The Effects of Zinc and Copper Supplementation on Double Transgenic (App/Tau) Mice Models of Alzheimer's Disease

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts at George Mason University

by

Tatiana Gervase
Bachelor of Arts
George Mason University, 2020

Director: Jane Flinn, Associate Professor
George Mason University

Fall Semester 2021
George Mason University
Fairfax, VA

DEDICATION

This is dedicated to my parents Philip and Michelle Gervase for their continued support and love.

ACKNOWLEDGEMENTS

I would like to thank the entirety of the Flinn lab who assisted and supported me throughout this process. I would specifically like to thank Karin Pedemonte, Rachel Barkey, and Erin Doherty who all mentored me in different aspects of the thesis process along the way. Further, I would like to thank all of the RAs who helped collect behavioral data for the five cohorts, especially Sam Neff who I could reliably turn to. I would like to thank my family and boyfriend who allowed me to rant when the project was overwhelming or frustrating, as well as my committee members, Dr. Brielmaeir and Dr. Murdoch for their support with the writing of this thesis. Finally, I have so much appreciation for Dr. Flinn for her mentorship during my undergraduate and graduate projects. I was pushed to develop skills and undertake projects I would not have imagined when I first joined the lab.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND SYMBOLS

Activities of daily living……ADL
Alzheimer's disease…….AD
Amyloid beta……..……..Aβ
Amyloid precursor protein…….APP
Copper……...Cu
Human tau……..h-tau
Morris water maze……..MWM
Transgenic……Tg
Triple transgenic…….3xTg-AD
Wildtype……..…WT
Zinc……Zn

ABSTRACT

THE EFFECTS OF ZINC AND COPPER SUPPLEMENTATION ON DOUBLE TRANSGENIC (APP/TAU) MICE MODELS OF ALZHEIMER'S DISEASE

Tatiana Gervase, MA

George Mason University, 2021

Thesis Director: Dr. Jane Flinn

Alzheimer's disease (AD) accounts for 70% of dementia, but there is no known cure for the disorder so many non-drug therapeutic treatments include changes in diet and maintaining a healthier lifestyle (Kametani & Hasegawa, 2018). There have been many behavioral studies examining the effect on zinc supplementation into AD mice models. However, while there is some contradicting evidence, overall, the evidence suggests that zinc supplementation results in a negative effect on behavior which may be a result of the diet causing a copper deficiency, another trace metal that plays a crucial role in the development of AD. This thesis examined the role of zinc and zinc and copper supplementation on 3.5-month dual transgenic (APP/h-tau) AD mice models to investigate if the addition of copper alleviated the negative behavioral effect. Results found that zinc and copper supplementation still had an overall negative effect compared

to wildtype (WT) mice in all four tests: Morris water maze, nesting, burrowing, and circadian activities, as well as no overall metal supplementation effect. Additionally, it was also found that female mice were more anxious than males were and performed worse at the activities of daily living (ADL) tests, with females on the metal supplements displaying the worst behaviors of anxiety. These findings suggest metal supplementation is not an acceptable treatment for AD patients.

INTRODUCTION

As the life expectancy of humans increases, the number of people diagnosed with dementia also increases. While there are many types of dementia, the most common is Alzheimer's disease (AD), and the prevalence of the neurological disease is expected to triple within forty years (CDC, 2020). There is no specific known cause, but it is characterized by senile amyloid-beta plaques and neurofibrillary tau tangles (Kametani & Hasegawa, 2018). The overproduction of amyloid-beta outside the cells contributes to neurodegeneration which further propagates the formation of intracellular tau tangles that accelerate cellular death (Geppert, Losy, Przedpelska-Ober, & Kozubski, 2010). Together, the two neurological proteins begin with low numbers in the entorhinal cortex until they slowly corrupt the entire brain (Braak & Braak, 1991).

