# THE EFFECT OF DIET MANIPULATION ON LEARNING, MEMORY AND ANXIETY

by

Karin A. Pedemonte
A Thesis
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The Requirements for the Degree
of
Master of Arts
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Committee:

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Date: _____ Summer Semester 2018
George Mason University
Fairfax, VA

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## DEDICATION

This is dedicated to my mom, Alicia, who has motivated me to continue with me education and has been there to support me in emotionally and financially; my grandmother, Rebeca, has always been there for me; Rebecca and Leroy who have guided me in all my endeavors and Jose, who has supported me emotionally in this past year.

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I would like to thank the many friends, relatives, and supporters who have made this happen. Drs. Flinn, Shaw and McDonald who were on my committee and provided invaluable help.

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## ABSTRACT

THE EFFECT OF DIET MANIPULATION ON LEARNING, MEMORY AND ANXIETY

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The focus of this research was on the effects of both autoclaving lab feed and soy content in lab feed. Autoclaving sterilizes and cleans equipment through heat and steam. However, autoclaving animal feed can not only affect key nutrients, but also increase the "hardness" of food pellets and produce toxic and carcinogenic products. An additional dietary factor that is often overlooked is soy (phytoestrogen) content. Evidence has found that phytoestrogens have anxiolytic effects in both males and females when assessed in an elevated plus maze (Lephart et al., 2002) and open-field testing (McCarthy et al., 1997). Therefore, it is important to consider both autoclaving and soy content in animal feed when conducting behavioral neuroscience research. Ninety-five C57BL/6J male and female mice were assigned to one of four diet groups prenatally: (1) a non-autoclaved standardized Teklad 7012 (containing soy); (2) an autoclaved standardized Teklad 7012; (3) soy-free Teklad 2020SX; and (4) low-soy Teklad 2018SX. Neither the soy-free nor

the low-soy diets were autoclaved. At 4 months of age, the mice were examined on behavioral tests including: Morris water maze (MWM), fear conditioning (FC), nesting, elevated zero maze (EZM), open field (OF), and grooming. Collapsed across sex, there were no significant differences in MWM, EZM, and OF between dietary groups. However, animals on Teklad 7012 and autoclaved Teklad 7012 extinguished the learned tone/shock pairing in FC significantly slower than animals on low-soy and soy-free diets. Animals on the standard Teklad 7012 diet created significantly better nests than the animals on any other diet. Additionally, animals on standard Teklad 7012 diet groomed less than the animals on low-soy and soy-free, but more than the animals on the autoclaved Teklad 7012. These data show that the dietary effects including autoclaving feed and amount of soy in diet have a significant effect on behavior and are therefore an important factor for behavioral researchers to consider.

# INTRODUCTION

In behavioral neuroscience research, diet is often a factor that is overlooked. Diet manipulation, such as autoclaving and soy content, can have significant effects on animal behavior including memory. These changes in memory are largely due to functional and structural changes in the brain that often involve the frontal cortex, hippocampus and amygdale, all of which have been related to genetic and modifiable lifestyle factors. One important lifestyle factor is diet.

Autoclaving animal feed can produce large effects on behavioral neuroscience research. Autoclaving is a common lab process that uses heat and steam to eliminate microorganisms and therefore sterilizes and cleans equipment (e.g. cages, enrichment objects, water spigots). Autoclaving causes a decrease in water-soluble vitamins, calcium, ascorbic acid, fat-soluble vitamins (Taciak et al., 2011), fat, starch and phosphorous as well as an increase in fiber and ash content (Barszcz et al, 2014). In addition, starch content becomes partially soluble and proteins are denatured (Voragen, Gruppen, Marsman, & Mul, 1995). Furthermore, it has been found that as a consequence of autoclaving, there are observable physical changes to the animal feed, the "hardness" and toughness in food pellets increase. In addition to destroying key nutrients, the autoclaving process produces acrylamide, a toxic and carcinogenic product (Erkekoglu & Baydar,

2010). All these changes in nutrients and addition of toxic chemicals can negatively affect behavior in animal research.

Another varying factor within diet that can impact behavioral performance is soy. Soy contains phytoestrogens, plant-derived compounds that mimic estrogen structurally and functionally (Lund & Lephard, 2001; Lephard, West, Weber, Rhees, Setchell, Adlercreutz & Lund, 2002; Patisaul & Jefferson, 2010). Phytoestrogens are found in fruits, vegetables, legumes, whole grains, flaxseed, clover, and soy products (Lephard, et al., 2002; Lund & Lephard, 2001). Isofavones, a type of phytoestrogens found in diets, have been identified to have effects on the brain and behavior (Lephart, et al., 2002).

Furthermore, studies have found evidence suggesting that phytoestrogens have anxiolytic effects in both males and females when assessed in an elevated plus maze (Lephart et al., 2002). In regard to other behaviors, researchers suggest that supplements with estrogen-like properties have anxiolytic effects causing decreased anxiety levels in open-field testing (McCarthy, Schwartz-Giblin & Wang, 1997), social interaction testing (Johnson & File, 1991), and as mentioned elevated plus-maze testing (Friedman & Frye, 2011; Patsiaul, Blum, Luskin & Wilson, 2005).

Research has shown that phytoestrogens cross the placenta, but there were no significant differences in aromatase levels, an enzyme responsible for the synthesis of estrogen. There were, however, differences in weight of pups, where the animals bred on the phytoestrogen-low diet had higher birth weights than those in the phytoestrogen-high diet (Weber, Setchell & Lephart, 2001). It has been found that phytoestrogen level manipulation can affect anxiety levels in as little as 25 days (Lund & Lephart, 2001).

However, other studies have shown that phytoestrogens may additionally have detrimental effects on behavior. It has been shown that administering phytoestrogens, via a soy-based diet, can cause decreased weight, delayed puberty, and neural alterations in hypothalamic structures such as the sexually dimorphic nucleus in rats (Lund et al, 2001). Researchers found that by administering a soy-free diet and the same soy-free diet infused with genistein (a type of estrogen) to dams, phytoestrogens hindered hippocampal-dependent learning in the Morris water maze, but they failed to find significant effects on freezing during fear conditioning (Ball et al., 2010). It is important to note, however, that the soy was infused in the diet by researchers rather than manufactured at an animal research facility such as Teklad, Inc.

Soy products store phosphorus as phytic acid and when it is bound to minerals it becomes phytate. When soy products are digested, phytates are hydrolyzed and the phytic acid can bind to minerals in the stomach. It has been shown that high levels of phytates inhibits non-heme iron absorption (Venderly, 2006) and has been found to interfere with the absorption of zinc (Lonnerdal, 2000). Zinc is important in hippocampal-based learning and memory (Yang, et al., 2013). Zinc deficiency has been found to impair hippocampal learning in the Morris water maze (Ceccom, Bouhsira, Halley, Daumas, Lassalle, 2013). Zinc supplementation has been shown to increase levels of BDNF and improve hippocampal learning. However, extremely high levels of zinc supplementation cause an overdose which also have negative effects of hippocampal-based learning and decreased levels of BDNF (Yang, 2013).

