

AN ABSTRACT OF THE THESIS OF

Emily J. Thompson for the degree of Honors Baccalaureate of Science in Biology presented on May 12, 2014. Title: The accumulation of mitochondrial DNA deletions as *Caenorhabditis elegans* ages

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Though previous genetic research studies performed on *Caenorhabditis elegans* found that high levels of endogenous oxidative stress resulted in decreased health, and that mitochondrial DNA deletions accumulated with age in these organisms, no studies have been conducted to link these two separate findings. This study correlated these separate results by confirming the hypotheses that (I) mitochondrial DNA deletions occur more frequently in older *C. elegans* than in younger *C. elegans*, and finding that (II) mitochondrial DNA deletions occur more frequently in *C. elegans* that experience high endogenous oxidative stress.

A *gas-1* mutant strain of *C. elegans* with known mitochondrial dysfunction and resultant increased levels of endogenous oxidative stress was used in comparison to an N2 wild-type strain. These strains were analyzed in a lifespan assay. Samples at different age-points were taken of each strain and surveyed for mitochondrial DNA deletions using polymerase chain reaction and gel electrophoresis techniques. Statistical analysis of the results found significant support for the hypotheses (I and II).

Keywords: Mitochondria DNA deletions, *C. elegans*, Free Radical Theory of Aging, reactive oxygen species, electron transport chain, *gas-1*, N2

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The accumulation of mitochondrial DNA deletions as *Caenorhabditis elegans* ages

by

Emily J. Thompson

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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The accumulation of mitochondrial DNA deletions as *Caenorhabditis elegans* ages

INTRODUCTION

The Free Radical Theory of Aging

The Free Radical Theory of Aging was developed in 1956 and proposed that free radicals produced during aerobic respiration can accumulate and cause cell damage and death (1, 2). Specifically, free radicals called reactive oxygen species (ROS) are a class of small oxygen-containing molecules with one or more unpaired electrons that are involved in many enzymatic reactions in the body (3). Although always present, ROS particles pose a threat to healthy cells when cells lack proper antioxidant capability, lack appropriate repair mechanisms, are exposed to exogenous sources of ROS, or are produced in excess concentrations within the cell or body (4). ROS can cause oxidative damage to important molecules such as lipids, proteins, and DNA (5). ROS are produced by the mitochondrial electron transport chain as a by-product of normal metabolism.

The Mitochondrion

The mitochondrion is a membrane-bound organelle that exists within the cell cytoplasm of eukaryotic organisms. This organelle is unique in that it contains its own self-replicating circular DNA (mtDNA), which controls the function and replication of the mitochondrion (6). Approximately 92% of the mitochondrial genome consists of coding DNA and the mitochondrial genome of *C. elegans* differs from that of humans by

only one gene (ATP8) and a small amount of noncoding DNA (7). The mitochondrion is responsible for cell energy production by oxidative phosphorylation via the electron transport chain (ETC), as well as the synthesis of a range of important molecules vital to the normal function of cells (8). Oxidative phosphorylation is performed by the ETC, which consists of a series of proteins and molecules that transfer electrons to fuel hydrogen pumps, which in turn promote the production of usable cell energy (6). These molecules are called Complexes I-V, ubiquinone, and cytochrome c (7).

The mitochondrion is also known as a major source of ROS production in cells because it produces ROS as a by-product of typical oxidative phosphorylation. One such ROS molecule is O_2^- , which is produced via the ETC. This molecule is unstable and potentially harmful to cell components, but is normally quickly converted to the more stable molecule H_2O_2 , which is used in cell signaling or is disposed of using cellular enzymes (9).

Mitochondrial DNA Damage and Aging

The Mitochondrial DNA Damage Theory of Aging, a branch of the Free Radical Theory of Aging, proposes that accumulation of mitochondrial DNA damage with time leads to increased rate of ROS production, which then can do more DNA damage. As this deleterious cycle is repeated, an increased level of DNA damage is a possible result (10, 11). Mitochondrial DNA damage can present as mitochondrial DNA deletions (dmtDNAs) (12).

Mitochondrial DNA damage has been implicated in many human diseases. One example of mitochondrial DNA deletions causing negative health effects includes humans with mitochondrial myopathies (13). Mitochondrial myopathies cause patients to have a range of skeletal muscular and neurological problems with a range of severity. A study done

on people with mitochondrial myopathies showed that a proportion of affected patients had dmtDNAs of 7 kilobases in length (13).

