Caenorhabditis elegans’ chemotaxis response to attractants has been shown to decline with age. However, research has shown that α-lipoic acid, a potent antioxidant, increases neural function and chemotaxis in older worms, but at higher dosages the beneficial antioxidant properties are reversed, causing prooxidizing effects. In this study, C. elegans were staged to ensure uniform age and treated with various dosages of α-lipoic acid from larva until assayed at various days of adulthood. A chemotaxis assay was used to determine the antioxidant’s effects on the worm’s response to the olfactant 2,3-butanedione. I confirmed that chemotaxis declines with respect to age; however unlike what was hypothesized, both the low dose of 25 μM and high dose of 50 μM α-lipoic acid treatment significantly increased chemotaxis when compared to untreated worms for day 1 and day 5 adult worms with 50 μM also significantly increasing the chemotaxis of day 3 worms. The difference between using 25 μM and 50 μM α-lipoic acid is insignificant, and no prooxidizing effects were observed using these dosages. The results of my day 1 control contradict with the prior research study conducted by Brown, et al. Thus, I suggest further research be conducted regarding this. My study contributes to research concerning C. elegans aging and the roles antioxidants play in this process.

Introduction

A Model Organism

Caenorhabditis elegans is considered a model organism, used extensively in a variety of research areas. Its genome has been completely sequenced with thousands of strains with various mutated genes available for purchase for research purposes (Ardiel and Rankin, 2010). The simplicity of this nematode makes it an ideal organism for study. For example, its nervous system shares many similar functions, neurotransmitters, and sensory pathways with the human nervous system, making it applicable to future research and findings for the human body (Chen, et al., 2013). Anatomy, developmental stages, and reproduction have all been deciphered. The life span of C. elegans is very short lived, including four separate larval stages that take around two and a half days to complete (Braeckman and Vanfleteren, 2006). Many generations can be produced in a short amount of time, versus the long generational periods of humans. This allows for experimental opportunities like knocking out genes or drug testing that may not be possible with or unethical using humans as subjects. At about 1.2-1.5 mm in length and transparent, the nematode can easily be observed and counted under a dissection microscope (Braeckman and Vanfleteren, 2006). Lastly, C. elegans is very easy to grow in the lab. It can grow on an agar plate and feed on the bacteria Escherichia coli as its main source of food. With its easy management, short generation time, and the immense amount of scientific knowledge regarding its biology, C. elegans is an ideal specimen for undergraduate research settings.
Chemotaxis: A Growing Research Field

From the time *C. elegans* became an experimental organism about fifty years ago until now, it has been applied to many facets of biology from medicine to genetics, neuroscience to chemistry. Its nervous system has by far been studied the most extensively. With 302 total neurons, the *C. elegans* nervous system can be subdivided into different classes based on structure, including interneurons, sensory, and motor neurons (Bargmann, 1993). Turning our attention to the sensory neurons, various chemosensory neurons that take part in the process of chemotaxis and chemosensation where the worm moves towards or away from a chemical substance have been deciphered. Many water-soluble and volatile chemicals have been identified as either attractants or repellants for *C. elegans*. The worm detects chemicals via olfaction, with over 5% of the worm’s genes devoted to sensing chemicals within its environment (Bargmann, 2006). Something even more interesting is the fact that these sensory neurons not only have a role in chemical sensation but also play a regulatory role in the worm’s lifespan (Bargmann, 2006). Thus, it would be interesting to look into neuronal function in regards to chemotaxis and its relationship to the age of the worm.

α-lipoic acid in relation to chemotaxis and the aging process

Studies have shown that a variety of natural compounds can increase the lifespan of *C. elegans*. Some examples include studies performed by Yu, et al and Brown, et al using *ginkgo biloba* extract, epigallocatechin gallate (EGCG), and α-lipoic acid (LA). The neurological degeneration that often accompanies the natural aging process has been shown to drastically slow down when the worm is treated with these natural compounds (Brown and Luo, 2010). Studies using herbal supplements like *Cinnamomum cassia* bark and *ginseng* root have also shown evidence in lengthening life span in *C. elegans* (Yu, et al, 2010). With increasing research on what may increase lifespan in the worm, this could potentially be applicable to humans since neural research on the worm has revealed to us a lot about the human nervous system. In this research, I explored the antioxidant α-lipoic acid and its effects on olfactory chemotaxis of *C. elegans*.