Male and female rats have shown significant differences in visual-spatial memory which has been attributed to sex-specific hormones such as estrogen. In visual-spatial memory tasks, males perform superiorly to female rats (Lund, West, Tian, Bu, Simmons, Setchell, Adlercreutz & Lephart, 2001). Female rats exhibit inferior performance in visual-spatial memory tasks than males do, which has been attributed to estrogen and other ovarian hormones found to affect visual-spatial memory. Since phytoestrogen mimics estrogen, it has been tested in rats to investigate its effects in visual-spatial tasks (radial arm maze) and acquisition and working memory. It was found that female rats that were on a phytoestrogen-rich diet performed superiorly to females on a phytoestrogen-free diet in visual-spatial memory, however, in males, the effect was flipped, and males on a phytoestrogen-rich diet performed worse than males on a phytoestrogen-free diet in visual spatial memory (Lephard, West, Weber, Rhees, Setchell, Adlercreutz & Lund, 2002).

Research has found that rats on high-fat diets (beef tallow or soy bean oil based) performed worse on the variable-interval delay alternation, a learning and memory task that measures the function of different brain regions, when compared to the rats that were on the regular control diet (Greenwood & Winocur, 2001). Similarly, it was found that rats on diets that were high in saturated fatty acids (lard based) and polysaturated fatty acids (soybean oil based) performed worse on radial arm maze, Hebb-Williams maze and variable-interval delay alternation when compared to the animals on the regular control diet (Greenwood & Winocur, 1990). It has been found that high-fat diets alter an animal's circadian rhythm. Mice fed a high-fat diet have a longer circadian rhythm than those on a

low-fat diet. The high-fat diet also alters eating habits and corticosterone levels, which are important in stress behavior. The rats fed the high-fat diet not only increased their body weight when compared to those on a control diet, but also increased their activity during the day (Pendergast, Branecky, Yang, Ellacott, Niswender & Yamazaki, 2013).

Additionally, nest building has been used to assess health and welfare on male and female mice. (Deacon, 2002; Deacon, 2003) Nest building is an innate behavior in many animals. Birds and fish use nests for reproductive purposes while great apes build nests for shelter and hibernation. Small rodents, including mice, build nests for reproductive purposes, heat conservation and shelter from predators. (Deacon, 2006) Laboratory mice, even though removed from their natural environment and natural hazards, continue to build nests when raised in laboratory settings. Changes to these behaviors indicate a change in their health or welfare, including temperature, pain, illness or stressors. (Gaskill, 2013)

With these important implications in mind, this study examined the behavioral effects of dietary manipulation such as autoclaving and soy content in the diet. To study the behavioral effects of autoclaving diet, this study compared standard Teklad 7012 diet (7012) with an autoclaved standard Teklad 7012 ($7012_A$). Additionally, this study compared a non-soy diet, Teklad 2020SX (2020) with a low-soy diet Teklad 2018SX (2018). No previous studies have examined the $7012_A$ diet and the 2020SX with the 2018SX.

This study examined the effects of autoclaving and the presence of soy (phytoestrogens) on observable measurements of behavior by administering C57 mice on

their assigned diets the day that they arrive to the animal facility. The mice were bred while they were on their assigned diets and continued the assigned diets throughout behavioral testing. At four months, they were tested on multiple behavioral assays: grooming, as a measure of depression and activity of daily living; open field, as a measure of general locomotor activity as well as anxiety; elevated zero maze, as a measure of anxiety; and fear conditioning and extinction as a measure of learning.

## Hypotheses

With those implications in mind, it was hypothesized that animals on the 7012 diet would perform better on all behaviors than the animals on the $7012_A$ diet since important nutrients are being lost through the autoclaving process. Additionally, it was hypothesized that animals on the 7012 diet would perform better compared to the 2018 diet and the 2020 diet on the Morris water maze, fear conditioning and extinction, nesting and grooming as well as show lower levels of anxiety on the open field task and the elevated zero maze.

It was expected that these data would show that the dietary effects including autoclaving feed and amount of soy in diet would have a significant effect on behavior and are therefore an important and potentially confounding factor for behavioral researchers to consider.

# METHODS

Ninety-five C57BL/6J mice were tested. These animals were evenly divided among 4 diet groups and further divided by male and female. The mice were introduced to their assigned diet prior to breeding to ensure that each animal that was tested was introduced to their given diet prenatally and remained on the specified diet until euthanasia. The diet groups included (1) a non-autoclaved standardized Teklad 7012; (2) autoclaved standardized Teklad 7012; (3) soy-free Teklad 2020SX; and (4) low-soy Teklad 2018SX. The non-autoclaved standardized Teklad 7012 served as a control group to compare the effects of autoclaving as well as the effects of soy in the diet. The animals were tested in a grooming test, open field paradigm elevated zero maze, Morris water maze as well as fear conditioning and extinction before they were sacrificed to analyze their brains.

## Mice

Ninety-five wild type mice of the C57BL/6J (B6) background mice were used in the study. These animals were evenly divided between the 4 diets; there were 24 mice in each diet group, except the $7012_A$ which did not breed enough mice, therefore there were only 23 mice in the $7012_A$ group.

## Food and Water

The mice were on standard lab water dispensed from a lixit in each cage. The breeder mice were placed on their assigned diets prior to breeding to ensure that the mice that were tested were introduced to their assigned diet prenatally. As seen in table 1, the four diets that were tested, were Teklad 7012 (7012), autoclaved Teklad 7012 ($7012_A$), low-soy Teklad 2018 (2018) and soy-free Teklad 2020 (2020).

**Table 1 Diets**

| Diet | Abbreviation | Contents | Isoflavone concentration |
|---|---|---|---|
| Teklad 7012 | 7012 | Ground corn, **soybean meal**, wheat middlings, alfalfa meal, corn gluten meal, and brewer's yeast | 300-500 mg/kg |
| Autoclaved Teklad 7012 | $7012_A$ | Ground corn, **soybean meal**, wheat middlings, alfalfa meal, corn gluten meal, and brewer's yeast | 300-500 mg/kg |
| Teklad 2018 | 2018 | Ground wheat, ground corn, wheat middlings, corn gluten meal, brewer's yeast and **soybean meal** | 150-250 mg/kg |
| Teklad 2020 | 2020 | Ground wheat, ground corn, wheat middlings, corn gluten meal, and brewer's yeast | 0-20 mg/kg |

The 7012 diet was designated as the control diet and was given to the control group. The main ingredients in the 7012 diet are ground corn, soybean meal, wheat middlings, alfalfa meal, corn gluten meal, and brewer's yeast and it contains 300-500 mg/kg of isoflavones. The $7012_A$ diet was be administered to group 2. To obtain the $7012_A$ diet, the 7012 diet was autoclaved in the Krasnow Institute at George Mason University. The 2018 diet was administered to group 3. The main ingredients in the 2018

diet are ground wheat, ground corn, wheat middlings, corn gluten meal, brewer's yeast and soybean meal; it has an added source of fat from soybean oil and contains 150-250 mg/kg of isoflavones. The 2020 diet was administered to group 4. The main ingredients in the 2020 diet are ground wheat, ground corn, wheat middlings, corn gluten meal, and brewer's yeast; it has many of the main ingredients as the 7012 diet without the soybean meal and alfalfa meal; it has an added source of fat from soybean oil and contains 0-20mg/kg of isoflavones. (Harlan Laboratories, Inc., 2009).