The proteins' origin of dissemination in the entorhinal cortex helps to explain the hallmark symptom of AD, memory loss, given that the entorhinal cortex is the input and output routes to the hippocampus (Braak & Braak, 1991). The hippocampus is the storage location for both declarative semantic memories - fact knowledge - and spatial memory (Nestor, Fryer, Hodges, 2005). Spatial memory is stored in the entorhinal cortex and hippocampus, and the former contains grid cells which encode spatial location. As a result of the early tangle pathology in this area, dysfunction in the grid cell firing, which

then in cause leads to dysfunction of spatial navigation, is one of the first symptoms AD patients develop (Fu et al., 2017).

Unfortunately, spatial navigation is not the only symptom patients develop as the disease progresses. Neuropsychiatric symptoms are also evidenced. Some of these symptoms include depression, agitation, apathy, irritability, and other behavior and mood changes. Further, severe disruptions to the circadian sleep-wake cycle are common (Lyketsos et al, 2011). It is important to investigate these disruptions because 45% of AD patients experience worse disturbances compared to normal aging and they affect both patient and caregiver. Additionally, sleep disruptions are often overlooked in favor of memory symptoms AD researchers focus on (Peter-Derex, Yammine, Bastuji, & Croisile, 2015). Not only is there a direct correlation between sleep deprivation and a decrease in cognitive functions (Pilkington, 2013), but there is a relationship between sleep deprivation and the amount of amyloid-beta cleared from the brain. As humans sleep, one of the culprit proteins associated with AD, amyloid-beta, is cleared from the brain, so disrupted sleep impacts the amount of protein in the brain (Spira et al, 2013; Peter-Derex, Yammine, Bastuji, & Croisile, 2015).

Sleep is an everyday behavior, but other impairments in daily functioning also occur in Alzheimer's. These activities of daily living (ADL) vary in complexity from basic, dressing and feeding, to intricate, handling finances and shopping. These disruptions often vary from case-to-case but are thought to be a result of executive dysfunction. Executive functioning is an underlying term for high-level cognitive processes, including working memory, attentional control, and concept generation which

are concepts controlled by the neocortex (Martyr & Clare, 2012). Overall, there are multiple symptom domains associated with Alzheimer's that have been investigated.

In order to investigate behavioral symptoms of AD, many researchers use AD mice models. In mice models, these mice have been manipulated to have genes that reflect AD symptoms. This can be done by genetically modifying the mice so they have human tau genes and the amyloid precursor protein (APP), a protein that leads to the amyloid beta development. There are many different mutations, but because this study used a dual transgenic mouse model (tau and APP), only studies that use dual transgenic (dual Tg) and triple transgenic (3xTg-AD) mice models (tau, APP, and presenilin-1, also known as PS1) are evaluated in this section. PS1 affects how APP is cut and often indicates early-onset AD (Janus et al, 2000). Since PS1 is a mutation of APP, when broken down, the triple transgenic mice only feature APP and tau. The current literature often features triple transgenic mice or single mutation mice models. In fact, the Flinn lab developed this model of the dual Tg mouse, so this mouse model is still very novel in the research community (Lippi et al, 2018).

Spatial Navigation

To investigate spatial navigation impairments using transgenic mice models, researchers often include tests of spatial memory. One of the most commonly used behavioral test to measure spatial memory is the Morris Water Maze (MWM). The MWM is comprised of a circular pool divided into four equal quadrants with visual cues placed on the inner wall that the mouse can see. A platform is placed in one quadrant,

slightly submerged below the surface, and the mouse is placed in the water and allowed to freely swim to find the platform. The number of trials done each day and how long the mouse stays on the platform often varies study to study. MWM also can feature an Atlantis probe trial in which the platform is submerged and the amount of time mice swim in the target quadrant is measured.