α-lipoic acid, or 1,2-dithiolane-3-pentanoic acid, is naturally synthesized in the mitochondrion, playing a role in mitochondrial energy metabolism. It is also known to accumulate in many of the human body’s tissues, serving other functions as a modulator of inflammatory pathways (Shay, et. al, 2009). It is known that worm chemotactic behavior declines with age; however, research has shown that while treatment with α-lipoic acid has no significant effect on younger worms, it greatly increases the chemotaxis of aged *C. elegans* (Brown, et al, 2006). In Brown, et al’s research, only day 1 and day 5 adult worms’ chemotactic behavior was measured. To extend this area of research, I contributed by collecting data from day 1, 3, and 5 treated adult worms in order to see if α-lipoic acid has a slow, increasing effect on each stage of the worm’s adulthood. In a study done by Wolkow on *C. elegans* aging, it was shown that day 8 adult worms barely moved from the initial spot they were placed on in a chemotaxis assay plate due to the substantial decline in their locomotive ability, so I considered this an upper age limit and did not collect any data close to this age since I would most likely not be able to obtain a control CI value (Wolkow, 2006). I stuck with data collection in the day 1-5 adult range. In addition to this, I wanted to see how different concentrations of α-lipoic acid affect the chemotactic behavior of *C. elegans*. Research has shown evidence of the “double-edged effects” of antioxidants. In a study by Bouayed and Bohn, they showed that at low concentrations, beneficial antioxidant properties were exhibited in humans, but at high concentration treatments, the end result reversed
itself as harmful, even fatal, as free radical promoting activity occurred (Bouayed and Bohn, 2010). In another study with a different antioxidant, PC12 cells were treated with different concentrations of the antioxidant EGCG, and similar results were produced in that higher concentrations of EGCG treatment caused increased levels of oxidative stress within the cells’ mitochondria (Raza and John, 2005).

Experimental Purpose
My research studied the effects of different concentrations of α-lipoic acid on the chemotaxis of C. elegans. As briefly mentioned above, Brown, et al. have shown that the effects of antioxidant treatment in the enhancement of chemotaxis are not evident in day 1 worms, however, chemotaxis is significantly enhanced in day 5 treated worms. My experimental purpose was to observe chemotaxis at various stages of adulthood by repeating day 1 and 5 and adding day 3 to see if antioxidant treatment causes significant effects on chemotaxis index in day 3 worms. By increasing treatment concentration from the initial 25 μM to 50 μM, I hypothesized that the higher dosage will reverse chemotaxis enhancement into a chemotaxis decline, possibly killing the worm before it even reaches day 5 of adulthood.

Materials and Methods

Reagents
α-lipoic acid (LA) (mw 206.33 g/mol) was obtained from Sigma, St. Louis, MO (Lot # SLBJ6083V). A stock solution was made in 100% ethanol by dissolving 206 mg of the powdered form in 100 mL ethanol, resulting in a concentration of 10,000 μM.

2,3-Butanedione, used as the olfactant for chemotaxis assays, was obtained from Sigma, St. Louis, MO and was diluted in ethanol for a final stock bottle concentration of 1 M.

The S basal stock was made by mixing 5.85 g NaCl, 1 g K2HPO4, 6 g KH2PO4, 1 mL cholesterol stock (5 mg/mL in ethanol), and water to equal 1 L total. It was autoclaved and stored in 4°C (Stiernagle, 2006).