***C. elegans* as a Model Organism**

Caenorhabditis elegans (*C. elegans*) serves as an exceptional model organism for genetics research for many reasons. *C. elegans* is a small (1 mm in length) transparent nematode worm that can be found living freely in a variety of temperate soils. It feeds on microorganisms such as *Escherichia coli*. *C. elegans* is eukaryotic with a genome of 9.7×10^7 base pairs in length, which has been fully sequenced. About 35% of this genome contains human homologs, so research done using *C. elegans* is potentially useful in human applications (14, 15). Its life cycle from egg to sexually mature adult is 3 to 4 days and life span from birth to death is 2 to 4 weeks depending of environmental conditions (14).

C. elegans can be easily cultivated in large numbers in laboratories on agar plates seeded with *E. coli* as a food source (15). *C. elegans* can be self-fertilizing hermaphroditic or male (16). Adult hermaphrodites typically lay hundreds of eggs. This high fecundity and short generation time is useful for examining large numbers of *C. elegans* as well as many generations in a relatively short amount of time (15). The life cycle of *C. elegans* consists of four distinct larval developmental stages named L1 through L4. The L1 larvae stage occurs just after hatching and can be maintained for several hours when no food is present. The transition into the other larval stages is achieved by growth and molting. The specific larval stages can be discerned under a light microscope by referencing their size and distinct internal features. *C. elegans* is also

readily available in many different mutant strains with known genetic dysfunction or other deviation from wild-type in order to test certain metabolic hypotheses (17).

***gas-1* Mutant**

This experiment examined two strains of *C. elegans* that have been shown to have different metabolic function. N2 is the wild-type strain, and *gas-1* is the mutant strain with a dysfunctional *gas-1* gene (18, 19). “Wild-type” refers to the normal form and function of the worms as would happen in natural populations. The *gas-1* gene in *C. elegans* encodes a subunit molecule of mitochondrial Complex I, a molecule in the electron transport chain. Mutation in this gene has been shown to negatively affect the lifespan and fecundity of *C. elegans* (19). *gas-1* mutants have reduced metabolic function of Complex-I and increased Complex-II-dependent metabolism (18,19).

A Summary of Previous Research

This experiment was designed to bring together two research projects performed by other groups. One of those projects was on *gas-1* gene dysfunction and mitochondrial function in *C. elegans*. The other was on how dmtDNAs accumulated with age in *C. elegans*.

In their publication titled *Mitochondrial expression and function of gas-1 in Caenorhabditis elegans* (19), Kayser et al. found that mutation in the *gas-1* gene of *C. elegans* mtDNA had a negative effect on the health of this organism due to high endogenous oxidative stress. *gas-1* mutants showed increased sensitivity to volatile

anesthetics used in general anesthesia, reduced fecundity (reproduction of offspring), and reduced life span as compared to wild-type. They also discovered the negative affect on Complex I of the ETC.

The second publication titled *Increased frequency of deletions in the mitochondrial genome with age of Caenorhabditis elegans* (11), Melov et al. found that there was a significant difference in the number of dmtDNAs between young and old *C. elegans*. Their study suggested that, on average, older wild-type worms contained more deletions than younger wild-type worms. They also found that this difference in deletions was more numerous in wild-type worms than between young and old worms of a mutant strain that had a longer life span.

Experimental Design

The N2 wild-type strain was used as a comparison group to the *gas-1* mutant strain. Based on the idea that high ROS production from a dysfunctional electron transport chain in the mitochondria leads to the accumulation of mitochondrial DNA deletions as the organism ages, the hypotheses were that counts of mitochondrial DNA deletions would (I) increase over time with age, and (II) occur at higher rates in the mutant strain. Known mitochondrial dysfunction and resultant increased ROS production in *gas-1* led to the prediction that dmtDNAs would be more numerous in *gas-1* mutant worms than in N2 worms, and that older worms of both strains would have more dmtDNAs than young worms of both strains. Both strain lines were cultured in unison, carefully tracked, and systematically sampled in order to track when and where mitochondrial DNA deletions started to happen. Individual worm samples were taken and

individually stored as both strains aged. The mtDNA of these samples were then individually analyzed using polymerase chain reaction (PCR) and gel electrophoresis. Pictures of the stained gels were taken and analyzed in order to determine if there were any deletions in the mtDNA of the worm samples. Any deletion bands were counted and sized. Statistical analysis was done to prove whether or not the results of this experiment significantly supported the hypotheses.