While the Morris water maze test is the most common, Filali et al (2012) used a water T-maze to evaluate 12–14-month female 3xTg-AD mice compared to wildtype (WT) controls. In this test, a platform is submerged below water level at the end of the target arm. Mice are placed in the stem to find the submerged platform and guided to it if they do not find it. After five consecutive errorless trials, mice are believed to have learned the correct placement. Two days after trials are run, a reversal learning phase was conducted which follows the same protocol except the escape platform is on the opposite side. Filali et al (2012) found that 3xTg-AD mice were significantly slower to reach criterion during the reversal phase and were significantly more likely to take a more complicated path instead of a direct path. Simply, they found that transgenic mice had significantly worse performance compared to wildtype mice.

In a different study that used a 3xTg-AD mouse model, Halagappa et al (2007) investigated MWM using four trials per day for eight days and were tested at ten months. 3xTg-AD mice swam significantly longer path lengths compared to controls, meaning they took more convoluted paths to find the platform. However, there was no significant difference in goal latency times between groups. Furthermore, 3xTg-AD mice spent less time in the target quadrant than controls during probe trials (Halagappa et al, 2007).

These results indicate that while triple transgenic mice may display different behaviors than control mice, they are still young enough to not be significantly worse. Samaey, Schreurs, Stroobants, and Balschun (2019) also had four trials per day, except they had only had five acquisition days. All mice, aged three to six months, displayed successful learning of the platform position and spent similar times in the target quadrant. There were no significant differences, though the dual Tg mice needed a greater number of days to reach learning criterion. Both studies indicate that when the mice are young, there may not be statistically significant differences in time it takes to reach the platform, but there still may be significant differences in speed and learning curve for both genotypes.

Activities of Daily Living & Circadian Activities

While in humans eating and dressing are considered activities of daily living, nesting and burrowing are the ADL measurements for mice models. Different materials can be used in the nesting behavioral test. For instance, researchers in Filali et al (2012) put two pieces of cotton into the home cage as nesting material, and the quality of the nest was rated one day later. They found there was no intergroup difference in nest-building, and both groups had an average score around 4 on a scale of five. In contrast, Samaey et al (2019) placed a single paper towel as nesting material and evaluated quality of nesting after 23 hours. They found significant differences between dual Tg and WT mice in which dual Tg mice built poorer nests than controls. These two studies introduce contradicting results, one in which there are significant genotypic differences and another where there is not. This could be a result of the material that was used for nesting or could be age-related as Filali et al (2012) used older mice. Nonetheless, both studies

indicate that activities of daily living may not result in significant results given the age of the mice. Flinn lab ADL results are reported later as findings were pulled from studies that used metal supplementation.

In consequence of how AD can affect sleep cycles, Wu et al. (2018) measured circadian activities using running-wheel activity. Mice circadian patterns are nocturnal, so it would be expected that the most activity would be during lights-off and night times while limited activity would occur during lights-on and the day. Active hours are generally reported as 8pm-8am while non-active hours are 8am-8pm; this study also uses these parameters. All activity was recorded in five-minute intervals during a two-week period of 12 hours of light followed by 12 hours of darkness. They found that six-month 3xTg-AD had increased activity during lights-on and scattered activity during lights-off compared to wildtype mice, which had activity beginning at the time of lights-off and ceased at lights-on. Ultimately, the triple transgenic mice had significantly lower activity when compared to the WT mice, and there were no sex differences (Wu et al., 2018).

Another study also used triple transgenic mice and found that 3xTg-AD mice had significantly increased daytime activity and a decrease of activity during the night (Sterniczuk, Dyck, LaFerla, & Antle, 2010). In contrast to Wu et al. (2018), Sterniczuk, Dyck, LaFerla, and Antle found that females had a decrease in activity levels during active hours whereas males had more activity during non-active hours. These differences could be a result of testing the male mice before and soon following AD pathology, and female mice post-AD pathology, indicating differences in ages. Furthermore, they found that the 3xTg-AD mice ran significantly shorter periods than wildtype mice.