OP50 Maintenance
An OP50 strain of E. coli was obtained through the Caenorhabditis Genetics Center (CGC). OP50 food source was prepared using methods from WormBook’s “Maintenance of C. elegans” (Stiernagle, 2006). An initial colony was streaked using aseptic technique onto a LB agar plate for colony isolation. LB agar consisted of 10 g Bacto-tryptone (Fluka, St. Louis, MO Lot # SLBJ2446V), 5 g Bacto-yeast (Sigma, St. Louis, MO Lot # SLBF7650V), 5 g NaCl (Fisher Scientific, Waltham, MA S271-500), and 15 g bacteriological agar (Sigma-Aldrich, St. Louis, MO Lot # SLBK4421V), all dissolved in 1 L of water with an adjusted pH of 7.5 and sterilized by autoclaving before pouring 10 cm diameter plates. After incubating a colony isolation plate overnight at 37°C, a tube of L broth was inoculated with one OP50 colony and incubated overnight at 37°C. L broth consisted of 10 g Bacto-tryptone, 5 g Bacto-yeast, and 5 g NaCl, dissolved in 1 L of water with an adjusted pH of 7, using 1 M NaOH to adjust, and sterilized by autoclaving in racks of individual glass test tubes. OP50 plates and broths were stored at 4°C throughout experimentation and were made fresh throughout the experimentation.
Nematode Maintenance

N2 Bristol worms were obtained from CGC. Nematode growth media (NGM) plates were made by mixing 3 g NaCl, 17 g agar, 2.5 g peptone, and 975 mL of water in a 2 L flask. After autoclaving, 1 mL of 1 M MgSO4 (stock: 120.4 g MgSO4 in 1 L of water; autoclaved), 1 mL of 5 mg/mL cholesterol in ethanol, 1 mL of 1 M CaCl2 (stock: 55.5 g CaCl2 in 1 L of water; autoclaved), and 25 mL 1 M K2HPO4 buffer (stock: 108.3 g KH2PO4, 35.6 g K2HPO4, and 1 L water; pH 6; autoclaved) was added before pouring into 10 cm diameter plates (Stiernagle, 2006). Plates were left in room temperature overnight and were then seeded using an OP50 culture (methods under “OP50 Maintenance”). Under sterile conditions, about half a milliliter of OP50 broth was pipetted to the middle of the NGM plate and then lawned using a sterile metal “hockey stick”. The lawn was limited to the center area of the plate to prevent worms from crawling up the sides of the plate and drying out. Seeded plates were incubated overnight at 37ºC before storing at 4ºC for up to 3 weeks. The initial plate of worms was chunked, using a sterile scalpel blade to cut a 1x1 cm square, onto new NGM+OP50 plates to grow. Chunking onto seeded NGM was done every 4-7 days to continue the worm lineage. Growth plates with worms were maintained at 21ºC.

Staging of Nematode Cultures

The method of staging worms was critical to ensure uniform age. To stage a seeded NGM plate, about 20 adult hermaphrodite worms were individually picked from a chunked NGM growth plate using a platinum worm pick and transferred to a new seeded NGM plate. Transferred worms were allowed to lay eggs for 2-3.5 hours before being removed off the plate. On the second day after the initial staging, worms were washed with 1 mL of S basal twice and transferred into a 1.5 mL microcentrifuge tube. Worms were allowed to settle into a pellet before pipetting the supernatant off. The microcentrifuge tube was then washed with fresh S basal an additional time and then washed a final time with deionized water to completely clear worms of any outside contaminants. The final pellet of worms was then transferred to a 6 cm diameter 5-fluoro-2′-deoxyuridine (FUdR), obtained from Sigma, St. Louis, MO, NGM plate (3 mL of 1 M FUdR was used for every liter of NGM). The FUdR NGM plates were made exactly the same as the NGM plates mentioned above with the addition of FUdR in a ratio of 3 mL per 1 L NGM as the last step before pouring 10 mL per 6 cm diameter plate. FUdR chemically prevents the hatching of eggs, lessens egg production, and inhibits cell division, so the worms on the plate do not lay any further eggs, and thus the population remains uniform in age (Sutphin and Kaeberlein, 2009). FUdR NGM plates are stored at 4ºC for up to a week.

Treatment Groups

I began by running a control week where worms were not treated with any LA. For treated groups, serial dilutions of the LA stock with the Escherichia coli strain OP50 broth (0.25:100, 0.5:100) gave final concentrations of 25 μM and 50 μM (Brown, et al, 2006). The groups were plated onto the LA or control OP50 lawns from the L1 larval stage until assayed (refer to Table 1). Three independent replicates for each measured day were performed each week; so 3 chemotaxis index (CI) numbers (“see below”) for each treatment and measured day can be averaged. It takes about 3 days for larva to reach adulthood; for example, lay the eggs on Friday, then perform adult day 1 chemotaxis assay on Monday.
Table 1: Experimental layout for weekly data collection. LA was diluted with the *Escherichia coli* food source of the worms before seeding plates.

