

The Effects of Omega-3 Fatty Acid and Exercise on Olfaction and Memory on Alzheimer's Disease in Mice

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A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the
Honor Code
and to maintain the integrity of both myself and the College community as a
whole.***

Dana Bacharach
Pledge

Approval by the Senior Thesis Committee:

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Acknowledgments:

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Abstract

Alzheimer's disease (AD) is categorized as type of dementia that is recognized post-mortem by neurofibrillary tangles and beta-amyloid plaque build-up in areas of the brain associated with memory. The main symptoms include: confusion, mood swings, and short-term memory deficits; the incidence rate of AD is predicted to more than double by 2050. Prior research has found that either omega-3 fatty acid and or regular exercise slow the progression of AD. I hypothesized that the combination of omega-3 supplementation and exercise would prevent olfactory and memory decline associated with AD more than one of the treatments alone. My study included twenty-four Alzheimer's model mice broken down into four groups. The Control group was given no treatment and the three experimental groups were given: exercise only, supplemented omega-3 fatty acid rich diet only, and both exercise and omega-3 supplementation. Groups that received exercise treatment ran on a treadmill three days a week at 13 meters/minute for twenty minutes. The groups that received omega-3 fatty acid were given food composed of 2.83% omega-3 fatty acid, as compared to 0.30% in the normal food that is in normal mouse chow. The progression of Alzheimer's was measured before and after treatment through a y- maze test, which measured spatial working memory and a hidden cookie test, which measured olfaction. A paired t-test did find a significant increase in time it took the mice in the Control group to find the hidden cookie after the treatment and a significant decrease in the time in both the exercise and omega-3 only mice spent in the novel arm of the y-maze after treatment. This study found no significant difference between treatments, but a general trend showed the experimental groups performed better than the Control group. It appears that any of the experimental treatments are equally beneficial for AD patients compared to no treatment.

Introduction

Dementia is defined as a set of symptoms that including language difficulty, loss of short-term memory, or poor judgment not due to a vitamin deficiency. Roughly 60% - 70% of dementia cases can be attributed to Alzheimer's disease (AD); 30% are due to other forms of dementia, which include: Frontotemporal dementia or atrophy of the frontotemporal lobes that typically occur early in life, vascular dementia that is caused by stroke or damage to blood vessels in the brain, Lewy body dementia named after the protein deposits in nerve cells, and Parkinson's Disease (Lunde 2007; Mayo Clinic Staff 2016). Roughly 1 in 8 people over the age of 65 currently suffer from AD; according the U.S. Census Bureau, the incidence rate is expected to more than double by 2050 due to the aging Baby Boomers generation (NIH 2016). There is currently no research that shows the causative factors of AD. However, it is thought that there is a higher risk of developing AD when there is high apolipoprotein E (apoE) present in the blood (Rasmussen 2016). There are three different isoforms of the apoE protein named E2, E3, and E4 (Rasmussen 2016). Of the three, the E4 allele is commonly linked to late-onset familial AD (Rasmussen 2016). Studies have found the predisposition to AD stems from the apoE protein's ability to facilitate the formation of neurofibrillary fibers in the brain, which are found postmortem in AD patients (Rasmussen 2016). Additionally, AD has also been associated with high blood pressure and acquired head injuries (Senelick 2015). Although there is no way to predict if a person will develop AD, there are some known symptoms that include: trouble focusing, confusion, mood swings, physical

problems, trouble communicating, and trouble remembering recent events (Senelick 2015). Typically, the first symptoms are declining memory and attention deficits without any apparent cause (National Institute on Aging 2016). Alzheimer's disease can be recognized post-mortem by neurofibrillary tangles, or tangled nerve cells and protein deposits called beta-amyloid plaque build-up in the brain (Senelick 2015). Beta -amyloid plaque, or a buildup of amyloid precursor protein (APP) has been proven toxic to neurons and leads to cognitive delays (Aaseth et al. 2016). Consequently, there has been a large amount of research on how to prevent or reduce amyloid plaques, but nothing has shown notable improvement of cognitive functioning (Aaseth et al. 2016). Furthermore, neurofibrillary tangles are phosphorylated forms of microtubule tau proteins that interrupt signaling cascades in the brain (Brion 1998). Unlike amyloid plaques, which are located outside of neuronal cells neurofibrillary tangles accumulate inside of the cell (Brion 1998).

Similar to the direct causative factors of AD, there is no known cure. Currently, there are medications and lifestyle changes that can be made to slow the progression of the disease. These medications function by regulating neurotransmitters that relay messages between neurons (National Institute on Aging 2016). For mild to moderate AD, patients are typically prescribed cholinesterase inhibitors such as Aricept®, Razadyne®, or Exelon®, which prevents acetylcholine from breaking down inside the synaptic cleft (Čolović et al. 2013; National Institute on Aging 2016). Treatment for more severe AD includes the drug Namenda®, which delays the progression of AD through glutamate regulation (National Institute on Aging 2016). Researchers are still working to

find new and more effective treatments through immunization therapy, drug therapy, and cognitive training (About Alzheimer's Disease). For example, there have been studies on the effects of amyloid beta protein antibodies that have improved cognitive functioning in mice (Tabira 2010). The follow up study on humans utilized a similar protocol and measured cognitive functioning using a mini-mental state examination (MMSE) and a disability assessment scale of dementia (DAD) (Tabira 2010). There were multiple cases of autoimmune encephalitis and the treatment showed no benefit with respect to the MMSE, but the antibody treatment did show a significant difference in the DAD (Tabira 2010). The researchers believed this was due to the method the immunization was given to human because it caused a different immune response than it did with mice (Tabira 2010).

Omega 3- fatty acids have been researched as a more holistic and safe method to slow the advancement of AD (Klodos 2015). In addition to managing heart disease, lowering blood pressure, and reducing triglycerides, fish oil supplements are believed to be beneficial for people suffering from AD (Klodos 2015). Hamazaki et al. (2005) have linked omega-3 fatty acid containing phospholipids to increased blood catecholamine levels, which work against decreased blood catecholamine levels that are linked to AD progression. Catecholamines are chemicals that are typically made by nerve tissue and the adrenal gland to modify vascular activity and play an essential role in regulation of the central nervous system (Martin 2015; Glowinski & Iversen 1966; Hamazaki et al. 2005). The patients who were given a daily dosage of omega-3 fatty acids had a significantly larger plasma norepinephrine concentration than the patients who received

a placebo (Hamazaki et al. 2005). Their findings could be linked to an earlier study that used the vitamins selegiline and alpha-tocopherol to examine the effects on patients with moderate to severe AD. The researchers found that the experimental vitamin group experienced elevated levels of catecholamine similar to what was seen in the study with Omega-3 fatty acid supplements (Sano et al.1997). These findings together suggest fish oil supplements in the correct dosage could have neurological protective properties that may slow cognitive damage and delay the progression of AD.

In addition to omega 3- fatty acid, exercise is a heavily researched has been tested as a treatment for AD. One study found that five months of voluntary exercise significantly decreased extracellular amyloid proteins in both the frontal cortex and the hippocampus of human Alzheimer's patients (Adlard et al. 2005). The frontal cortex and hippocampus are the areas of the brain that function as in short-term memory, mood, attention and planning, and have shown to be affected as AD progresses (Adlard et al. 2005), and have been show to be affected as AD. Researchers believe the decreased extracellular amyloid precursor occurs either through a change in the processing of amyloid precursor protein (APP) or a change in the neuronal metabolism that is thought to change the way the body processes APP (Adlard et al. 2005). Exercise is also advantageous with respect to depression (Cooney et al. 2014). A randomized clinical trial conducted in 2014 revealed that both resistance training and cardiovascular exercise showed significant improvement in depression scores over a 10-week period (Cooney et al. 2014). Depression is a well-known byproduct of AD (Ownby et al. 2005). A meta-regression analysis found AD patients had a disproportionately greater chance of

developing depression than their healthy counterparts (Ownby et al. 2006). Therefore, exercise's anti-depressant properties can provide additional benefits to the overall delay of AD progression. Therefore exercise's anti-depressant properties can provide additional benefits to the overall delay of AD progression.