HYPOTHESES

Two hypotheses were tested in this experiment:

- I. Mitochondrial DNA deletions will be more numerous in older *C. elegans* worms compared to younger worms.
- II. Mitochondrial DNA deletions will occur more frequently in the high ROS producing mutant strain *gas-1* as compared to the wild-type N2.

MATERIALS AND METHODOLOGY

All of the materials and methods used in this experiment are described in detail in Worm Book (20). Any variations on these procedures used or other specific details relevant to the materials and methods applied are listed in this section, as well as basic descriptions of the materials and methods used. Refer to Worm Book for in-depth procedure descriptions (20).

***C. elegans* Strains and Initial Culture Protocols**

The *C. elegans* strains used in this experiment were *N2* (with normal life-span and function), and *gas-1* (with mutant characteristics). The *gas-1* strain was created by exposure to random mutagenesis and genetic screening for mutation within the *gas-1* gene. The Denver lab back-crossed this *gas-1* mutant strain with *N2* worms ten times in order to dilute the presence of mutation in other portions of the genome that could have occurred after the random mutagenesis procedure. The strain progenitors were thawed from Denver lab frozen stocks and cultured on standard Nematode Growth Medium (NGM) plates seeded with *Escherichia coli*. *N2* and *gas-1* strains were kept separate throughout the experiment. A small number of worm strain progenitors were moved to new seeded NGM agar plates and allowed to produce eggs until a sufficient number of eggs were laid on the plates at one time (hundreds of eggs). The eggs were isolated and extracted using standard bleaching protocols (20). Eggs were allowed to hatch on non-seeded NGM agar plates in order to keep the newly hatched worms arrested in the L1 larval stage. After several hours when most of the eggs were hatched, L1 worms were

moved to seeded NGM agar plates and worms were allowed to proceed through their life cycles in synchrony. Worms from these age-synchronized strains were used in all further analyses.

Lifespan Assay and Picking Protocols

Age-synchronized worms were allowed to proceed through their life cycle until the age of 19 days. Throughout this life cycle, individual worm samples were taken at 2-day intervals for the first 11 days of growth, and a final late-stage sample at the age of 19 days. Twelve individual worms of each strain were randomly picked from the NGM agar plates on each sample date and placed in individual micro centrifuge strip-tubes containing 15 μ L of worm lysis buffer (WLB). The strip-tubes were then stored at -80°C until all of the sample dates were completed.

The first collection point was the L1/Day1 stage, the day immediately following bleaching. The process of picking 12 individual worms of each strain into individual PCR tubes and stored at -80°C freezer was repeated on development days 3, 5, 7, 9, 11 and 19, giving a total of 168 worms sampled (12 worms per two strains at 7 time points). The strains were monitored daily so that the age-synchronized worms were moved away from their offspring onto new seeded NGM agar plates in order to ensure that the second generation (F1) progeny of the age-synchronized worms would not be included in the life span analysis.

DNA Extraction

DNA extraction of the total genomic DNA of the single-worm samples was performed according to standard Denver lab protocol (20, 21). This process involves freezing and thawing the sample in succession in order to break up the worm bodies and release the DNA into the buffer. Five freeze/thaw cycles were performed on the worm sample strip-tubes for 7 minutes per each freeze and each thaw. This process was performed on all single worm samples after the final worm sample collection on day 19 was completed. These DNA samples were kept in the original PCR tubes and stored at -20°C.