<table>
<thead>
<tr>
<th>Week</th>
<th>Treatment Group</th>
<th>Day(s) Measured</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>1, 3, 5</td>
<td>n=3</td>
</tr>
<tr>
<td>2</td>
<td>25 μM LA</td>
<td>1, 3, 5</td>
<td>n=3</td>
</tr>
<tr>
<td>3</td>
<td>50 μM LA</td>
<td>1, 3, 5</td>
<td>n=3</td>
</tr>
</tbody>
</table>

Chemotaxis Assay
I obtained data for day 1, 3, and 5 adult worms treated with LA. Chemotaxis assays were run according to the methods of Bargmann, et al (Bargmann, et al, 1993). 5 assay plates were prepared by autoclaving 1.6 g of agar in 100 mL of water. The autoclaved flask cooled in a 50°C water bath before 500 μL 1M potassium phosphate, 100 μL 1M CaCl2, and 100 μL 1M MgSO4 were added. Immediately, 10 cm diameter plates were poured. Chemotaxis plates were left at room temperature overnight before storage at 4°C.

For the experimental assay, a spot was marked 1 cm below the center of a chemotaxis plate, and another two spots were each marked 1 cm from the left and right sides of the plate, one for the olfactant and one for the control (refer to Figure 1).

Figure 1. Standard chemotaxis assay plate where “A” and “B” denote where chemicals will be diffused and worms counted. A= control: 1 μl of 1 M sodium azide; B= olfactant: 1 μl of 2,3-Butanedione + 1 μl drop of sodium azide. “X” is where the worms are initially placed.

Staged worms of two separate 6 cm diameter FUdR NMG plates were washed with 0.5 mL of S basal twice and combined into a single 1.5 mL microcentrifuge tube. Worms were allowed to settle into a pellet before pipetting the supernatant off. The microcentrifuge tube was then washed with fresh S basal an additional two times and then washed a final time with deionized water to completely clear worms of any outside contaminants. During this time, 1 μl of 1 M sodium azide (a paralyzing solution) obtained from Aldrich was added to each of the two peripherally marked spots. The final pellet of worms was then transferred with a 10 μl micropipette to the center spot of the marked chemotaxis plate. 1 μl of the attractant 1 M 2,3-Butanedione was added on top of the azide of the right spot, leaving the left spot alone as the control. Using a Kimwipe corner to dab excess water, the worms were freed to crawl and
allowed to chemotax for 1 hour in room temperature before being counted. A chemotaxis index was used to quantify the results: 

$$\text{chemotaxis index} = \frac{\# \text{ of worms at the attractant location} - \# \text{ of worms at the control}}{\text{total # of worms on the plate}}$$

(Brown, et al, 2006).

**Statistical Tests**
The student t-test, which assumes parametric data, was used to indicate significant differences between the three treatment groups for each age measured. It was also used to compare day 1 and day 5 worms of each treatment group. A p-value of less than 0.05 was considered significant.

**Results**

Day 1 adults treated with 25 μM LA showed a significantly greater increase in chemotaxis than those treated with 50 μM (Figure 2A); however there was no difference observed between the 25 μM and 50 μM treatments in the other two ages. Both LA concentrations significantly increased chemotaxis in day 1 adults. As for day 3 adult worms, 50 μM significantly increased chemotaxis.
but 25 μM treatment did not (Figure 2B). Lastly for day 5 adult worms, both 25 μM and 50 μM LA treatments significantly increased chemotaxis compared to the untreated day 5 worms, but there was no difference observed between 25 μM and 50 μM treatments.

Discussion

In Comparision to the Results of Brown, et al.

In Brown, et al.’s chemotaxis assay experiment, LA increased the chemotaxis index in the day 5 adult worms and produced significant p-values. When the calculated chemotaxis indexes were plotted into a bar graph, the control was the lowest bar with a CI value of about 0.2 and the LA data was the highest bar at about 0.5 CI value. However, the day 1 adult worms’ chemotaxis index did not increase for the treated groups. Both control and treated had CI values around 0.95. In a bar graph, the bars were about the same height.