There are many ways in which the progression of AD AD can be monitored. Multiple studies have linked olfactory deficits to the more severe cases of AD case of AD (Woodward et al. 2017). A neuroanatomical study found that the severities of AD symptoms are correlated with olfaction (Murphy et al. 1990). As the severity of Alzheimer's increased, the olfactory detection threshold also increased (Murphy et al.1990; Woodward et al. 2017). The exact mechanism of how this occurs is not established, but it is a valuable detection tool when assessing the progression of Alzheimer's (Woodward et al. 2017).

Another behavioral symptom of AD is the tendency for patients to favor familiar places and things (Huntley & Howard 2010). In mice, the spatial working memory can be tested using a Y-maze with one arm of the maze defined as the familiar path, which would be established during a preliminary exploration period and the other arm defined as the novel path. In theory, the mice with more progressed Alzheimer's would spend more time in the familiar arm of the y-maze compared to the unfamiliar arm (Bartlett et al. 1995). A decrease in time spent in the novel arm would indicate a decline of working spatial memory. In my study, both working memory and olfaction were measured to determine the extent to which AD had progressed in mice.

While both exercise and fish oil supplements have shown, individually, to effectively prevent neurological decline associated with AD, there has been no research on the combination of these two treatments. I hypothesized that exercise and Omega-3 fatty acid rich food would treatment would have a synergistic effect and slow the decline of memory and olfaction due to the advancement of ADAD more effectively than each treatment alone.

Methods

Animals

The Animal Review Committee (ARC) at Allegheny College approved the protocol used in this study. A total of 24 two to four month old Alzheimer's disease model mice (Jackson Lab: Bace1^{tm1Pcw}) were used in this study. Six mice were used as a control and 18 were randomly assigned to one of the three experimental groups (exercise only, Omega-3 only, exercise and Omega-3). The Control group was composed of AD mice that received no treatment and were provided with standard mouse chow and water *ad libitum*. The first experimental group included AD mice that performed exercise and were provided with standard mouse chow and water *ad libitum*. The second experimental group was composed of AD mice that were given Omega-3 rich food and tap water, but did not perform exercise. The final group included AD mice that were exercised, given Omega-3 rich food and tap water (Figure 1). All mice used in this study were kept under their given conditions for five weeks in a room with a 12/12 hour light/

dark cycle at approximately 21 °C. The mice were housed in pairs based off of their sex and which food they were assigned to eat.

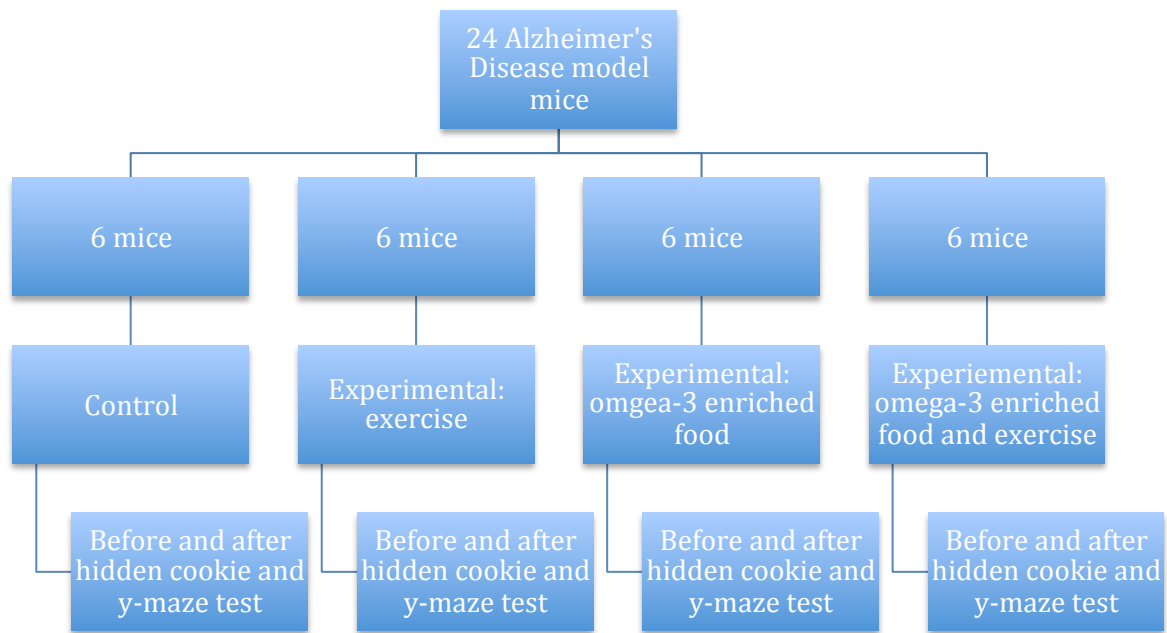


Figure 1. Visual Representation of control and experimental group treatments

Mice receiving omega-3 fatty supplementation were administered omega-3 fatty acid rich food with 2.38% omega-3 (Appendix A) in addition to plain tap water. The groups that did not receive omega-3 fatty acid food were only given standard food with 0.03% omega-3 (Appendix B) and tap water. The mice assigned to an exercise treatment were conditioned to exercise on a standard treadmill that was blocked off by clear plastic into 4 sections (Figure 2). The mice were exercised at a moderate intensity of

approximately 13-meters/ minute for 20 minutes twice a week (Figure 2). The conditioning began by running the mice at 13-meters/ minute for one minute. Each time the mice were exercised, the exercise time was increased by roughly five minutes until they reached the desired 20-minute time period. Following each session, the mice were given a small piece of a peanut butter cookie as a reward. The mice were assigned to their given diet and treatment when they were between two and three months of age and assigned to their groups for the duration of the project.



Figure 2. *Mouse treadmill set-up*

Protocol

The progression of AD was measured using timed olfaction and working spatial memory tests that theoretically, should both decline as the severity of AD increases. Olfaction was tested using the olfactory sensitivity task called the “hidden cookie test”. Mice were placed in a modified 10-gallon aquarium with one end equally divided into 4

quadrants (Figure 3). The walls of the arena were covered with paper to avoid visual distractions. A cookie was randomly placed in one of the four quadrants, and all four quadrants were covered with 1-inch standard pine mouse bedding. Prior to being placed in the modified aquarium, the mice were prompted to smell the cookie so they were familiar with the smell they were expected to find. The measurement was defined as the time it took the mice to successfully find the cookie. The time was recorded once a mouse had dug up the cookie and touched it with one of their paws. If the mice could not find the cookie within ten minutes, the trial was stopped and recorded as 600 seconds. In between each trial, the bedding was replaced to prevent confounding smells.



Figure 3. *Hidden Cookie test to determine olfactory detection thresholds*

Working spatial memory and novel environments were tested using a Y-maze where the left arm was defined and the familiar arm and the right arm as the novel arm (Figure 4). Individually, each mouse was allowed to explore the maze with the right arm blocked off for one minute. After they gained familiarity with the left side of the maze,

the barrier blocking the right arm was removed. The mouse was then allowed to explore the entire maze for two minutes. Once all of the mice completed testing, they were given a piece of a peanut butter cookie as positive reinforcement. The Y-maze measurement was the time the mouse spent in the right, or novel arm in seconds in the two minutes allotted to explore the entire maze. The less time the mice spent in the novel arm indicated the advancement of AD.



Figure 4. *Picture of the Y-maze*

Data and Statistical Analysis

Measurements of olfaction and memory were recorded two times for each mouse: once before treatment, and once after treatment. The differences between before and after measurements in each group were examined using a one-tailed paired t-test. A one-tailed test was chosen over a two-tailed t-test due to the results of similar past study that used behavioral tests to measure AD progression in mice (Robinson 2015). The study showed olfaction ability and memory decrease due to the progression of AD (Robinson 2015). Furthermore, the differences between the Control and experimental groups were tested using a one-way ANOVA on the percent change and the percent of the baseline between before and after treatment measurements. Percent of the baseline was defined as the after measurement divided by the before multiplied by 100 ($(\text{After/Before})(100) = \% \text{ of baseline}$). If the ANOVA test was found to be significant with a p -value less than 0.05, a Tukey post hoc t-test was run to determine if there were significant differences between groups.

Results

Qualitative Behavioral Data:

Although there appeared to be a difference in the weight of the mice between dietary groups after treatment, a t-test found the difference was not significant ($p = .3176$) (Appendix C). Nearly all of the mice were able to complete the exercise regimens excluding two separate occasions where two different mice failed to complete the allotted 20-minute exercise session. In this case, they were removed from the

treadmill and placed back in their cage while the other mice completed their exercise regimen.