## Behavioral Testing

The mice were bred on their assigned diet and therefore introduced to their assigned diet prenatally. Additionally, the mice were handled 3 times a week until testing at 4 months to ensure that there was no increased anxiety at the time of testing from lack of handling. The animals underwent behavioral testing to measure their memory, anxiety, and behaviors of daily living. The behavioral tests included Morris water maze, fear conditioning, open field, elevated zero maze, nest building and grooming.

### Morris Water Maze

The Morris water maze is a behavioral task for rodents that tests hippocampal-dependent spatial learning and long-term spatial memory, which is largely a function of the hippocampus. The maze consisted of a 4-foot circular pool of water with four different visual cues around the pool that assist the animal in learning where the "escape" platform is located and a removable "escape" platform at one side of the pool. The task required that the animals learn the location of the hidden platform in relation to the visual cues that were placed around the pool. (Puzzo, Lee, Pelmeri, Calabrese & Arancio, 2014)

The animals underwent 8 days during which they swam in the pool and learned where the hidden platform was placed related to the cues around the pool; the platform was hidden by adding white paint to the water and submerging the platform below the water. During days 1 through 6, each animal underwent 3 trials to find the hidden platform from 3 different starting points in the pool. On day 7, each animal only had 1 trial to find the hidden platform. From days 1 through 7, the hidden platform remained in the same location in the pool; however, the starting position of the mice varied from trial to trial. Day 8 serves to verify that the animals were not blind and the platform was on the other side of the pool but was no longer submerged under the water and had as a large landmark for the animals to see, as long as they were not blind. The primary variables measured in the Morris water maze task were latency, the average amount of time required by each animal to locate the escape platform, the percentage of time spent in the quadrant containing the platform, and thigmotaxis, the percent time spent around the edges of the pool.

**Fear Conditioning and Extinction**

Fear conditioning is a task that measures the effects of associative learning. Throughout the entire experiment, freezing behavior was the primary variable measured. Animals underwent fear conditioning, contextual retention, and cued extinction and recall. During fear conditioning, the animals were placed in the testing chamber for 360 seconds. At 180 seconds, 240 seconds and 300 seconds, there was a 20 second tone that co-terminates with a 2 second 0.5 mA shock. Twenty-four hours later, on day 2, the animals underwent contextual fear retention. For contextual fear retention, the animals

were placed in the original testing chambers without a tone or shock, this was used to examine if the animal associated the chamber with the fear of being shocked. Day 3 and 4 of testing were cue based extinction and extinction recall learning; the testing chambers were altered by using black Plexiglass and bedding to cover the shock bars. The animals were placed in the altered testing chambers for 1280 seconds during which eighteen 20 second tones played starting at 180 seconds with 40 seconds of silence between each tone; none of the tones were paired with a shock. Fear extinction recall learning is an extension of fear extinction learning and served to gauge the effect of diet on longer-term associative memory.

**Nesting**

Nesting was used as a measure of activity of daily living in mice. For the nesting assay, each cage was lined with corn cob bedding, and 2.5 g of shredded paper were added and evenly distributed throughout each cage. The shredded paper was made from clean white computer paper that had been shredded in a paper shredder and then trimmed into 2-3-inch strips. Each animal was individually housed during the nesting trial which lasted 24-hours. The nesting cages only had corncob bedding and shredded paper; there were no running wheels or igloos in these cages. All the animals continued their assigned diet and standard laboratory water. At the end of the trial, each nest was scored on a 7-point likert scale by blind raters.

**Elevated Zero Maze**

The elevated zero maze is a measure of anxiety. The elevated zero maze is similar to the elevated plus maze such that both mazes have an open portion and a closed portion.

However, since the elevated zero maze is an "O" shaped platform that is raised from the ground and is divided into two sections with high walls surrounding the edges and two open sections with no surrounding walls and does not have the middle intersection found in the elevated plus maze, this apparatus eliminates error by forcing the animal to be in either an open portion or a closed portion of the maze (Braun, Skelton, Vorhees & Williams, 2011). Once the animal was placed in the maze, the animals were recorded for 5-minutes to measure the time spent in the closed arms versus time spent in the open arms.

**Open Field**

The open field test is a measure of anxiety as well as exploratory behavior and general activity and is commonly used as a "control" assay for other behavioral tests that involve activity such as freezing and motor coordination. This test served as a proper control for fear conditioning procedures by measuring any impact these diets used in this study could have on general locomotion, which would then impact freezing behaviors in fear conditioning and extinction. This lack of movement/impairment in motor coordination may be incorrectly interpreted as a learning, rather than motor impairment. Open field testing permits more "natural" motor activity and eliminates the potential effects of stress from being placed in a fear conditioning chamber. Each animal underwent a 10-minute trial where percent time spent in the center of the apparatus as well as speed of each animal were recorded. Percent time spent in the center of the apparatus was used as a measure of decreased anxiety-like behavior. Speed of the animal was a measure of general activity and locomotion.

### Grooming

The grooming test, or splash test, is a measure of apathetic behavior, similar to what is seen in depression (Willner et al., 2005; Rosa et al., 2014). Animals were placed in clear plexiglas cylinders 12 inches in height with a camera attached above the tube. Each animal was sprayed 10 times with distilled water, via a spray bottle, on the dorsal area of its body (Kyzar, E., et al, 2011). Grooming is an innate behavior, therefore immediately after being sprayed, animals should have begun to groom their fur. The grooming behavior was recorded for 5-minute intervals after they had been sprayed with distilled water. Blind scorers manually recorded time spent grooming using a stopwatch. The amount of grooming behavior within the five minutes indicated self-care and motivated behavior, and lack thereof would indicate symptoms of depression.

## Statistical Analysis

After the completion of behavioral testing, SPSS was used to analyze the results between the animals on the 7012 diet and $7012_A$, 2018 and 2020. In all analyses, overall ANOVAs were conducted, but in order to specifically test the hypotheses that 7012 diets would be significantly different from all other diets, additional planned comparisons were run comparing 7012 with $7012_A$, 2018 and 2020.