Both studies indicate that circadian activities are likely to result in genotypic differences. They also conducted tests when mice were around six months of age, and while the mice used in the present study will be younger than that, these findings seem to indicate results may be similar to the Morris Water Maze results in which genotypic differences should be present. While there may not be genotypic differences, there may be trends that can demonstrate how the dual Tg mice will differ from control mice.

Role of Metals

Despite the range of symptoms, with no known cure, many treatments focus on a broad approach to slow the progression or make the patient comfortable (National Institute of Aging, 2016). Often, these treatments include dietary supplements or lifestyle changes (Reiman et al, 2016), and most medication somewhat unsuccessfully targets amyloid-beta (Kametani & Hasegawa, 2018). Newer treatments are investigating the impact of trace metals in the brain, including zinc and copper.

Zinc is most prevalent in the hippocampus, where amyloid-beta and tau are also the most abundant in areas in and around (Constantinidis, 1992). Zinc plays an essential role in axonal and synaptic transmission as well as brain tubulin growth (Pfeiffer & Braverman, 1982). Further, zinc can selectively inhibit NMDA receptors by inducing inhibition. NMDA receptors are one of the main receptors for glutamate, a neurotransmitter essential for memory (Nuttall & Oteiza, 2014). Zinc's role in NMDA receptors illustrates its importance in memory, and in consequence, Alzheimer's.

Zinc levels are also critically crucial in Alzheimer's disease because of its specific function with amyloid-beta. There are two main pathways in which amyloid-beta is formed: the disease-resulting amyloidogenic and the healthy nonamyloidogenic pathway. These specific pathways are determined by where amyloid precursor protein (APP), the precursor to amyloid-beta, is cleaved. In the nonamyloidogenic pathway, APP is cleaved by α-secretase within the Aβ region and γ-secretase at C83 at the C terminal. α-secretase activity is attributed to a family of zinc metalloproteases and have a long zinc-binding sequence.

Further, any amyloid-beta that is developed in a healthy brain is controlled by amyloid-beta degrading enzymes, many of which are zinc metalloproteases. Then, even the amyloid-beta plaque itself often has zinc binding to it demonstrating the universal effect zinc can have on amyloid (Watt, Whitehouse, & Hooper, 2010). Zinc also binds to amyloid when it is released into the synaptic cleft which prevents the protein's clearance. The amyloid interacts with copper which can lead to the production of hydrogen peroxide and can cause neuronal damage (Bush et al, 2008).

In AD patients, amyloid plaques disturb the blood-brain barrier which can cause other metals to reach the cerebral cortex; this results in redistributed cerebral zinc levels. This includes lowering levels of zinc on amyloid-beta degrading enzymes, and higher concentrations of zinc on amyloid-beta plaques. Therefore, while healthy elderly populations often have lower levels of zinc, AD patients often have excess zinc in the brain which largely results in higher concentrations of amyloid plaques (Nuttall & Oteiza, 2014).

There are often low concentrations of zinc in elderly, hospitalized patients which can be explained by dietary changes, including reduced motivation or ability to cook (Nuttall & Oteiza, 2014; Watt, Whitehouse, & Hooper, 2010). Zinc supplementation is often given to these populations, and has shown benefits (Nuttall & Oteiza, 2014). Adding the metal to small clinical trials resulted in decrease of positive symptoms in both atherosclerosis and depression because of zinc's anti-inflammatory and antioxidant effects (Bao et al, 2010; Lai et al, 2012).

This demonstrates the complications with elderly populations given zinc as a universal treatment: AD patients will receive them too, which will further cause their zinc levels to rise. Surprisingly, there have also been small clinical trials that indicate zinc supplementation could be beneficial for AD patients. Zinc-aspartate supplementation improved cognitive performance in eight out of ten patients with AD, and a six-month randomized placebo-controlled trial of reaZin conducted in sixty AD patients found a prevention of cognitive decline, though this did not reach statistical significance (Constantinidis, 1992; Brewer, 2012). In both studies, the sample size was small, and the effects were limited.