Hidden Cookie Test:

The data from the hidden cookie test showed a noticeable increase in time to find the cookie after treatment as opposed to before in all groups excluding exercise (Appendix D). On average, there was a large increase in the time to find the hidden cookie over the course of this study for the Control group, a decrease in the exercise group, a small increase in the omega-3 group, and a very slight increase in the omega-3 and exercise group (Figure 5). The Control group was the only group that showed statistical significance ($p=0.025^{**}$) ($p=0.025$) using a paired t-test, while the three experimental groups were not significant ($p>0.025$) paired t-test while the three experimental groups were not significant ($p>0.025$). On average, the mice in the Control group took 62.7 seconds to find the cookie compared to 262.4 seconds after the treatment (Appendix D). On average, the mice in the control group took 62.7 seconds to find the cookie compared to 262.4 seconds after treatment (Appendix D). The results were further examined through a one-way ANOVA on for the percent of the baseline ($p>0.05$) (Appendix E & Figure 6).

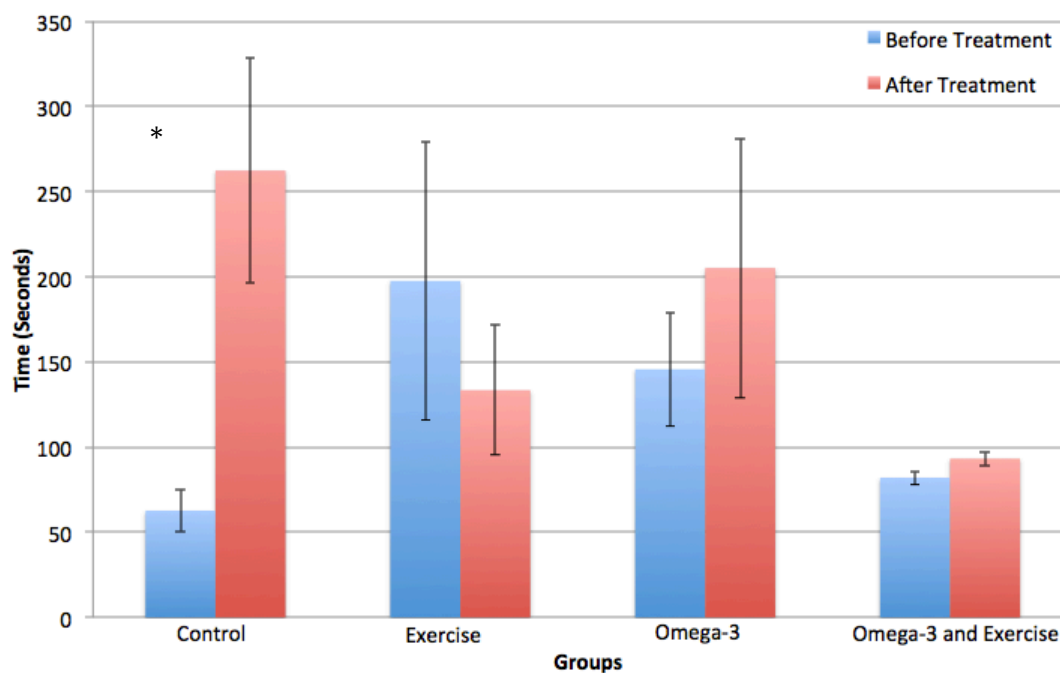


Figure 5. Average time spent finding the cookie before and after assigned treatment.

Error bars depict the standard of the mean.

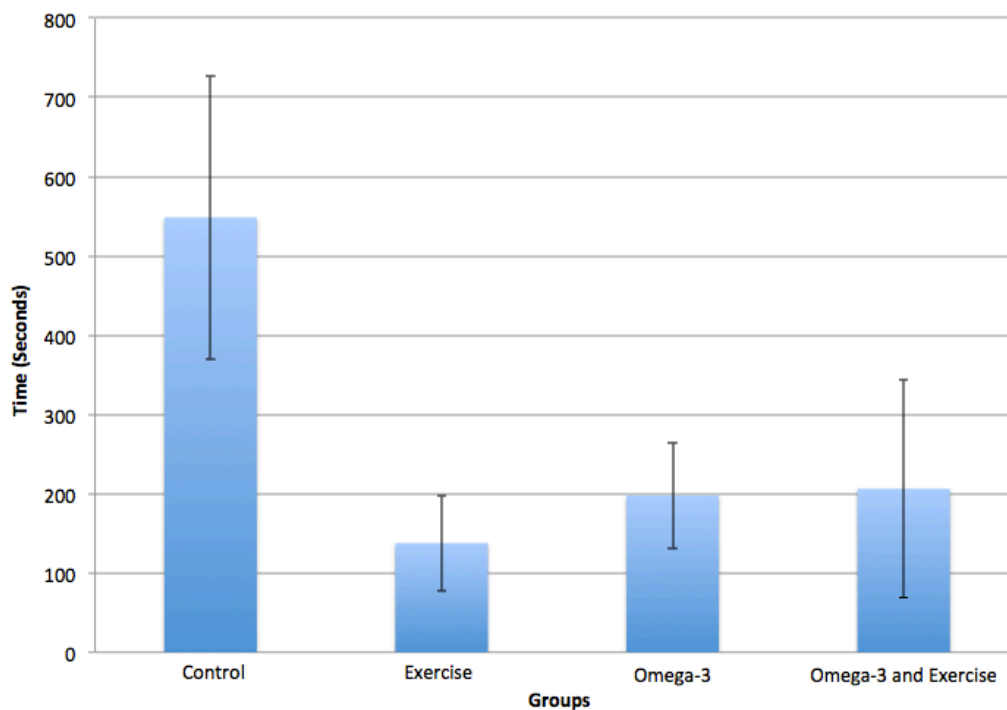


Figure 6. Normalized average change in time for mice to find the cookie before and after treatment measured as percent of baseline.

Y-maze Test:

The Y-maze test showed a predominantly decreasing trend in time spent in the novel arm after treatment compared to before treatment for nearly every mouse (Appendix E). On average, there was a decrease in time, but the difference did not appear to be distinct when the groups were compared (Figure 7). A paired t-test analysis found that the that groups were not were not significantly different ($p>.025$) before and after treatment except the Omega-3 only group ($p=.0152^*$) and the Exercise only group ($p=0.0484$). On average, the Omega-3 group decreased the time they spent in the novel arm from 35.4 seconds to 21.0 seconds and the Exercise group decrease from 33.1 seconds to 25.0 seconds (Appendix F). Between groups comparison showed no statistical difference when the percent of the baseline ($p>.05$) was examined using a one-way ANOVA (Appendix G & Figure 8).

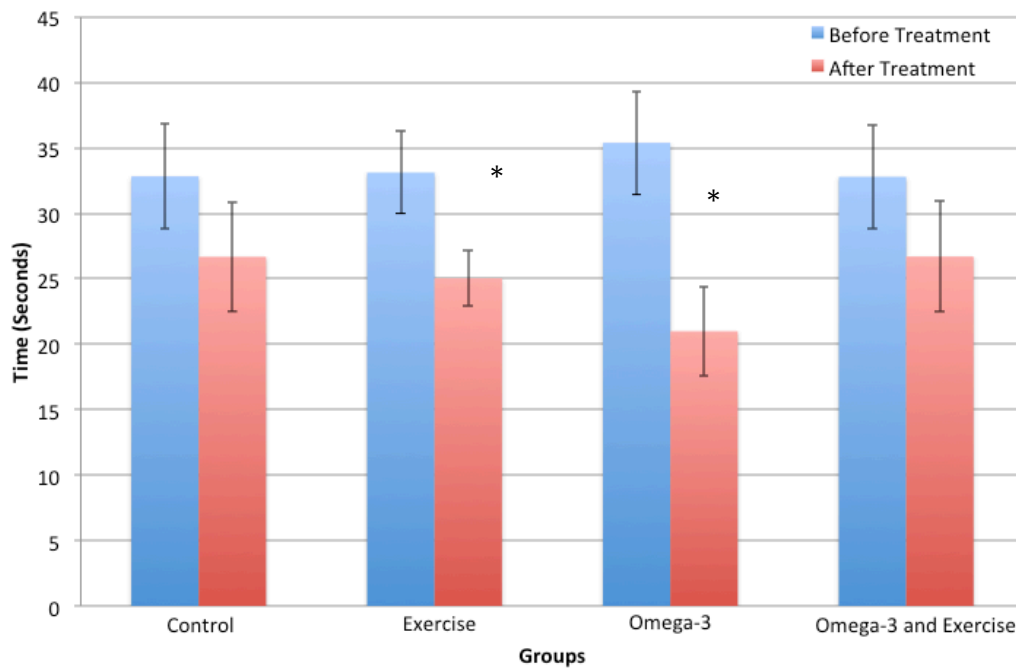


Figure 7. Average time spent in the right, or novel side of the y-maze before and after assigned treatment. Error bars show the standard of the mean.