## RESULTS

Ninety-five wild type mice of the C57BL/6J (B6) background mice were used in the study. These mice were bred while on their assigned diets, therefore to account for mother cannibalization and pup death and ensure proper quantities of mice in each diet group, overbreeding of mice was planned. There difference between live births between diets was analyzed with planned comparisons. The comparisons found that while there was no significant difference between diets, the difference in litter sizes between 7012 ($4.44 \pm 0.38$) and $7012_A$ ($3.20 \pm 0.65$, $p = 0.08$) was trending. There were 24 mice in each diet group, except the $7012_A$ which did not have enough pups in the second cohort of animals, therefore there were only 23 mice in the $7012_A$ group. Standardized Teklad 7012 was used as a control and was compared with autoclaved Teklad 7012 as well as with low-soy Teklad 2018 and soy-free Teklad 2020. Autoclaved Teklad 7012 was not compared with low-soy Teklad 2018 and soy-free Teklad 2020. There were no significant differences in weight of mice at time of sacrificing, which was analyzed with a one-way ANOVA, $F$ (3, 91) = 0.96, $p = 0.32$. There were no significant differences in mortality; no mice died before testing.

### Morris water maze

To analyze latency over 6 days in the Morris water maze, a mixed ANOVA 4 (diet) x 6 (days) was run as seen in Figure 1. The main effect of diet was not statistically

significant, $F$ (3, 91) = 1.86, $p$ = 0.14; collapsing across days, the animals on different diets did not learn at significantly different rates. The main effect of day was significant, such that latency was significantly reduced across days, $F$ (5, 455) = 65.60, $p < 0.01$. However, the interaction of diet and latency was not significant, $F$ (15, 455) = 0.84, $p$ = 0.63. To specifically test the hypothesis that animals on 7012 should perform better, planned comparison were run comparing animals on 7012 with animals on $7012_A$, 2018 and 2020. The planned comparison revealed that animals on 7012 (17.00 ± 4.44) averaged a significantly lower latency than animals on 2018 (20.08 ± 5.67, $p$ = 0.04), but were not significantly different from the mice on 2020 (18.03 ± 6.19, $p$ = 0.49) or the mice on $7012_A$ (17.09 ± 3.78, $p$ = 0.95).

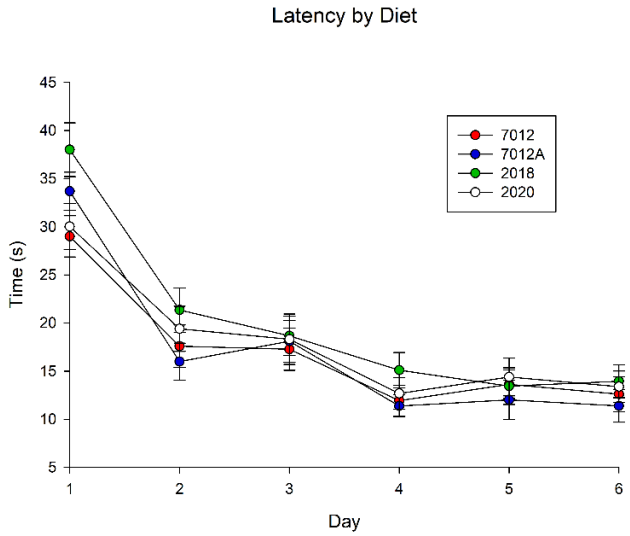


**Figure 1.** Morris water maze: latency over 6 days of testing

In terms of latency, the greatest learning occurred between days 1 and 2 of Morris water maze; therefore, further analysis was run on latency to analyze the latency from day 1 to day 2 using a mixed ANOVA 4 (diet) x 2 (days) as seen in Figure 2. The main effect of diet was statistically significant; there was a significant difference in learning between the diets, ($F$ (3, 91) = 2.94, $p$ = 0.04). The main effect of day was significant, such that latency was significantly reduced across days, $F$ (1, 91) = 78.05, $p < 0.01$. However, the interaction of diet and latency was not significant, $F$ (3, 91) = 1.26, $p$ = 0.29. To further test the hypothesis that animals on 7012 should perform better, planned comparisons were run comparing animals on 7012 with animals on $7012_A$, 2018 and 2020. This test revealed that animals on 7012 (23.30 ± 7.23) averaged a significantly lower latency than animals on 2018 (29.68 ± 9.97, $p$ = 0.01), but no significant differences between animals on 2020 (24.72 ± 8.52, $p$ = 0.54) or $7012_A$ (24.84 ± 5.19, $p$ = 0.51).

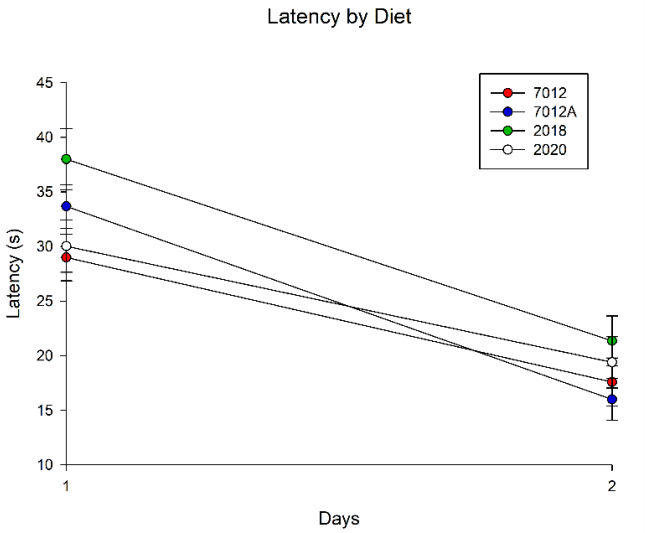


**Figure 2.** Morris water maze: latency over first 2 days of testing

To analyze percent time spent in quadrant, the correct quadrant in the Morris water maze on probe days, as shown in Figure 3, a mixed ANOVA 4 (diet) x 3 (days) was run. The main effect of diet was not statistically significant, $F$ (3, 91) = 0.39, $p$ = 0.76; collapsing across days, there was no significant difference between the diets. The main effect of days was significantly different, such that the time spent in the correct quadrant was significantly different across days, $F$ (2, 182) = 4.39, $p$ = 0.01. However, the interaction between diet and time spent in correct quadrant was not statistically significant, $F$ (6, 182) = 0.97, $p$ = 0.45. To further analyze the hypothesis that animals on 7012 should perform better than animals on $7012_A$, 2018 and 2020, planned comparisons were run. This test showed that there were no significant differences between animals on 7012 (29.05 ± 5.85) vs. 2018 (28.18 ± 6.84, $p$ = 0.61), 2020 (27.59 ± 4.82, $p$ = 0.39) or $7012_A$ (27.37 ± 5.60, $p$ = 0.33).