One mouse model study also demonstrated positive effects by preventing cognitive deficits. Corona et al (2010) gave 30 p.p.m. of Zn^{2+} to 3xTg-AD male mice (mice with APP, tau, and PS1 pathology) starting at one month old and continuing for 11 to thirteen months. Mice underwent a three-day training period, and then spatial reference probe trials at 1.5 and 24 hours. 3xTg-AD mice displayed no impairment in short term memory but had spent significantly longer to find the platform for long-term memory.

Zinc treatment prevented the long-term decline seen in the mice. They also evaluated the mice using the novel object task and found there was no difference between treated and untreated mice, which made them conclude that zinc only helped in the hippocampus (Corona et al, 2010). They could have found that zinc was helpful in this study due to the older age mice were when tested, which could indicate that zinc at first results in a decline but then ultimately helps.

Zinc & Copper in Previous Research

However, most mice model behavioral research indicates that zinc supplementation often results in negative behavioral effects. For example, this project was, in part, based on the study conducted by Lippi, Smith, and Flinn (2018). They also used dual transgenic mice (dual Tg) breeding together the rTg4510 tau mouse and the J20 hAPP mouse, the same mice models that were used in the present study and also tested at 3.5 months and later at 7 months. Half of the mice received zinc water while the other half received normal tap water. To test spatial memory, they used the Barnes Maze, which is a spatial memory test similar to the Morris Water Maze test except the Barnes Maze features “escape” holes on a circular platform. Researchers found wildtype mice were significantly faster at finding the escape hole and spent more time in the target quadrant than dual Tg mice. While they did not observe any main effects with zinc, they observed that male mice on zinc water took more time to find the escape hole than male mice on lab water and made more primary errors. Thus, while there were no main effects of water, male mice on zinc water performed significantly worse than males on lab water for multiple measures.

Lippi et al (2018) also investigated measures activities of daily living through burrowing and nesting. After burrowing for two hours, dual transgenic mice burrowed significantly less pea-gravel compared to control mice. For nesting, dual Tg mice built significantly poorer nests than control mice. There were no main effects of zinc for either burrowing or nesting, nor for circadian activities though dual Tg mice on zinc water trended towards running less than mice of the same genotype on lab water (Lippi, Kakalec, Smith, Flinn, 2020). Data from the model study indicate effects of zinc may be overshadowed by the Alzheimer's effects that a mouse model displaying tau and APP would show. However, if there are any effects, male mice would have worse effects largely evidenced by the results from the MWM test.

Another study to explore zinc's behavioral effects outside of spatial memory was conducted by Craven, Kochen, Hernandez, and Flinn (2018). Comparing tau-only mice, half of the tau and WT mice were given 10 ppm of zinc water while the other half received regular tap water at eight weeks of age. To conduct the MWM test, mice ran three trials of 60 seconds per day for six days. Researchers found tau mice performed significantly worse in learning the platform, had significantly fewer crossings of the target platform, and spent more time in the outer ring of the pool compared to WT mice. Like Lippi et al, (2019) there were no main effects of water for MWM.

To test nesting, Craven et al (2018) used 2.5 grams of shredded paper and mice were left undisturbed for two days. At the end of the two-day period, researchers found that tau mice constructed significantly worse nests than WT mice, and that tau mice on zinc water constructed significantly worse nests than all other groups. Circadian rhythm

was measured by placing mice in individual running wheel cages for seven days while running wheel activity was assessed using ClockLab. Researchers found that tau mice were significantly delayed in starting activity compared to wildtype mice, and that tau mice on zinc also demonstrated the largest delay compared to tau on lab water and WT on zinc water. Wildtype mice with zinc water also had a trend for a delayed onset time compared to WT mice on lab water. Neither test showed a main effect of water.