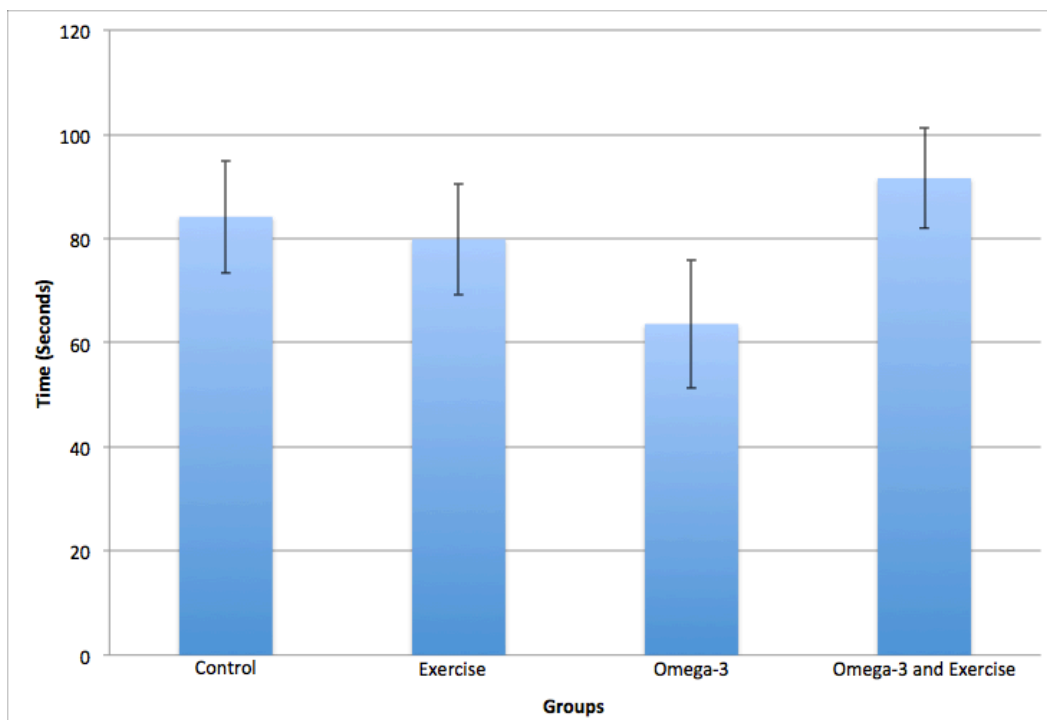


Figure 8. Normalized average change in time spent in the novel arm of the Y-maze, measured as percent of baseline

Discussion

The synergistic effect of omega-3 and exercise on the progression of AD was examined through four groups including a Control group, Exercise group, Omega-3 group, and Omega-3 and Exercise group. Analysis using a one-way ANOVA showed that there was no statistical difference in the effect of treatment for the four groups. The results refute the hypothesis that the omega-3 and exercise together would more effectively delay AD progression than omega-3 or exercise treatment alone.

When examining the results of the hidden cookie test using a one-way ANOVA, there was no significant difference before and after treatment between groups (Appendix E & Figure 6). Furthermore, a paired t-test showed there was a statistical difference before and after treatment in the AD Control group. There seemed to be a smaller difference in time in the experimental groups compared to the Control group, it was not significant (Figure1). A major concern of this study was the uncertainty of how closely correlated olfaction is to AD progression (Murphy 2009). While past studies have shown a correlation between olfactory detection thresholds and AD progression (Woodward et al. 2017), little is known about how olfactory function is affected by neurodegeneration (Murphy 2009). A past study at Allegheny College used a hidden cookie test that tested the mice from three weeks to four months (Gruskiewicz 2013). The time it took each of the mice to recover the hidden cookie closely resembled my results for the beginning portion of the study (Gruskiewicz 2013; Appendix F). The last four measurements during weeks 98, 105, 112, and 119 all were 600 seconds, which

was consistently higher than the average times it took my control mice to complete the test (Gruskiewicz 2013). Additionally, genetic studies have shown discrepancies when comparing olfaction across species (Young et al. 2002). Since the average life span of a typical mouse is roughly two years and 71.4 years for humans, it is difficult to apply mouse studies to humans with respect to time (WHO 2015). Although previous research has established that olfactory deficits indicate AD progression in mice (Woodward et al. 2017), it is unclear what stages of AD progression olfactory deficits begin to occur humans (Ottaviano et al. 2016).

Past studies have found spatial learning memory declines as AD progresses (Bartlett et al. 1995). Under the conditions of this study, there was found to be no significant difference between treatments. Follow-up paired t-tests only found a significant difference in the recorded times before and after treatment in the Omega-3 group. It was hypothesized that the Control group would have the greatest decline of time in the novel side of the y-maze compared to the Omega-3 and Exercise only groups, with the Omega-3 and Exercise group to have the smallest decline in time. These results could be accounted for by the relatively young age of the mice. A previous study conducted at Allegheny College (Robinson 2015) used mice that were 3-4 months old for 16 weeks. On average, her control AD mice spent around 40 % of the time in the novel arm, while mine spent on average 81.3% (Robinson 2015; Appendix D). Both my study and the study conducted by Robinson (2105) did not show any consistent decline in the Control group. Another study found no difference in Y-maze results from the age of 5 to 7 months, but found a significant change in mice from 15 months to 17 months

(Arendash et al. 2001). Over the course of my study the mice ranged in age from 2 to 5 months old. It is highly likely that the mice in my study were not old enough to exhibit spatial learning deficits, which is evident in the results of the Control group. Had my study been conducted on mice that were older, the symptoms of AD would have been more pronounced and a greater difference in time measurements would have been more probable (Arendash et al. 2001). Future studies measuring spatial learning should look at mice through 17 months of age.

Although no significant difference was found between treatments, this could be reflective of the subjectivity that comes with behavioral tests (Ferguson et al. 2013). Since each of the tests was only conducted once before treatment and once after treatment, chance events were more likely to skew my data. For instance, in the hidden cookie test, a mouse could have coincidentally walked right to the cookie and found it by luck rather than solely relying on olfaction. In the Y-maze test, mice often times would defecate during the exploratory portion of the test. They then would exhibit avoidance patterns that could have skewed the measurement. While olfaction and spatial working memory are common indicators of AD progression, the disease symptoms can manifest in a variety of ways (Martinson et al. 1995). A study done on humans included 17 distinct symptoms that were noted by caregivers and many of the participants showed symptoms at different times and in different orders (Martinson et al. 1995). If this were consistent with mice, the two symptoms of AD progression measured in my study would not be sufficient to accurately monitor the severity of the disease.

It is also important to note that for both the Y-maze and hidden cookie test results there are a notable number of outliers. For example, in the hidden cookie Exercise group, all of the mice had increasing times or they stayed roughly the same except one mouse whose time decreased from 600 seconds to 38 seconds to find the cookie (Appendix D). This could have been due to a number of reasons. The anxiety due to the pathology of AD results in behavioral abnormalities starting as early as 1-2 months of age (Webster et al. 2014). Additionally, anxiety disturbances have been found to be more common in AD model mice (Webster et al. 2014). Environmental anxiety could have been caused due to their handling in unfamiliar environments such as the Y-maze, hidden cookie test apparatus and/or, the exercise regimen. Non-systematic behavioral abnormalities from confounding stress would have resulted in skewed data points. Since there is no legitimate way to monitor behavioral abnormalities caused by AD related anxiety as opposed to environmental anxiety, it is not possible to know the cause of an outlying data point.

