08	6.186	6.606	7.717
Exp	F	95%	77	84	6.41	5.53	5.445	7.891	4.58
Exp	F	95%	77	85	7.75	6.34	7.131	8.496	8.619
Exp	M	95%	77	86	3.48	4.57	3.161	4.645	3.238
Exp	M	95%	77	87	3.41	4.78	3.038	4.545	2.463
Exp	M	95%	77	88	6.15	5.33	5.168	7.197	5.474
Exp	M	95%	77	89	3.86	4.9	3.341	5.295	2.41
Exp	M	95%	77	90	5.85	4.55	6.086	7.589	7.234

APPENDIX B

STATISTICAL ANALYSIS TABLES

Table B.1. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene ATP synthase between control and experimental groups paired by mortality indicator subset and gender with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Female Experimental	1.0000
0%	Male Control	Male Experimental	1.0000
25%	Female Control	Female Experimental	1.0000
25%	Male Control	Male Experimental	0.0039
50%	Female Control	Female Experimental	1.0000
50%	Male Control	Male Experimental	0.9995
75%	Female Control	Female Experimental	1.0000
75%	Male Control	Male Experimental	1.0000
95%	Female Control	Female Experimental	0.9244
95%	Male Control	Male Experimental	0.9994

Table B.2. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene ATP synthase between male and female groups paired by mortality indicator subset and treatment with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Male Control	0.1440
0%	Female Experimental	Male Experimental	0.1440
25%	Female Control	Male Control	1.0000
25%	Female Experimental	Male Experimental	0.0033
50%	Female Control	Male Control	0.9978
50%	Female Experimental	Male Experimental	0.9832
75%	Female Control	Male Control	0.2888
75%	Female Experimental	Male Experimental	0.1469
95%	Female Control	Male Control	0.2841
95%	Female Experimental	Male Experimental	0.0566

Table B.3. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene 16S between control and experimental groups paired by mortality indicator subset and gender with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Female Experimental	1.0000
0%	Male Control	Male Experimental	1.0000
25%	Female Control	Female Experimental	1.0000
25%	Male Control	Male Experimental	0.7328
50%	Female Control	Female Experimental	1.0000
50%	Male Control	Male Experimental	0.0400
75%	Female Control	Female Experimental	0.0070
75%	Male Control	Male Experimental	1.0000
95%	Female Control	Female Experimental	0.2887
95%	Male Control	Male Experimental	0.9993

Table B.4. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene 16S between male and female groups paired by mortality indicator subset and treatment with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Male Control	< .0001
0%	Female Experimental	Male Experimental	< .0001
25%	Female Control	Male Control	0.9981
25%	Female Experimental	Male Experimental	1.0000
50%	Female Control	Male Control	1.0000
50%	Female Experimental	Male Experimental	0.0292
75%	Female Control	Male Control	1.0000
75%	Female Experimental	Male Experimental	0.0187
95%	Female Control	Male Control	0.9980
95%	Female Experimental	Male Experimental	0.2365

Table B.5. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene tick actin between control and experimental groups paired by mortality indicator subset and gender with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Female Experimental	1.0000
0%	Male Control	Male Experimental	1.0000
25%	Female Control	Female Experimental	0.9424
25%	Male Control	Male Experimental	0.5160
50%	Female Control	Female Experimental	1.0000
50%	Male Control	Male Experimental	1.0000
75%	Female Control	Female Experimental	1.0000
75%	Male Control	Male Experimental	0.7433
95%	Female Control	Female Experimental	0.0122
95%	Male Control	Male Experimental	0.9911

Table B.6. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene tick actin between male and female groups paired by mortality indicator subset and treatment with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Male Control	0.7900
0%	Female Experimental	Male Experimental	0.7900
25%	Female Control	Male Control	1.0000
25%	Female Experimental	Male Experimental	0.0398
50%	Female Control	Male Control	1.0000
50%	Female Experimental	Male Experimental	1.0000
75%	Female Control	Male Control	0.8832
75%	Female Experimental	Male Experimental	0.1138
95%	Female Control	Male Control	0.9654
95%	Female Experimental	Male Experimental	0.0060

Table B.7. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene peroxireodixin between control and experimental groups paired by mortality indicator subset and gender with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Female Experimental	1.0000
0%	Male Control	Male Experimental	1.0000
25%	Female Control	Female Experimental	1.0000
25%	Male Control	Male Experimental	0.0048
50%	Female Control	Female Experimental	0.0004
50%	Male Control	Male Experimental	1.0000
75%	Female Control	Female Experimental	0.0009
75%	Male Control	Male Experimental	1.0000
95%	Female Control	Female Experimental	< .0001
95%	Male Control	Male Experimental	0.3027

Table B.8. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene peroxiredoxin between male and female groups paired by mortality indicator subset and treatment with statistically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Male Control	0.0210
0%	Female Experimental	Male Experimental	0.0210
25%	Female Control	Male Control	0.9940
25%	Female Experimental	Male Experimental	0.0305
50%	Female Control	Male Control	1.0000
50%	Female Experimental	Male Experimental	0.0065
75%	Female Control	Male Control	1.0000
75%	Female Experimental	Male Experimental	0.0609
95%	Female Control	Male Control	1.0000
95%	Female Experimental	Male Experimental	0.6922

Table B.9. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene dmaged DNA binding between control and experimental groups paired by mortality indicator subset and gender with statically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Female Experimental	1.0000
0%	Male Control	Male Experimental	1.0000
25%	Female Control	Female Experimental	1.0000
25%	Male Control	Male Experimental	0.5095
50%	Female Control	Female Experimental	0.9984
50%	Male Control	Male Experimental	0.3063
75%	Female Control	Female Experimental	0.9782
75%	Male Control	Male Experimental	0.9992
95%	Female Control	Female Experimental	0.4413
95%	Male Control	Male Experimental	1.0000

Table B.10. Results for an analysis of variance (ANOVA) test for statistical significance when α=0.05 for the *Amblyomma americanum* gene damaged DNA binding between male and female groups paired by mortality indicator subset and treatment with statically significant comparisons highlighted in gray

Mortality	Variance 1	Variance 2	P-Value
0%	Female Control	Male Control	0.5431
0%	Female Experimental	Male Experimental	0.5431
25%	Female Control	Male Control	1.0000
25%	Female Experimental	Male Experimental	0.2459
50%	Female Control	Male Control	1.0000
50%	Female Experimental	Male Experimental	1.0000
75%	Female Control	Male Control	0.8144
75%	Female Experimental	Male Experimental	0.9077
95%	Female Control	Male Control	1.0000
95%	Female Experimental	Male Experimental	0.2186

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