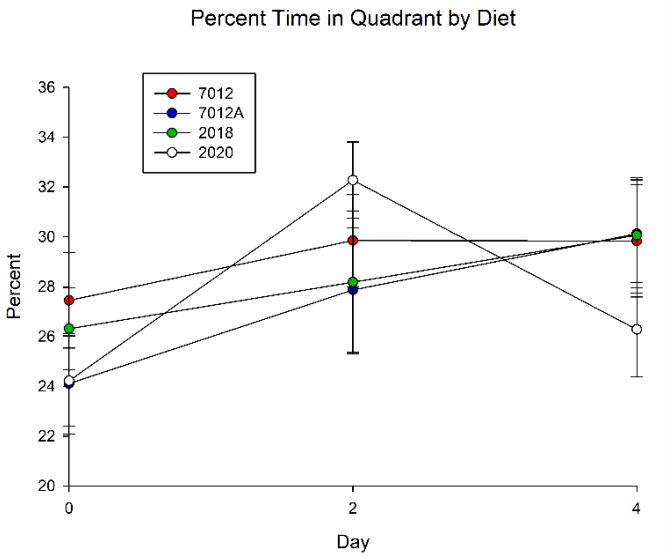


**Figure 3.** Morris water maze: percent time spent in quadrant of testing

To analyze thigmotaxis in the Morris water maze, a mixed ANOVA 4 (diet) x 6 (days) was run as seen in Figure 4. The main effect of diet was not statistically significant, $F$ (3, 91) = 0.41, $p$ = 0.75; collapsing across days, there was no significant difference between the diets. The main effect of days was significantly different $F$ (5, 455) = 132.58, $p < 0.01$, therefore the time spent along the edges of the Morris water maze pool decreased significantly across days. However, the interaction between diet and thigmotaxis was not statistically significant, $F$ (15, 455) = 0.46, $p$ = 0.96. To specifically test the hypothesis that animals on 7012 should perform better, planned comparison were run comparing animals on 7012 with animals on $7012_A$, 2018 and 2020. The planned comparison showed that there were no significant differences in thigmotaxis between animals on 7012 (13.71 ± 5.15) vs. 2018 (13.65 ± 3.53, $p$ = 0.97), 2020 (12.85 ± 3.85, $p$ = 0.61) or $7012_A$ (11.97 ± 5.02, $p$ = 0.33).

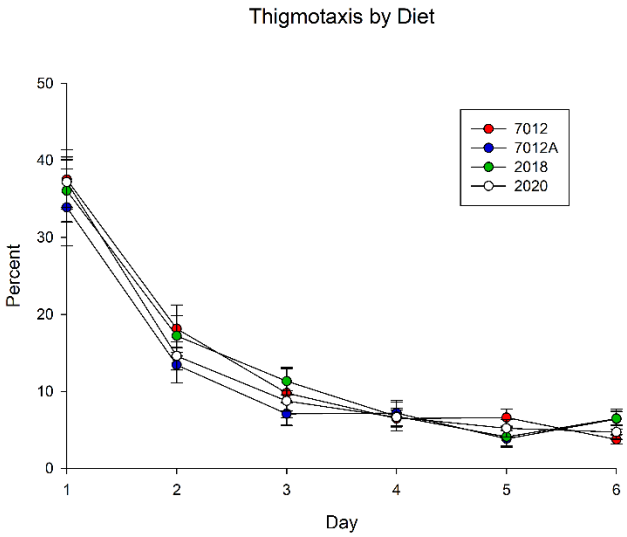


**Figure 4.** Morris water maze: thigmotaxis

## Fear Conditioning and Extinction

Day 1 (training day): To analyze day 1 (training day) in fear conditioning, as seen in Figure 5, a mixed ANOVA 4 (diet) x 3 (time) was run over the three tone presentations. The main effect of diet was not statistically significant, collapsing across times, there was no significant difference between the diets, $F$ (3, 91) = 1.003, $p$ = 0.395. The main effect of time was significantly different, $F$ (2, 182) = 252.066, $p < 0.01$ such that the freezing increased across time. However, the interaction of diet and time was not statistically significant, $F$ (6, 182) = 0.634, $p$ = 0.703. To specifically test the hypothesis that animals on 7012 would perform better, planned comparison were run between animals on 7012 and animals on 2018, 2020 and $7012_A$. This analysis showed that there were no significant differences between animals on 7012 (29.33 ± 11.41) and 2018

(26.06 ± 12.81, $p = 0.36$), 2020 (31.88 ± 12.11, $p = 0.48$) or $7012_A$ (30.88 ± 12.44, $p = 0.70$).

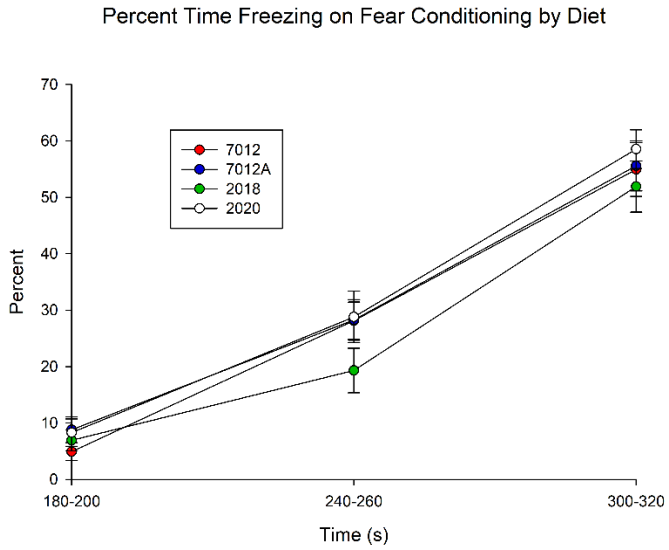


**Figure 5.** Fear conditioning: day 1 (conditioning day)

Contextual recall: To analyze contextual recall in fear conditioning, a one-way ANOVA was run to compare the average amount of time spent freezing as shown in Figure 6, during times when the tones were playing. The one-way ANOVA was not statistically significant, $F$ (3, 91) = 1.039, $p = 0.379$; collapsing across time there were no significant differences in freezing between the diet groups. Additionally, the times when tones played during the conditioning day were analyzed for freezing with a mixed ANOVA 4 (diet) x 3 (time) as seen in Figure 6. The main effect of diet was not statistically significant, $F$ (3, 91) = 1.19, $p = 0.32$. Furthermore, there was no significant main effect of time, $F$ (3, 91) = 1.53, $p = 0.22$. Lastly, the interaction between diet and

time was not significant $F$ (3, 91) = 0.83, $p$ = 0.48. To specifically test the hypothesis that animals on 7012 should perform better than animals on $7012_A$, 2018 and 2020, planned comparisons were run. This comparison showed that the animals on 7012 (59.35 ± 22.43) froze more of the time than those on 2018 (48.81 ± 21.19, $p$ = 0.08), which was a trend. However, there was no significant difference in freezing between the animals on 7012 (59.35 ± 22.43) and those on 2020 (57.06 ± 21.98, $p$ = 0.70) or $7012_A$ (53.04 ± 16.99, $p$ = 0.30).