Circadian rhythm disruptions could have acted as an added confounding variable. A study completed by Webster et al. (2014) found that human AD patients tend to wake frequently at night, which causes them to nap during the day. A disrupted sleep schedule could have led to a less effective exercise session or and an inaccurate behavioral test time. Throughout the study, it was noted the mice often times were napping when checked on and were most active in the evening. There were not particular mice that napped notably more than others, but since the mice were not constantly monitored, there is no way to definitively know. There were not particular

mice that napped notably more than the others, but since the mice were not constantly monitored, there is no way to definitively know.

Overall, this study did not find that omega-3 and exercise had a synergistic effect on AD, although it did raise many questions. Improved studies should further examine what constitutes an effective dosage of both omega-3 and exercise with a larger sample size to better balance outlying data. It would also be beneficial to measure the progression of AD through a more definitive means such as histology, cerebrospinal fluid (CSF) or brain imaging (Mueller et al. 2005). Although histology is very effective at examining AD progression, it can only be performed post mortem, while brain imaging and CSF can be used for a longitudinal study (Mueller et al. 2005). In summary, this study found a significant decrease in olfactory ability in the Control group of mice and a significant decrease in spatial working memory amongst the Omega-3 only group and Exercise only group. Additionally, there seemed to be decreased olfactory and spatial memory ability amongst all groups over time. Although it was not significant, all treatments seemed to delay the onset of AD symptoms as measured by olfaction and spatial memory (Appendix D; Appendix F). Within the protocol design and the number of animals in each group of my study, the results indicate there is no advantage to taking both omega-3 supplements and exercising regularly compared to one of the treatments alone.

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Appendix

Appendix A. Omega-3 enriched food nutrition information

(See link for clearer image:

http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/ml8x/nz uw/~edisp/36142_1750535.pdf)

EnerPro™ Supplement		5BFR	
DESCRIPTION		NUTRITIONAL PROFILE ¹	
EnerPro™ is a high-energy supplement designed for breeding and lactating rodents. The pellets are enriched with the antioxidant vitamin E and contain cod liver oil and flaxseed—both excellent sources of supplemental omega 3 fatty acids.		Protein, %	14.2
		Arginine, %	0.30
		Histidine, %	0.13
		Isoleucine, %	0.27
		Leucine, %	0.32
		Lysine, %	0.34
		Methionine, %	0.08
		Cystine, %	0.09
		Phenylalanine, %	0.20
		Tyrosine, %	0.15
		Threonine, %	0.22
		Tryptophan, %	0.07
		Valine, %	0.26
		Alanine, %	0.36
		Aspartic Acid, %	0.50
		Glutamic Acid, %	0.51
		Glycine, %	0.17
		Proline, %	0.16
		Serine, %	0.18
		Taurine, %	0.00
		Fat (ether extract), %	15.4
		Fat (acid hydrolysis), %	9.0
		Cholesterol, ppm	0
		Linoleic Acid, %	0.24
		Linolenic Acid, %	0.40
		Arachidonic Acid, %	0.14
		Omega-3 Fatty Acids, %	2.83
		Total Saturated Fatty Acids, %	2.58
		Total Monounsaturated Fatty Acids, %	5.76
		Polyunsaturated Fatty Acids, %	5.58
		Fiber (max), %	9.8
		Neutral Detergent Fiber ² , %	2.6
		Acid Detergent Fiber ³ , %	1.1
		Nitrogen-Free Extract (by difference), %	49.7
		Starch, %	2.32
		Glucose, %	0.00
		Fructose, %	0.00
		Sucrose, %	0.00
		Lactose, %	0.00
		Total Digestible Nutrients, %	9.5
		Energy (kcal/g)⁴	3.94
		From:	kcal %
		Protein	0.570 14.5
		Fat (ether extract)	1.386 35.1
		Carbohydrates	1.987 50.4
		Minerals	
		Ash, %	0.9
		Calcium, %	0.02
		Phosphorus, %	0.19
		Phosphorus (available), %	0.08
		Potassium, %	0.21
		Magnesium, %	0.07
		Sulfur, %	0.03
		Sodium, %	0.01
		Chloride, %	0.01
		Fluorine, ppm	0.2
		Iron, ppm	17
		Zinc, ppm	15
		Manganese, ppm	14
		Copper, ppm	3
		Cobalt, ppm	0.28
		Iodine, ppm	0.03
		Chromium (added), ppm	0.00
		Selenium, ppm	0.33
		Vitamins	
		Carotene, ppm	0.0
		Vitamin A, IU/g	88
		Vitamin D-3 (added), IU/g	8.8
		Vitamin E, IU/g	33
		Vitamin K, ppm	0.0
		Thiamin Hydrochloride, ppm	7
		Riboflavin, ppm	1.2
		Niacin, ppm	31
		Pantothenic Acid, ppm	7
		Folic Acid, ppm	0.7
		Pyridoxine, ppm	2.09
		Biotin, ppm	0.0
		Vitamin B-12, mcg/kg	0
		Choline Chloride, ppm	558
		Ascorbic Acid, ppm	0
		1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly. Nutrients expressed as percent of ration on an As-Fed basis except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.	
		2. NDF = approximately cellulose, hemicellulose and lignin.	
		3. ADF = approximately cellulose and lignin.	
		4. Energy (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate x 4, 9, 4 kcal/gm respectively.	
FEEDING DIRECTIONS			
We suggest to begin by including 1-2 pellets of EnerPro™ with current LabDiet® rodent food as a supplement in the bottom of each cage; increase or modify as recommended by attending veterinarian. Begin adding EnerPro™ to feeding program approximately 2 weeks before breeding begins and continue throughout lactation period.			
CAUTION: Perishable - store upon receipt. For laboratory animal use only; not for human consumption.			
9/22/2015			
		TestDiet www.testdiet.com	

Appendix B. Regular omega-3 enriched food nutrition information

(See link for clearer image:

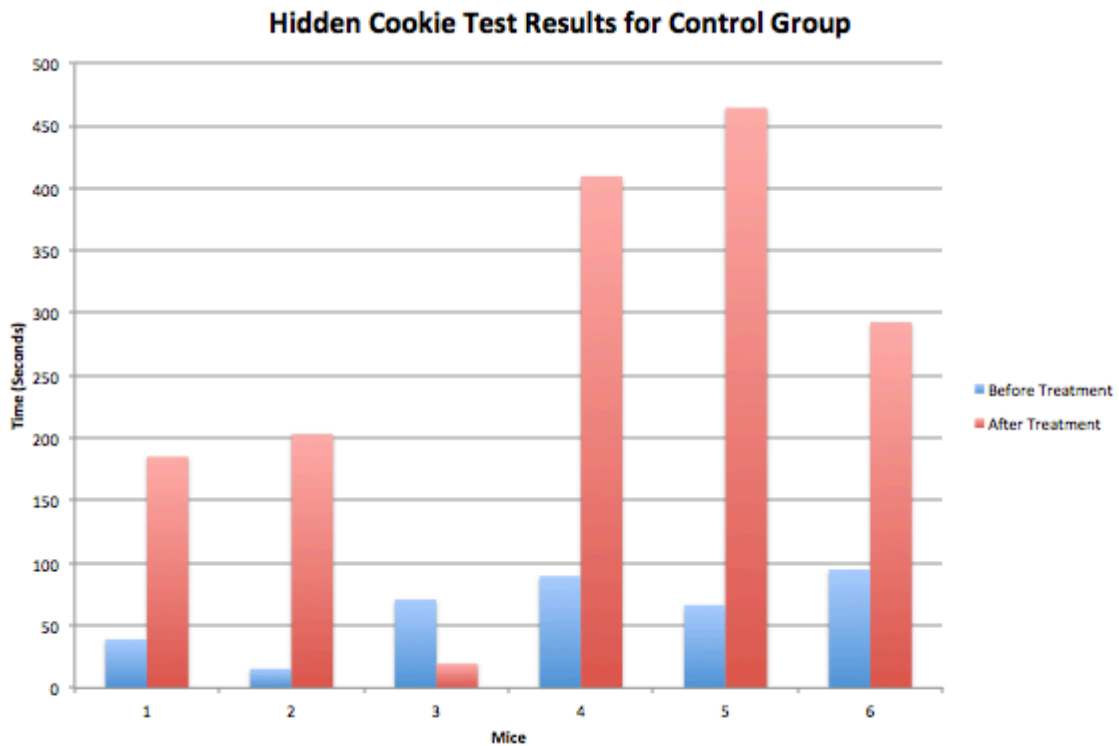
http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrf/midi4/~edisp/ducm04_028021.pdf)