PCR and Gel Electrophoresis Assay

The polymerase chain reaction (PCR) protocol used was described in the study by Hu et al. (22). The long extension program was designed for the PCR amplification of large fragments from small amounts of mtDNA (associated with the single worm analysis). This long PCR program allowed for the primers to target the mtDNA for amplification, even when that mtDNA was mixed with the total genomic DNA of the worm (22). PCR amplification was performed using MyTaq™ DNA Polymerase (Bioline) with 1 µL DNA sample per PCR reaction and four different sets of primers. Each primer set amplified a different quarter of the mitochondrial genome, approximately. No overlap in these primer sets occurred, so there were some small unsurveyed portions of the mtDNA. The specific primers used for the PCR are listed and

number coded in the Table 1. Gene location information was obtained from the NCBI Gene information database annotation of the *C. elegans* mitochondrial genome (23).

| Primer Set # | Forward and Reverse Primers | Sequence (5' to 3') | Expected Intact Product Size (base pairs) | Gene Location and Position (5' to 3' base) |
|--------------|-----------------------------|--------------------------|---|--|
| 1 | Ce-8852-F | GATTGGCTACATTATT TGGT | 3132 | COX1 (8833) to ND5 (11964) |
| | Ce-11945-R | GCCTAAACATTCTAC CTACG | | |
| 2 | Ce-1910-F | ATTTATGGGATTAGC ACAAG | 3035 | ND1 (1891) to CYTB (4925) |
| | Ce-4906-R | ACTGCTGCTCAAAT CTTAT | | |
| 3 | Ce-5373-F | TATTAGGGGTAATTG CTTTA | 3120 | CYTB (5354) to COX1 (8473) |
| | Ce-8454-R | GCTAAAACCGGTAGA GATAA | | |
| 4 | Ce-12431-F | CATGCTTAGCAAGTTT GGTC | 2880 | ND5 (12412) to 12S rRNA (1535) |
| | Ce-1516-R | ATTGATGGATGATTG TACC | | |

Table 1. Primer set and PCR product information

Each of the four primer sets used in this experiment amplified approximately a quarter of the mitochondrial DNA of *C. elegans*. This allowed for the survey of the entire circular mitochondrial DNA of each individual worm sample. Primer set 1 was used to amplify the mtDNA of *N2* Day1, *N2* Day19, and *gas-1* Day19 worms. Primer sets 2, 3, and 4 were used to amplify the mtDNA of *gas-1* Day19 worms. Primer set 4 was used to amplify *N2* Day1, *N2* Day19, and *gas-1* Day1 worm mtDNA.

Standard gel electrophoresis protocol was used to analyze all PCR samples immediately after amplification using 1% agarose gels stained with Ethidium Bromide. The 1 kb Plus DNA Ladder (Life Technologies - Invitrogen™) was used as the gel standard and 3 µL PCR sample was loaded per well. Negative and positive controls were used for each PCR amplification and gel electrophoresis. The negative control used was sterile molecular water. The positive control used was high quality N2 DNA from a large population of mixed stage worms.

Pictures were taken of the gels and used to score them. Scoring was based on size of DNA bands, in relation to the ladder used. The mtDNA deletion bands were smaller and migrated further down the electrophoresis gel than the expected size of intact mtDNA per primer set (Table 1). Any amplicon smaller in size than the expected PCR product size was counted as a deletion.

Statistical Analysis

Chi-squared (X^2) statistical tests were used to test the two hypotheses, termed alternative hypotheses in statistics. The X^2 tests assessed if an association existed between two variables. The null hypothesis was that the two proposed hypotheses were not true. This test involved using observed counts in comparison to expected counts to calculate a X^2 value that was then used to calculate a p-value, or the probability that the null hypothesis was true. This comparison calculation formula was: $\sum (\text{observed} - \text{expected})^2 / \text{expected}$. In order for the test to be accurate, all expected counts of dmtDNAs needed to be greater than or equal to 5. The degrees of freedom used in the p-value

calculations were calculated by the following formula: $(\text{number of variables}-1)(\text{number of categories}-1)$.

To test Hypothesis I, that mitochondrial DNA deletions would be more numerous in older *C. elegans* worms compared to younger worms, three χ^2 tests were done. The expected values used were Day1 deletion counts, and the Day19 deletion counts were used as the observed values. For test 1, N2 and *gas-1* deletion counts were combined to compare the total number of Day1 N2+*gas-1* dmtDNA deletion counts compared to Day19 N2+*gas-1* dmtDNA deletion counts. For test 1A, the number of Day1 N2-only dmtDNA deletions was compared to the number of Day19 N2-only dmtDNA deletions. Test 1B was done by comparing the number of Day1 *gas-1*-only dmtDNA deletions to the number of Day19 *gas-1*-only dmtDNA deletions. The categories of this analysis were Day1 and Day19. The variables for this analysis were counts of samples with dmtDNAs and counts of samples without deletion bands. This meant that the degrees of freedom for this test was equal to 1.