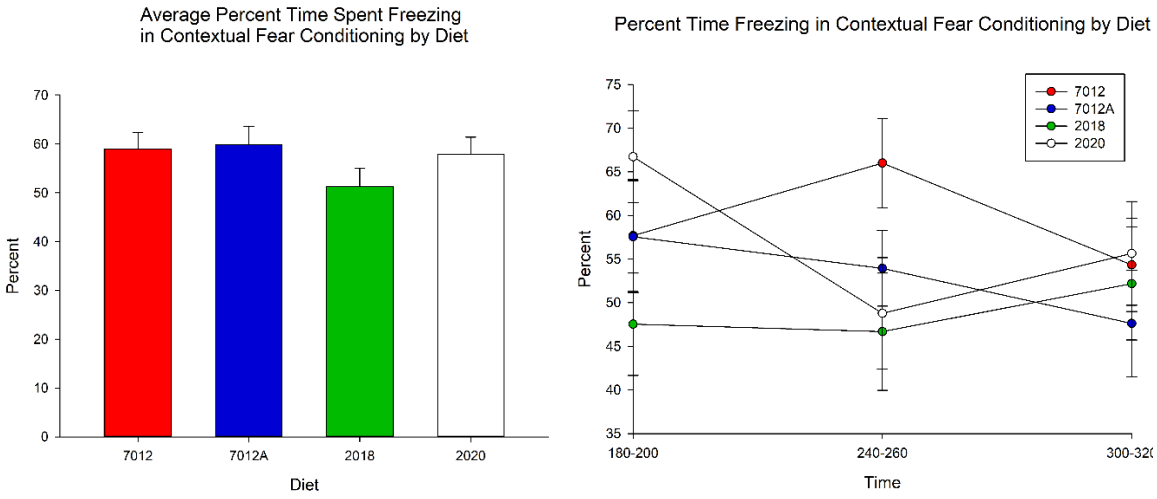


**Figure 6.** Fear conditioning: contextual retention

Cued extinctionl: To analyze cued recall in fear conditioning, a mixed ANOVA 4 (diet) x 6 (time) was run as seen in Figure 7. The main effect of diet was not statistically significant, so there was no significant difference between the diets, $F$ (3, 91) = 1.699, $p$ = 0.17. The main effect of time was statistically significant, $F$ (17, 1547) = 18.38, $p$ < 0.01, such that the animals learned to not pair the tone with the shock over time.

However, the interaction of diet and time was not statistically significant, $F$ (51, 1547) = 1.03, $p$ = 0.42. To specifically test the hypothesis that animals on 7012 would perform better than those on $7012_A$, 2018 and 2020, planned comparisons were run. This analysis showed that there were no significant differences between animals on 7012 (63.61 ± 17.27) and 2018 (69.67 ± 15.14, $p$ = 0.18), 2020 (69.62 ± 15.57, $p$ = 0.19) or $7012_A$ (61.42 ± 14.62, $p$ = 0.63). Note that while the analysis was conducted on all 18 time points, for illustrative purposes, what is displayed is the data across six averages of three-minute time periods.

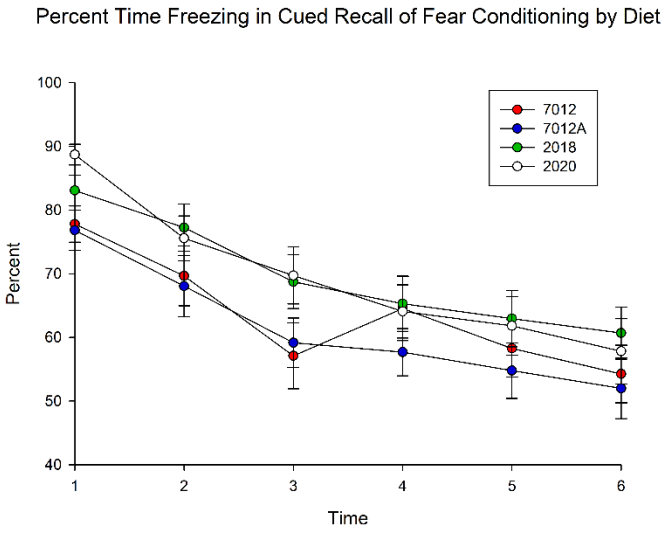


**Figure 7.** Fear conditioning: cued extinction

When analyzing extinction, the greatest learning occurred between the first three, time averages on the cued days of fear conditioning and extinction; therefore, further analysis was run on the first three, time averages during cued recall of fear conditioning

using a mixed ANOVA 4 (diet) x 3 (time) as seen in Figure 8. The main effect of diet was statistically significant between the groups, $F$ (3, 91) = 2.70, $p$ = 0.05; collapsing across times there was a significant difference between the diet groups. Additionally, the main effect of time was statistically significant, $F$ (2, 182) = 47.72, $p < 0.01$ such that the animals relearned to not pair the tone with the shock across time. However, the interaction was not statistically significant, $F$ (6, 182) = 0.61, $p$ = 0.73. Further analysis was run to test the hypothesis that animals on 7012 would perform better than those on $7012_A$, 2018 and 2020. The planned comparisons revealed that there was a significant difference in freezing time between animals on 7012 (68.20 ± 17.46) and those on 2020 (78.01 ± 14.05, $p$ = 0.03). While the freezing time between animals on 7012 (68.20 ± 17.46) and those on 2018 (76.36 ± 14.79, $p$ = 0.07) was not significant, it was trending towards significance. Lastly, there was no significant difference between animals on 7012 (68.20 ± 17.46) and those on $7012_A$ (68.03 ± 16.09, $p$ = 0.97).