Laboratory Rodent Diet		5001*
DESCRIPTION		CHEMICAL COMPOSITION¹
Laboratory Rodent Diet is recommended for rats, mice, hamsters and gerbils. This diet is a complete life cycle diet formulated using managed formulation, delivering Constant Nutrition [®] . This is paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. It is formulated for life-cycle nutrition; however, it is not designed for maximizing production in mouse breeding colonies. This product has been the standard of biomedical research for over 70 years.		Nutrients²
Features and Benefits		Sulfur, % .036
- Managed Formulation delivers Constant Nutrition[®] - High quality animal protein added to ensure a superior balance of amino acids for optimum performance - Formulated for multiple species for single product inventory - The rodent diet standard for biomedical research		Sodium, % .039
Product Forms Available		Chloride, % .064
- Chow pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1") - Meal (ground pellets)		Fluorine, ppm .15
Other Versions Available		Iron, ppm .240
SLIOD PicoLab[®] Laboratory Rodent Diet (Minimum order required)		Zinc, ppm .85
GUARANTEED ANALYSIS		Manganese, ppm .75
Crude protein not less than .23.0%		Copper, ppm .15
Crude fat not less than .4.5%		Cobalt, ppm .091
Crude fiber not more than .6.0%		Iodine, ppm .099
Ash not more than .8.0%		Chromium (added), ppm .001
Moisture not more than .12.0%		Selenium, ppm .041
INGREDIENTS		Vitamins
Defatted soybean meal, ground corn, dried beet pulp, fish meal, ground oats, dehydrated alfalfa meal, cane molasses, brewers dried yeast, wheat germ, whey, porcine animal fat preserved with BHA and citric acid, wheat middlings, porcine meat and bone meal, salt, calcium carbonate, DL-methionine, choline chloride, cholecalciferol, folic acid, vitamin A acetate, menadione dimethylpyrimidinol bisulfite (source of vitamin K), pyridoxine hydrochloride, thiamine mononitrate, biotin, nicotinic acid, calcium pantothenate, D-alpha tocopheryl acetate (form of vitamin E), vitamin B ₁₂ supplement, riboflavin supplement, ferrous sulfate, manganese oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.		Carotene, ppm .2.3
FEEDING DIRECTIONS		Vitamin K, ppm .1.3
Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times.		Thiamin Hydrochloride, ppm .1.6
Rats: All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.		Riboflavin, ppm .4.7
Mice: Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.		Niacin, ppm .120
Hamsters: Adults will eat up to 14 grams per day.		Pantothenic Acid, ppm .24
For information regarding shelf life please visit www.labdiet.com .		Choline Chloride, ppm .2250
		Folic Acid, ppm .7.1
		Pyridoxine, ppm .6.0
		Biotin, ppm .0.30
		B ₁₂ , mcg/kg .51
		Vitamin A, IU/gm .15
		Vitamin D ₃ (added), IU/gm .4.6
		Vitamin E, IU/kg .42
		Ascorbic Acid, mg/gm .—
		Calories provided by:
		Protein, % .29.829
		Fat (ether extract), % .13.427
		Carbohydrates, % .56.744
		*Product Code
		1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
		2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
		3. NDF = approximately cellulose, hemicellulose and lignin.
		4. ADF = approximately cellulose and lignin.
		5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (see Nitrogen Free Extract) x 4, 9, 4 kcal/gm respectively.
		LabDiet www.labdiet.com

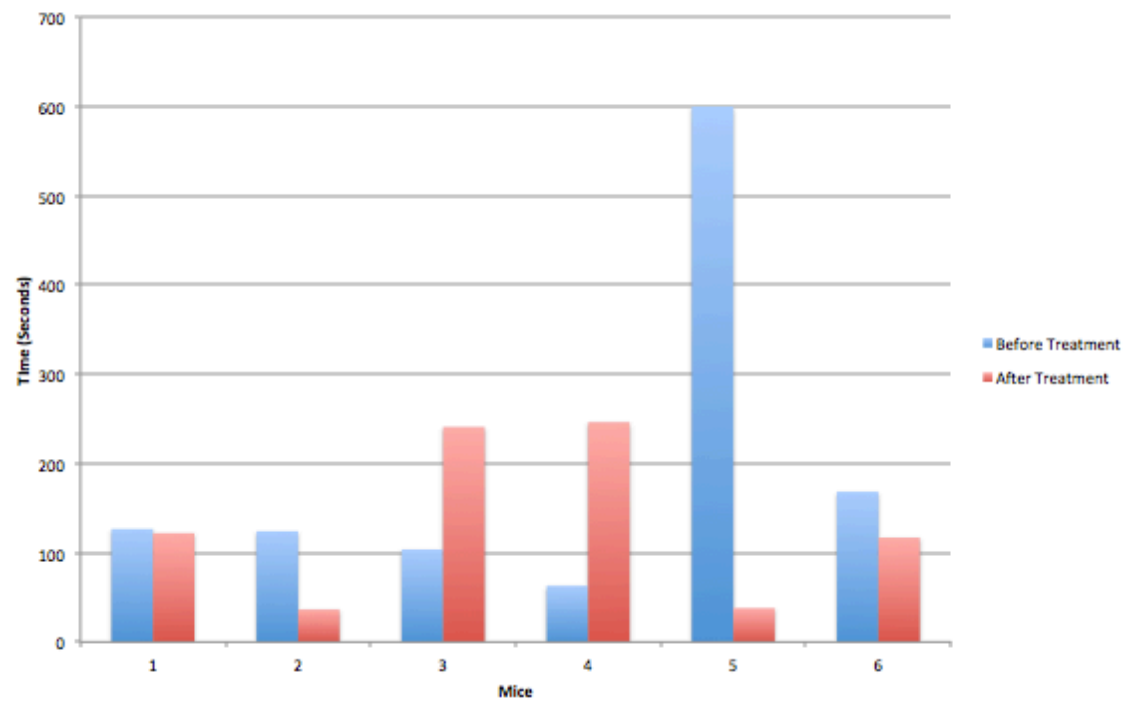
Appendix C. Randomly selected weight in grams of mice grouped by diet and the corresponding average

	M1	M2	M3	M4	Average
Omega-3 Enriched Diet	33.04 g	38.08 g	28.89 g	28.39 g	32.09 g
Regular Diet	25.92 g	23.04 g	28.06 g	30.91 g	26.98 g

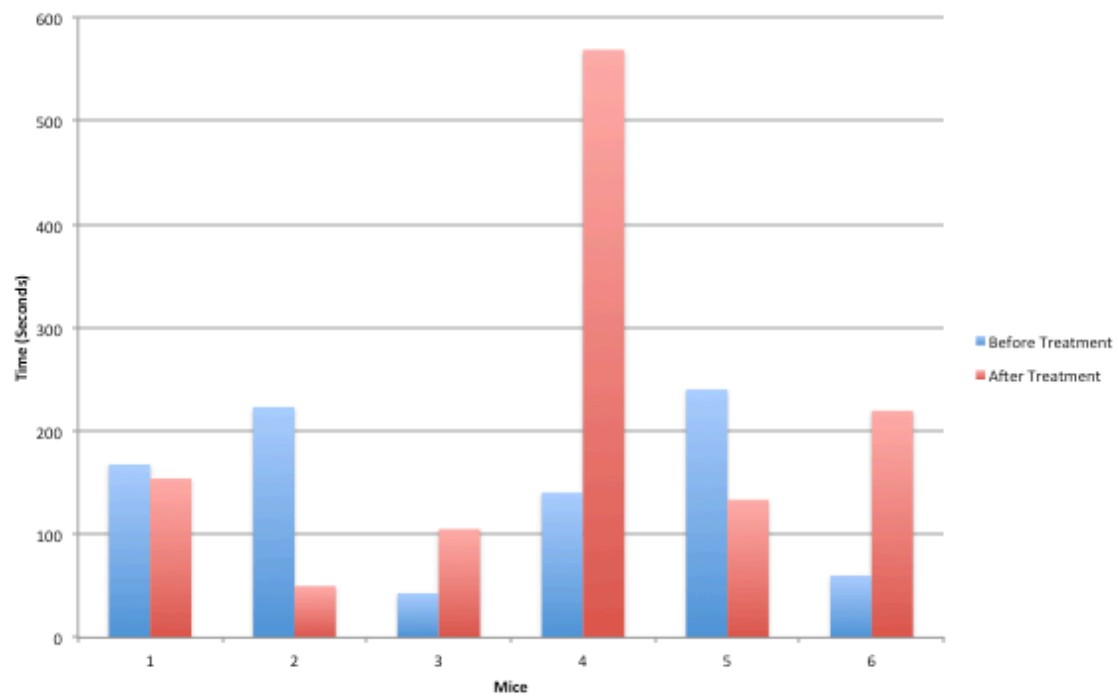
Appendix D. Hidden cookie test results from each individual mouse, grouped by treatment