To test Hypothesis II, that mitochondrial DNA deletions would occur more frequently in the high ROS producing mutant strain *gas-1* as compared to the wild-type N2, one χ^2 test was done. The expected values used were N2 deletion counts and *gas-1* deletion counts were used as the observed values. Day1 and Day19 deletion counts were combined to compare the number of N2 dmtDNA deletions to the number of *gas-1* dmtDNA deletions. The categories of this analysis were N2 and *gas-1*. The variables for this analysis were counts of samples with dmtDNAs and counts of samples without deletion bands. This means that the degrees of freedom for this test was equal to 1.

Counts and values for each χ^2 test were tabulated and calculated using a graphing calculator. P-values for each χ^2 test were calculated using a graphing calculator as well. The significance level used for this analysis was 0.05, so any p-value lower than this was statistically significant as support for the rejection of the null hypothesis. A value larger than this significance level was interpreted as evidence to not support the rejection of the null hypothesis, as support that the hypotheses of this experiment were true.

RESULTS

Mitochondrial DNA Deletions

In the region of the mitochondrial genome amplified by primer set 4, from gene ND5 to gene 12S rRNA (Table 1), many dmtDNAs were found at both Day1 and Day19 samples of both worm strains (Table 2). Refer to Table 2 for counts of deletion bands within the worm samples. The deletion band sizes were approximately 2500 base pairs approximately 500 base pairs smaller than expected (Figures 1 and 2). No deletion bands were detected in any of the worm samples amplified by primer sets 1, 2 and 3.

Primer Set 4 spanned a region of the mitochondrial genome of *C. elegans* from gene ND5 (12412 base) to gene 12S rRNA (1535 base). Primer Set 4 encompassed other coding and non-coding regions of the mitochondrial genome. Listed in order from approximately halfway in gene ND5 where the primer set started to a position near the end of the 12S rRNA coding region where the primer set ended, these regions were: an approximately 500 basepair noncoding region, gene ND6, and gene ND4.

| Primer Set # | Strain of <i>C. elegans</i> | Day1 Gel # Deletion Bands Present (out of 12 samples) | Day19 Gel # Deletion Bands Present (out of 12 samples) |
|---------------------|------------------------------------|--|---|
| 1 | N2 | 0 | 0 |
| | <i>gas-1</i> mutant | N/A | 0 |
| 2 | N2 | N/A | 0 |
| | <i>gas-1</i> mutant | N/A | 0 |
| 3 | N2 | N/A | 0 |
| | <i>gas-1</i> mutant | N/A | 0 |
| 4 | N2 | 5 | 11 |
| | <i>gas-1</i> mutant | 11 | 10 |

Table 2. Counts of deletion bands per each gel-electrophoresis: Primer set sample by age group (N/A = no sample was analyzed for this)