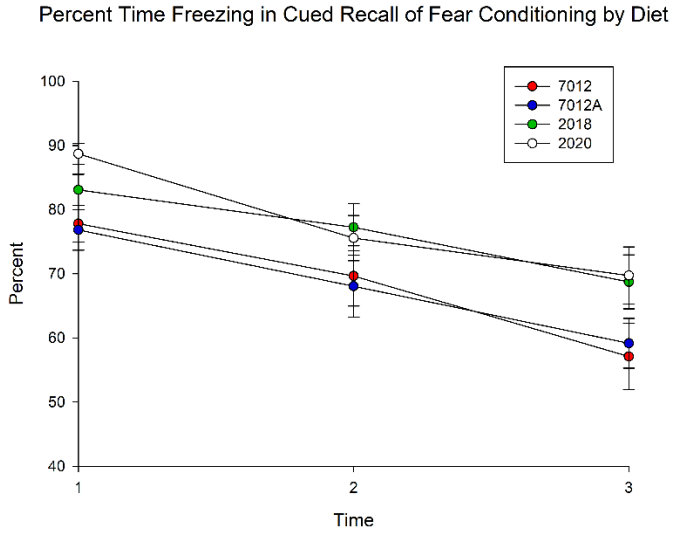


**Figure 8.** Fear conditioning: cued recall, first three time averages

## Nesting

To analyze the activities of daily living, as seen in Figure 9, a one-way ANOVA was run on the nesting scores. A one-way ANOVA showed that there was statistical significance between the groups, $F$ (3, 91) = 5.19, $p < 0.01$. To specifically test the hypothesis that animals on 7012 would perform better than those on $7012_A$, 2018 and 2020, planned comparisons were run. The planned comparisons found that animals on 7012 (2.90 ± 0.13) had significantly higher nesting scores than animals on 2018 (2.50 ± 0.29, $p < 0.01$), 2020 (2.63 ± 0.27, $p = 0.03$) or $7012_A$ (2.64 ± 0.30, $p < 0.01$).

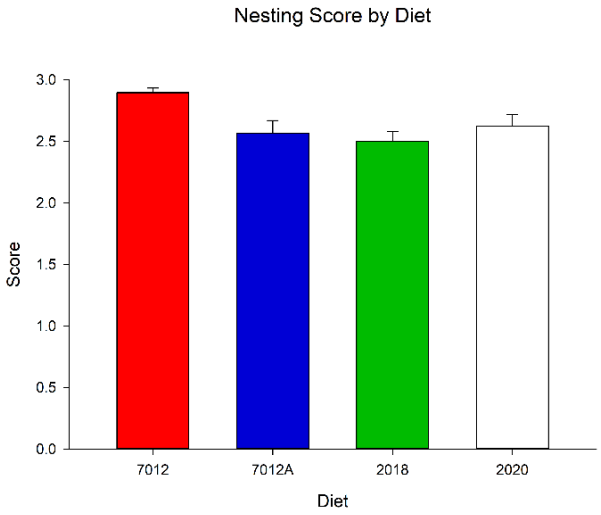


**Figure 9.** Nesting

## Elevated Zero Maze

To analyze anxiety in the elevated zero maze, in figure 10, a one-way ANOVA was run. The one-way ANOVA was not statistically significant, $F$ (3, 91) = 0.889, $p$ = 0.450. Therefore, there were overall significant differences in anxiety among the diet groups. To further test the hypothesis, planned comparisons were run to compare animals on 7012 and animals on $7012_A$, 2018 and 2020. This analysis showed that the time spend in the open arms of the elevated zero maze was not significantly different between animals on 7012 (39.24 ± 19.32) and those on 2018 (45.40 ± 28.09, $p$ = 0.43), 2020 (52.00 ± 32.18, $p$ = 0.11) or $7012_A$ (45.49 ± 27.12, $p$ = 0.45).

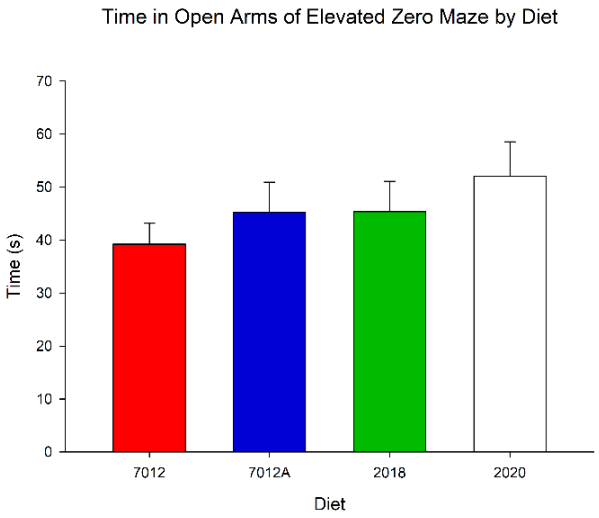


**Figure 10.** Elevated Zero Maze

## Open Field

To analyze anxiety in the open field test, as seen in Figure 11, a one-way ANOVA was run on the time spent in in the center of the chamber. The one-way ANOVA was not statistically significant, $F$ (3, 91) = 1.404, $p$ = 0.247. Therefore, there were no significant differences in anxiety levels among the diet groups. To further test the hypothesis, planned comparisons were run to compare animals on 7012 and animals on $7012_A$, 2018 and 2020. The planned comparison showed animals on 7012 (15.84 ± 10.00) had significantly lower anxiety than those on 2020 (12.18 ± 3.93, $p$ = 0.05). The test further revealed that there were no significant differences in anxiety between animals on 7012 (15.84 ± 10.00) and those on 2018 (13.84 ± 4.35, $p$ = 0.29) or $7012_A$ (14.90 ± 5.79, $p$ = 0.62).

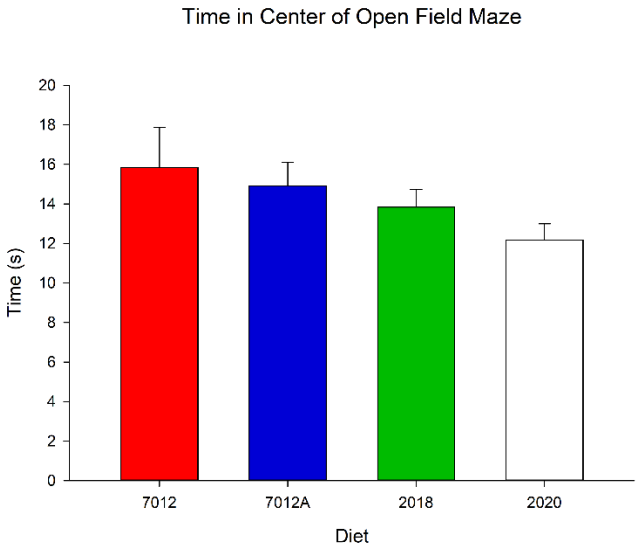


**Figure 11.** Open Field: time in center

To analyze speed of the animals in the open field maze, a one-way ANOVA was run as seen in Figure 12. The one-way ANOVA was not statistically significant, $F$ (3, 91) = 1.072, $p$ = 0.247. Upon further analysis of the hypothesis with planned comparisons of animals on 7012 with those on $7012_A$, 2018 and 2020. This comparison showed that there were no significant differences in locomotion between animals on 7012 (20.82 ± 9.57) and those on 2018 (23.33 ± 5.37, p = 0.29), 2020 (24.73 ± 7.33, p = 0.10), or 2012A (24.17 ± 9.81, p = 0.17).