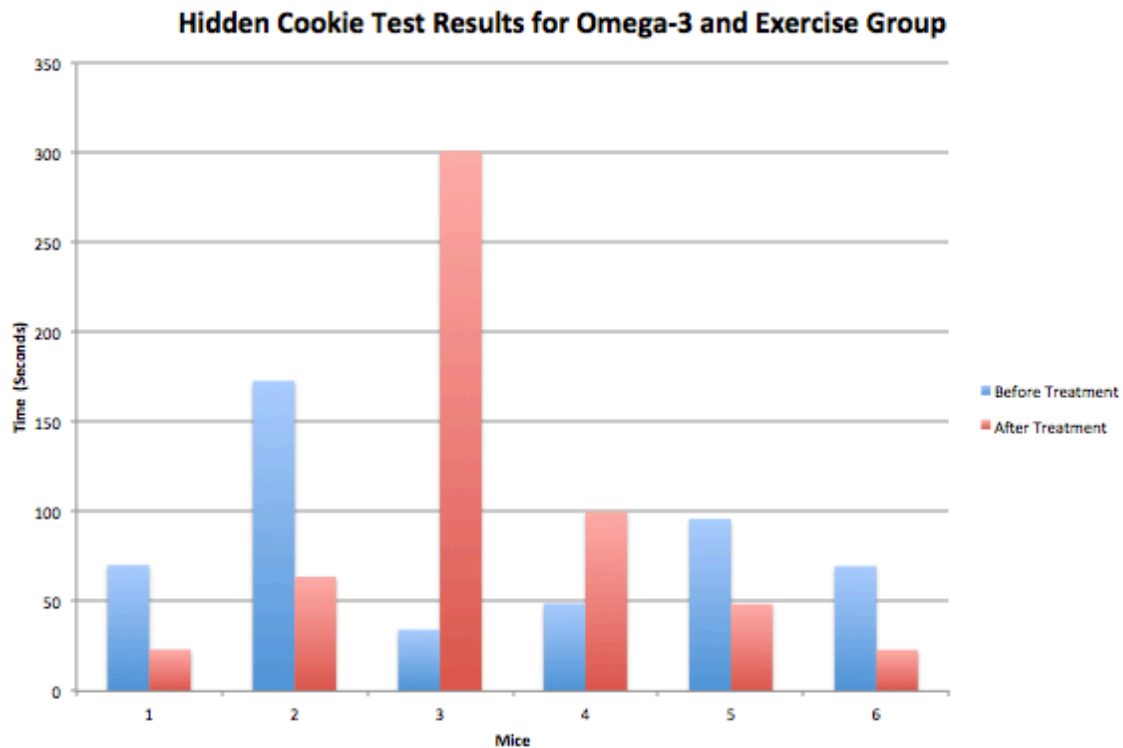


Hidden Cookie Test Results for Exercise Group



Hidden Cookie Test Results for Omega-3 Group





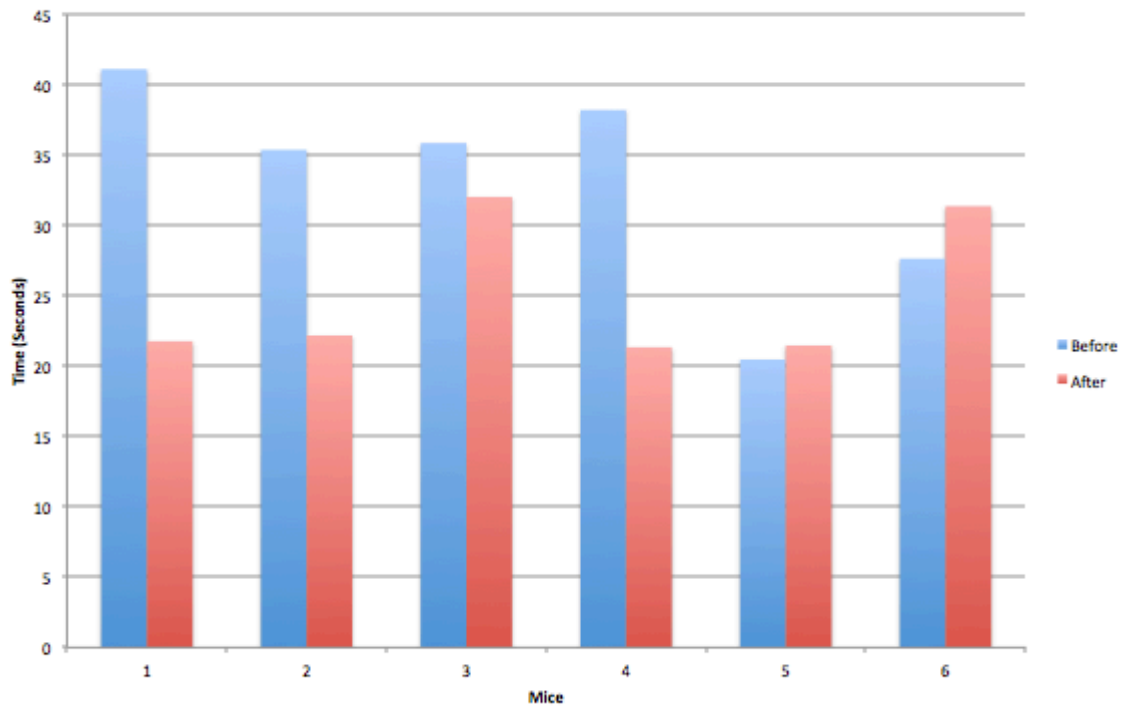
Appendix E. One-way ANOVA results of percent change and percent of baseline for the hidden cookie test

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
HC-Control	HC-Exercise	410.8125	171.9480	-70.459	892.0841	0.1116
HC-Control	HC-Omega	350.5912	171.9480	-130.680	831.8628	0.2074
HC-Control	HC-O and E	342.2140	171.9480	-139.058	823.4856	0.2247
HC-O and E	HC-Exercise	68.5984	171.9480	-412.673	549.8700	0.9779
HC-Omega	HC-Exercise	60.2213	171.9480	-421.050	541.4929	0.9848
HC-O and E	HC-Omega	8.3772	171.9480	-472.894	489.6488	1.0000

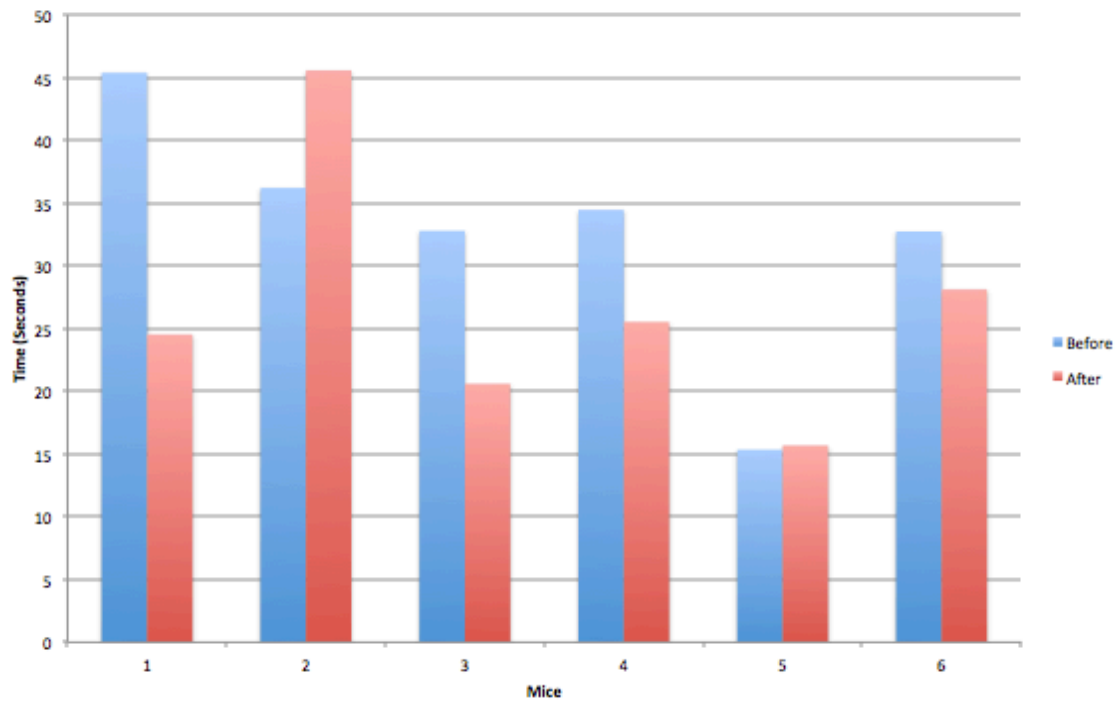
Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
HC-Control	HC-Exercise	410.8125	171.9480	-70.459	892.0841	0.1116
HC-Control	HC-Omega	350.5912	171.9480	-130.680	831.8628	0.2074
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HC-O and E	HC-Exercise	68.5984	171.9480	-412.673	549.8700	0.9779
HC-Omega	HC-Exercise	60.2213	171.9480	-421.050	541.4929	0.9848
HC-O and E	HC-Omega	8.3772	171.9480	-472.894	489.6488	1.0000