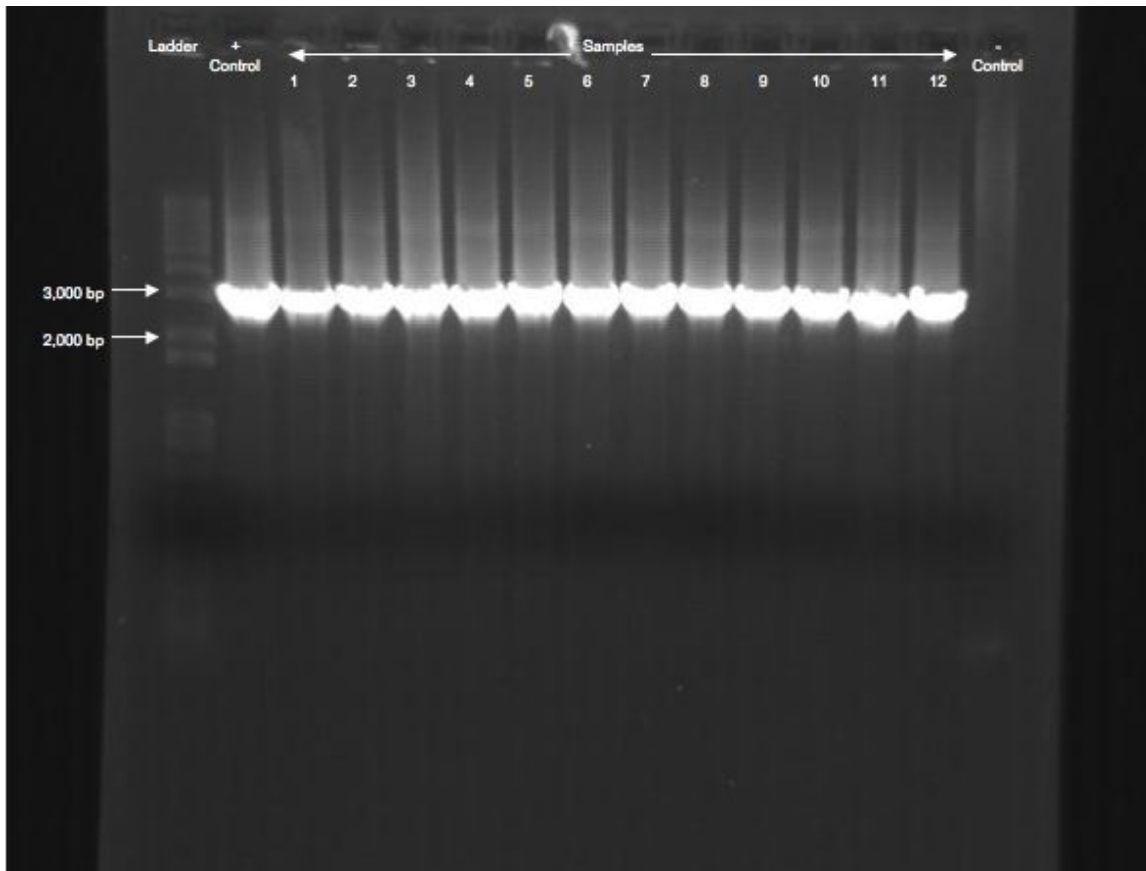


Figure 1. *gas-1* mutant strain Day19 *C. elegans* gel electrophoresis product for PCR amplification using primer set 2 (Ce-1910-F, Ce-4906-R) presenting an absence of deletion bands