The results I obtained for day 5 adult worms are similar in comparison to Brown, et al’s in that there is a significant difference between worms treated with LA compared to worms that were not treated with anything (p-value=0.024 for 25 μM and p-value=0.013 for 50 μM). The CI value of my day 5 worms was below 0.4 for the control and treatment with 25 μM LA greatly increased CI to about 0.65 (Figure 2C). However, my day 1 chemotaxis results differ from the day 1 results of Brown, et al. Brown, et al.’s day 1 control chemotaxis index was in the 0.9-1 range, thus there was no way for their day 1 treatment group to significantly increase in chemotaxis, and they cannot say whether or not LA has an effect on day 1 worms. On the other hand, my day 1 control chemotaxis index was around 0.6, and day 1 treatment significantly increased chemotaxis. It is interesting that my day 1 adults have seemingly lower CI index values than my day 3 control worms since day 1 worms are young in age and presumably should have the greatest neural function and chemotaxis index values out of all 3 measured ages.

LA Increases Chemotaxis In All Measured Ages, But Even With LA Treatment Chemotaxis Still Declines Over Time.

25 μM LA significantly increases chemotaxis in day 1 and day 5 adults, and 50 μM LA significantly increases chemotaxis in all three ages. Whether treated or untreated, CI values still significantly decline as worms age from day 1 to day 5 when comparing each CI value of day 5 to that of its corresponding day 1 worms through a statistical t-test (denoted by the double, triple, and quadruple * in Figure 2A and 2C). However, when comparing worms treated with LA to untreated worms of the same age, LA treatment increases chemotaxis. This suggests that the concentrations of LA I used cannot entirely prevent the neural decline of the aging process, but the concentrations of LA I used do help slow down the aging process of C. elegans.

The Difference Between Using 25 μM and 50 μM LA is Insignificant.

50 μM LA treatment was not detrimental to worms as hypothesized. In fact, it was just as beneficial as 25 μM. The 50 μM LA treatment of day 1 adult worms had a lower chemotaxis index value than the day 1 adult worms treated with 25 μM LA (p-value=0.028), but the 50 μM LA treatment still increased CI when compared to day 1 control (p-value=0.034). No difference between 25 μM and 50 μM dosages was observed for day 3 and day 5 worms. 25 μM LA concentration appears to increase AWA neuron function and chemotaxis in day 1 and day 5
worms, and 50 μM LA concentration appears to increase AWA neuron function and chemotaxis for all three ages.

What I should have performed at the start of this research project was a dose response curve for LA. Concentration of the antioxidant would be plotted on the x-axis and worm chemotaxis response on the y-axis. Unlike my experiment where I doubled the concentration of LA from 25 μM to 50 μM, a dose response curve is typically plotted on a logarithmic scale, and graph shapes vary based on different receptor binding mechanisms of different molecules (Motulsky, 1999). A dose response experiment could be of great help in determining the beneficial and possible detrimental effects of LA on chemotaxis and neural function of C. elegans.

**Significance and Further Research**

The significance of my research contributes to the field of developmental biology, adding to the pool of research concerning the aging process of C. elegans and the roles that LA plays in this process. Since I obtained contradictory results compared to that of Brown, et al. for my day 1 control, I suggest that for future research the day 1 control assays should be repeated to see if there is a substantial increase in CI values. CI data could also be gathered in the future for day 2 and day 3 adults. A dose response experiment for LA would be a good experiment to do in the future for this research. Finally, using a different olfactant in the future to examine another type of sensory neuron, for example the AWC neuron, would add to our pool of knowledge concerning the effects of LA on C. elegans neurons and chemotaxis.

**Acknowledgements**

I would like to thank the Belmont University Biology Department for supplies, lab space, and making this research project possible. Thank you members of Team Grammer (Amanda Bigness, Lauryn Bouldin, John Holt, Kyle Tran), members of Team Ragsdale (Chris Bowens, Jacob Dahm), Dr. Robert Grammer, and Dr. Nick Ragsdale for the assistance and guidance I have received throughout this research study. I would also like to acknowledge Ms. Barbara Ward of the Belmont Mathematics Department for her aid in statistical tests and analysis.
Literature Cited