Appendix F. Y-maze test results from each individual mouse, grouped by treatment

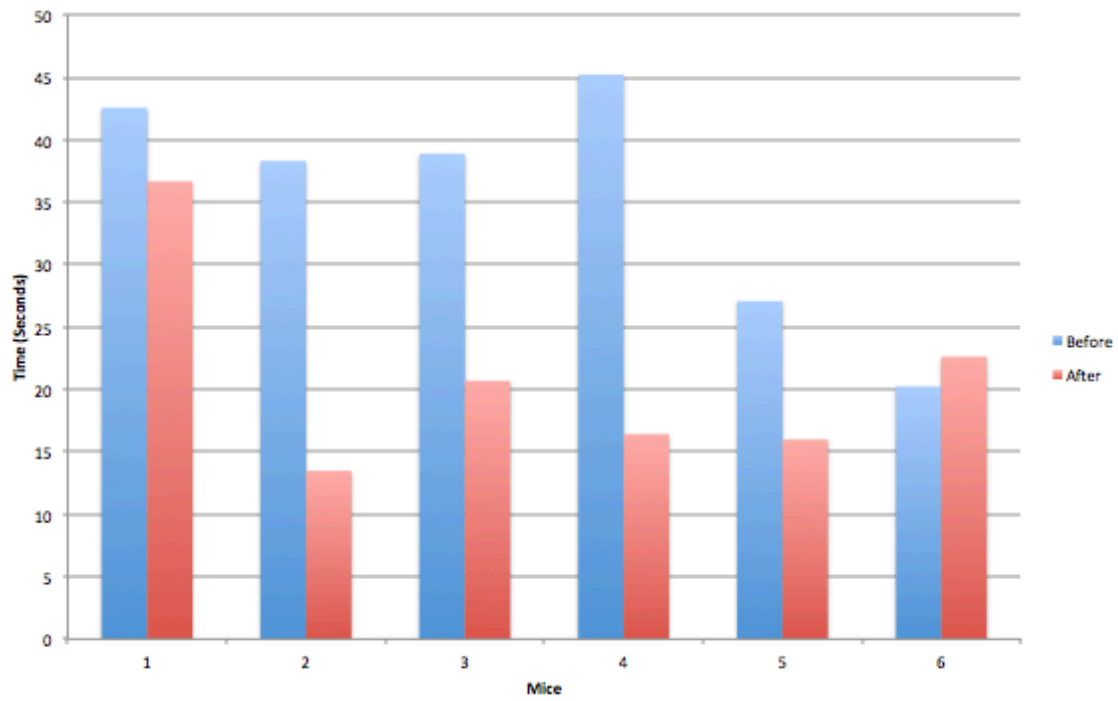
Y-Maze Test Result for Exercise Mice Group



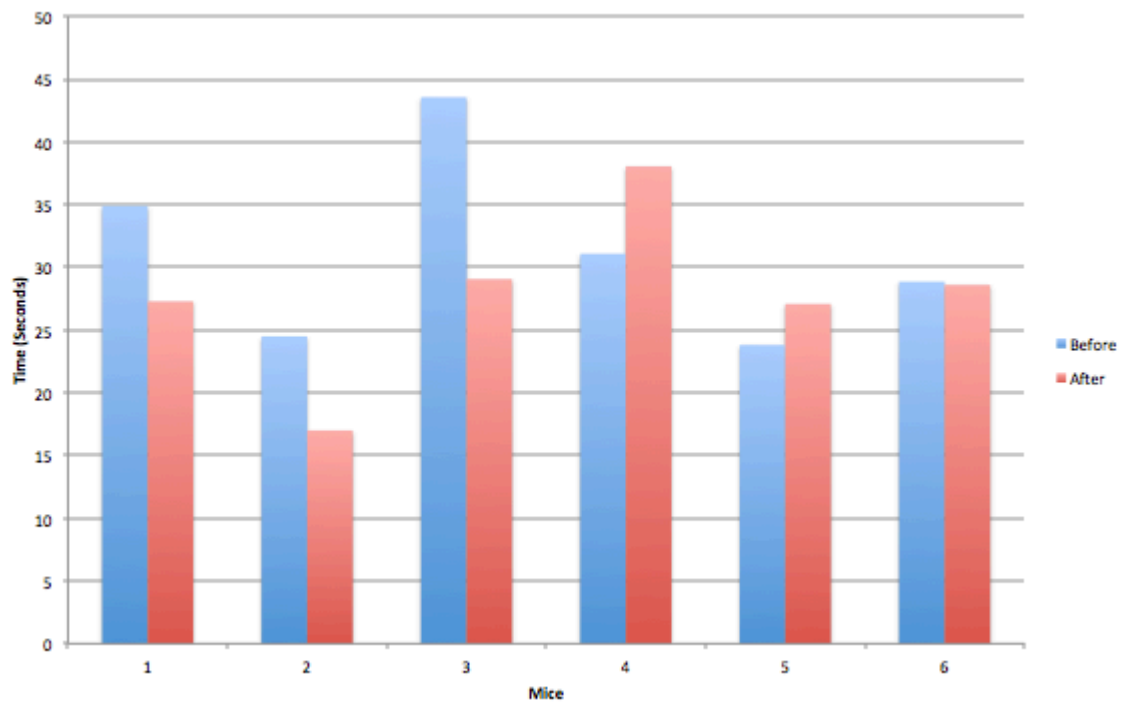
Y-Maze Test Results for Control Mice Group



Y-Maze Test Results for Omega-3 Mice Group



Y-Maze Test Results for Omega-3 and Exercise Mice Group



Appendix G. One-way ANOVA results of percent change and percent of baseline for the Y-maze test

Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
YM-O and E	YM-Omega	27.96707	15.42089	-15.1950	71.12916	0.2964
YM- Control	YM-Omega	20.52683	15.42089	-22.6353	63.68892	0.5548
YM- Exercise	YM-Omega	16.24765	15.42089	-26.9144	59.40974	0.7206
YM-O and E	YM- Exercise	11.71942	15.42089	-31.4427	54.88151	0.8713
YM-O and E	YM- Control	7.44024	15.42089	-35.7218	50.60233	0.9621
YM- Control	YM- Exercise	4.27917	15.42089	-38.8829	47.44126	0.9923
Level	- Level	Difference	Std Err Dif	Lower CL	Upper CL	p-Value
YM-O and E	YM-Omega	27.96707	15.42089	-15.1950	71.12916	0.2964
YM- Control	YM-Omega	20.52683	15.42089	-22.6353	63.68892	0.5548
YM- Exercise	YM-Omega	16.24765	15.42089	-26.9144	59.40974	0.7206
YM-O and E	YM- Exercise	11.71942	15.42089	-31.4427	54.88151	0.8713
YM-O and E	YM- Control	7.44024	15.42089	-35.7218	50.60233	0.9621
YM- Control	YM- Exercise	4.27917	15.42089	-38.8829	47.44126	0.9923