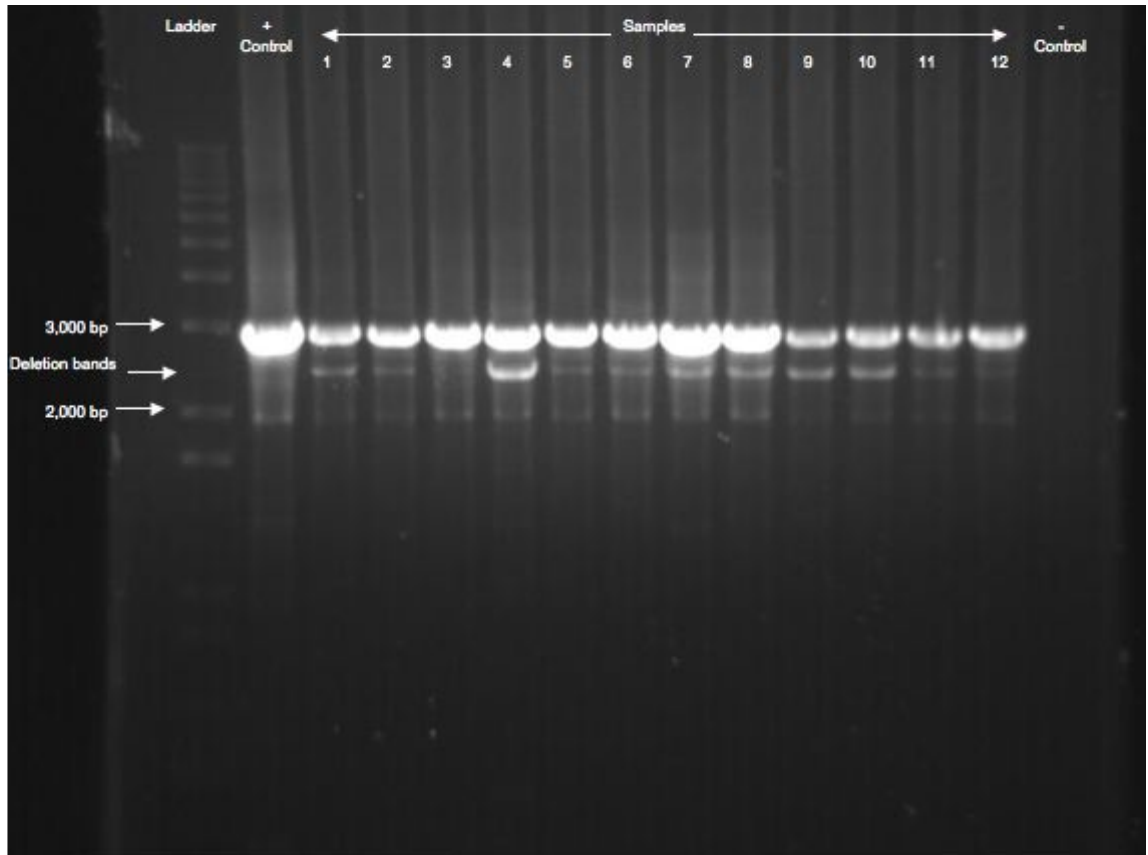


Figure 2. N2 strain Day19 *C. elegans* gel electrophoresis product for PCR amplification using primer set 4 (Ce-12431-F, Ce-1516-R) presenting deletion bands

Statistical analysis

Chi-squared (X^2) tests, with a p-value threshold of 0.05, were used to assess whether or not the hypotheses of this experiment were supported by the data. For data and calculated values for the X^2 value and the p-value, refer to Tables 3-6 and their captions.

The data used for the Hypothesis IX² test are represented in Table 3. The p-value of 0.03038 obtained from the X^2 test statistic strongly supported the rejection of the null hypothesis and supported the hypothesis that total mitochondrial DNA deletions were more numerous in older *C. elegans* worms compared to younger worms.

| | Day1 | Day19 | Total |
|--|------|-------|-------|
| N2 + <i>gas-1</i> # samples with deletions | 16 | 21 | 37 |
| N2 + <i>gas-1</i> # samples without deletions | 8 | 3 | 11 |
| Total | 24 | 24 | 48 |

Table 3. Hypothesis I chi-squared test data: $X^2=4.6875$, $df=1$, $p\text{-value}=0.03038$

The data used for the Hypothesis IA X^2 test is represented in Table 4. The p-value of 0.00044 obtained from the X^2 test statistic very strongly supported the rejection of the null hypothesis and supported the hypothesis that mitochondrial DNA deletions were more numerous in older *gas-1 C. elegans* worms compared to younger *gas-1* worms.

| | Day1 | Day19 | Total |
|---|-------------|--------------|--------------|
| <i>gas-1</i> # samples with deletions | 11 | 10 | 21 |
| <i>gas-1</i> # samples without deletions | 1 | 2 | 3 |
| Total | 12 | 12 | 24 |

Table 4. Hypothesis IA chi-squared test data: $X^2=12.3429$, $df=1$, $p\text{-value}=0.00044$

The data used for the Hypothesis IB X^2 test is represented in Table 5. The p-value of 0.29627 obtained from the X^2 test statistic did not support the rejection of the null hypothesis and did not serve as support for the hypothesis that mitochondrial DNA deletions were more numerous in older N2 *C. elegans* worms compared to younger N2 worms.

| | Day1 | Day19 | Total |
|---------------------------------------|-------------|--------------|--------------|
| N2 # samples with deletions | 5 | 11 | 16 |
| N2 # samples without deletions | 7 | 1 | 8 |
| Total | 12 | 12 | 24 |

Table 5. Hypothesis IB chi-squared test data: $X^2=1.0909$, $df=1$, $p\text{-value}=0.29627$

The data used for the Hypothesis II X^2 test is represented in Table 6. The p-value of 0.03038 obtained from the X^2 test statistic strongly supported the rejection of the null

hypothesis and supported the hypothesis that mitochondrial DNA deletions occurred more frequently in the high ROS producing mutant strain *gas-1* as compared to the wild-type N2.

| | N2 | <i>gas-1</i> | Total |
|---|-----------|---------------------|--------------|
| Day1 + Day19 # samples with deletions | 16 | 21 | 37 |
| Day1 + Day19 # samples without deletions | 8 | 3 | 11 |
| Total | 24 | 24 | 48 |

Table 6. Hypothesis II chi-squared test data: $X^2=4.6875$, $df=1$, $p\text{-value}=0.03038$

DISCUSSION

Statistical Significance

The results of this experiment and subsequent statistical analysis served as support for the two hypotheses tested. Hypothesis I was that mitochondrial DNA deletions were more numerous in older *C. elegans* worms compared to younger worms. Hypothesis II was that mitochondrial DNA deletions occurred more frequently in the high ROS producing mutant strain *gas-1* as compared to the wild-type N2.