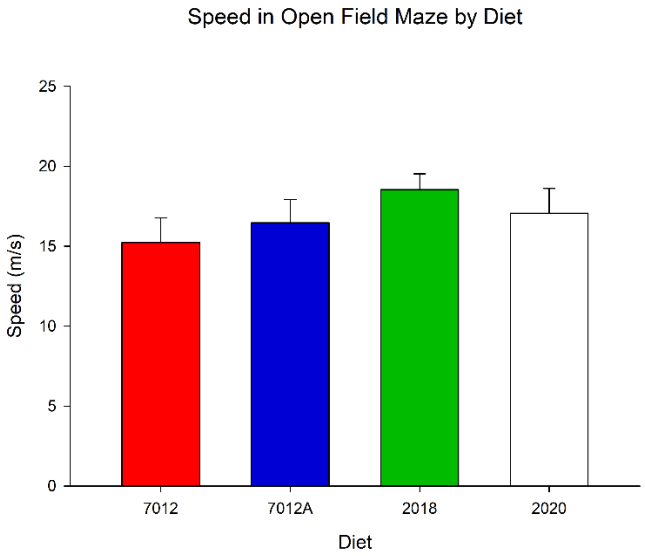


**Figure 12.** Open Field: speed

## Grooming

To analyze grooming behavior, as seen in Figure 13, a one-way ANOVA was run. There was a statistically significant difference between the groups as determined by the one-way ANOVA, $F$ (3, 91) = 5.45, $p$ < 0.01). Therefore, there were significant

differences in the time spent grooming between the diet groups. Additionally, the time spent grooming was not significantly different between 7012 (154.81 ± 9.44) and $7012_A$ (140.10 ± 8.12, $p = 1.00$). To specifically test the hypothesis, planned comparisons were run between animals on 7012 and those on $7012_A$, 2018 and 2020. This test showed that animals on 7012 (154.81 ± 46.23) groomed significantly less than those on 2018 (179.59 ± 37.61, $p = 0.03$) and 2020 (175.01 ± 27.25, $p = 0.05$). However, there was no significant difference in time spent grooming between animals on 7012 (154.81 ± 46.23) and those on $7012_A$ (140.10 ± 38.96, $p = 0.19$).

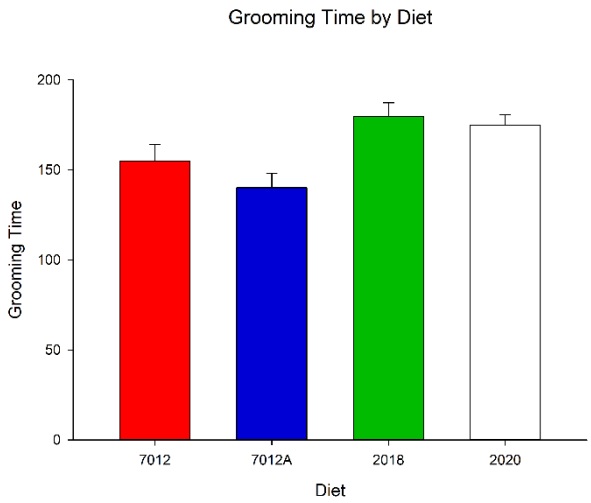


**Figure 13.** Grooming

## DISCUSSION

Changing dietary formulation can have a large effect on behavior and pose as a confounding factor in behavioral neuroscience. Differing soy composition in diet and autoclaving lab chow are two factors that can affect behavior. It was hypothesized that animals on the 7012 diet should perform better on all behaviors than the animals on the $7012_A$ diet since important nutrients are being lost through the autoclaving process. Additionally, it was hypothesized that animals on the standard 7012 diet when compared to the 2018 diet and the 2020 diet should perform better on the Morris water maze, fear conditioning and extinction, nesting and grooming as well as show lower levels of anxiety on the open field task and the elevated zero maze.

**Table 2 Key findings, X = p < 0.05; ^ = p < 0.1**

| | 7012 vs. $7012_A$ | 7012 vs. 2018 | 7012 vs. 2020 |
|---|---|---|---|
| MWM: Latency days 1-6 | | X | |
| MWM: Latency days 1-2 | | X | |
| MWM: Percent time in quadrant | | | |
| MWM: Thigmotaxis | | | |
| FC: Day 1 (training day) | | | |
| FC: Contextual recall | | ^ | |
| FC: Cued recall | | | |
| FC: Cued recall ($1^{st}$ 3 time points) | | ^ | X |
| Nesting | X | X | X |
| EZM | | | |
| OF | | | X |
| Grooming | | X | X |

As seen in Table 2, the data showed that contrary to expectations, animals on the 7012 diet did not perform significantly better than animals on the $7012_A$ diet except in nesting. The animals on the 7012 diet also produced more pups than animals on the $7012_A$ diet.

Additionally, as seen in Table 2, the animals on the 7012 diet performed significantly better than those on the low-soy 2018 diet in terms of latency in Morris water maze, nesting and grooming were trending on fear conditioning; animals on the 7012 diet performed better than those on the soy-free 2020 diet on fear conditioning, nesting, open field and grooming. It should be noted that the animals on the low-soy 2018 and soy-free 2020 diets spent less time in the center in the open field task and more time in the open arms of the elevated zero maze when compared to the animals on the 7012 diet, similar to the behavior of transgenic mice bred for Alzheimer's (Lippi, 2018). Therefore, the hypotheses, that animals on the 7012 diet would perform better than the 7012, 2018 and Teklad 2020 was partially supported.

Altering the composition of animal feed by autoclaving or changing their soy content is not only affecting widely used behavioral tasks like Morris water maze and fear conditioning and extinction, but also negatively impacting innate behaviors such as nest building and grooming behaviors. Animals on the Teklad 7012 diet performed better than animals on diets that are autoclaved or contain less or no soy on important behavior tasks that are widely used in behavioral neuroscience research.

These findings show that Teklad 7012 diet is better compared to autoclaved Teklad 7012, Teklad 2018 and Teklad 2020 diets in terms of showing baseline

performance for mice in multiple widely used measures including Morris water maze, nest building and grooming. This will allow researchers to eliminate a potential confound from these new low-soy (2018) and soy-free (2020) diets that Teklad has produced when studying effects on learning, memory or activities of daily living. In addition, Teklad 7012 was better able to maintain standard litter sizes; whereas there was a decrease in the number of pups produced when breeders were on autoclaved feed.

With the significant finding in nesting scores between diets, future research will compare the quantity of vasopressin in the brains of these mice since increased vasopressin is linked with better, more elaborately built nests.

Dietary manipulation of animal feed including autoclaving feed and amount of soy in diet have a significant effect on behavior in neuroscience research and should be under stricter scrutiny when planning and conducting an experiment since dietary composition is an important and possibly a confounding factor for behavioral researchers to consider.

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## BIOGRAPHY

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