The low probabilities that the two hypotheses were incorrect as calculated by the statistical analysis (Tables 3 and 6) served as evidence that the hypotheses were correct. Though the first χ^2 test (Table 3) was appropriately designed to test Hypothesis I, the individual testing of the two strains for a difference in the number of deletions by age was done in order to be thorough and to see if these numbers were different within each strain, as strain type may have had an effect on the results of χ^2 test 1. The high p-value calculated by the 1A χ^2 test could have been due to the fact that N2 wild-type worms didn't have known high levels of endogenous oxidative stress, and that a low number of deletions were naturally present in normal populations of *C. elegans*. The expected counts for the 1B χ^2 test (Table 5) were low (<5), and this low number of expected counts could have led to artificially small p-values. The individual testing of strains by age was not done because the Hypothesis II χ^2 test was sufficient, as age was not a potential influential variable in this hypothesis, that *gas-1* had higher counts of deletions than N2 overall.

Mitochondrial DNA Deletions in Primer Set 4 Region

This experiment showed that deletions were present in the mitochondrial DNA of both N2 and *gas-1* strains at both sampled time points in the region of mtDNA between genes ND5 and 12S rRNA. The deletions found in the region demonstrated that N2 and *gas-1* mtDNA may be naturally polymorphic in this region of the mitochondrial genome. This was a surprising and unexpected result, as it was not hypothesized that all deletions detected by this experiment would be exclusively within one region of the mtDNA of *C. elegans*. No deletions were detected in the other three regions of the mitochondrial genome of any of the samples.

Limitations

The DNA samples that were used were of small volume and pulled out of the freezer many times. This eventually led to some of the samples not amplifying as well as they should. One explanation for was that damage could have been done to the DNA after so many extra freeze/thaw cycles of the samples executed every time a round of PCR amplification was performed on the samples. As a result, the picture of the day 1 N2 strain gel for primer set 4 was very dim, and only 5 deletions were counted with confidence. There was a chance that more deletions were present in the samples but they were not seen clearly enough on the picture to accurately report. This may have skewed statistical data, however 5 deletions was still a significant amount.

The *gas-1* mutant strain used in this experiment was created by exposing the worms to random mutagenesis. These worms were then screened for mutation only

within the *gas-1* gene, so it is possible that other mutations could exist within the genome of this strain. Though the Denver lab back-crossed this strain with N2 to try and dilute the presence of these other mutations, some could still be present that were not detected by sequencing methods. It is possible that deletions, if present, could have affected the *gas-1* worms used in this experiment in some way.

Future Research

A possible next step to take after this research would be to identify the exact region of mitochondrial DNA that was deleted in the samples with dmtDNA bands present. The size of these smaller bands were all very consistent and it would be interesting to see if the deletions were or weren't in the same region of every worm's mitochondrial DNA. The etiology behind these dmtDNAs remains unclear and requires further study to uncover. A sequencing analysis of the region of the mitochondrial genome that contained deletions as well as further analysis on the gaps of unsurveyed mtDNA between primer sets would be helpful in revealing more information about the mitochondrial genome and about the deletions in it. Additionally, since the statistical analysis may have been skewed by low counts of deletions, ANOVA analysis may be a more thorough tool to use to obtain reliable statistics.

Significance

This experiment effectively brought together the research done by Kayser et al. (18) and Melov et al. (11), by finding a significant correlation between increased ROS levels and decreased health, with increased numbers of dmtDNAs with age in *C. elegans*. This correlation has not been made before and is evidence that ROS and oxidative stress on mitochondrial DNA is associated with decreased life span in this model organism. Also, the results of this experiment demonstrate that *C. elegans* are naturally polymorphic in the mtDNA region between the ND5 gene and 12S rRNA gene, which was not previously known, and thus adds to the knowledge of *C. elegans*. Since the genome of *C. elegans* contains human homologs, the findings of this research can be used to guide future research on the effects of oxidative damage on human mitochondrial DNA.

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