Thus, zinc effects were most emphasized within the genotype, though the transgenic and zinc water group displayed the worst activity overall. Results from Craven et al (2018) could indicate that zinc will have no effect on spatial memory but could impact activities of daily living and circadian activity. Further, synthesizing from Lippi et al (2019), the effects of both tangles and plaques in the AD mice model may result in the behavioral effects overshadowing the metal effects. Additionally, the mice are being tested when they are young, so differences may not be as apparent.

Linkous et al (2009) used the Tg2576 mice model that harbor APP mutations and only tested spatial memory. Each APP mouse model and the control were randomly assigned either zinc-enhanced water or lab water. The Tg2576 mice were given zinc water prenatally and continued after birth. Mice were tested on an MWM for 8 days with three trials per day. Every 6^{th} trial was a probe day. Zinc-enhanced mice swam in the incorrect outer-most and inner-most pool ring significantly more than lab water mice. Lab water mice swam in the correct quadrant the most often. During Atlantis trials, zinc-enhanced mice were more likely to swim in the incorrect rings compared to control mice. There was also an interaction in which Tg-mice on zinc water had significantly slower

escape latency compared to all other groups. Probe trials also indicated that zinc-treated mice were significantly more thigmotaxic than lab-water animals and spent significantly less time in the middle of the pool. Overall, Tg2576 mice performed significantly worse than control mice, and zinc-enhanced mice also performed significantly worse than lab water mice on multiple aspects of spatial memory (Linkous, Adlard, Wanschura, Conko, & Flinn, 2009).

Flinn, Bozzelli, Adlard, and Railey (2014) examined mice models with APP and used two types of transgenic mice: CRND8 with only APP and CRND8/E4 with both APOE4 and APP. Zinc supplementation began at six weeks and behavioral testing was at five months of age. Like the study above, only spatial memory was analyzed, this time using the Barnes Maze. Mice received three trials per day with day one as a habituation day and day seven as a probe trial. On days 1-5, wildtype mice had significantly shorter primary latencies compared to CRND8 mice but not CRND8/E4, who were also not significantly different than CRND8. There were no main effects of water for primary latency. There were also no main effects of genotype or water for primary errors, but CRND8/E4 mice on zinc water made significantly more primary errors than CRND8/E4 mice on lab water. Finally, there were also no main effects of genotype or water for the 24-hour probe trial, but zinc water wildtype mice spent significantly less time in the target quadrant compared to lab water wildtype mice. Thus, zinc affected the CRND8/E4 and wildtype groups, but not CRND8 mice, and there were also genotypic effects outside of water effects. Overall, zinc affected the mice within the genotype rather than comparing across genotypes.

Synthesizing data from studies that tested spatial memory, zinc will most likely result in a poorer performance and there should also be a genotypic difference. There is a possibility that zinc supplementation to control mice will result in worse performances in Morris Water Maze and circadian activities compared to non-treated WT mice. This is hypothesized to be a result of zinc's close interaction with copper. Excess zinc intake can result in a copper deficiency because zinc prevents the adequate absorption through the intestinal wall (Railey, Micheli, Wanschura, & Flinn, 2010). When working properly, copper affects the amyloid cascade, by helping to cut APP to the nonamyloidogenic pathway. However, copper can bind to amyloid-beta which can assist in the development of Aβ neurotoxicity. The metal can generate hydrogen peroxide which then produces oxidative stress ultimately resulting in the formation of plaques. Thus, by also supplementing copper, the extra intake of the metal could positively affect any deficiency that the addition of zinc may have had in the diet.

One study that tested both zinc and copper used male Sprague-Dawley rats that were raised pre- and post-natally on zinc carbonate or zinc carbonate and copper carbonate. These animal models do not have AD pathology but demonstrate the effect the addition of copper supplementation can have when already including zinc. They were tested using fear conditioning and Morris Water Maze. Railey et al (2010) found that there were no differences between water groups during training for fear conditioning, but when testing retention, zinc animals had significantly higher freezing rates than lab controls and zinc and copper rodents. For extinction, all animals had significantly lower freezing rates across all six days, but zinc animals had significantly higher freezing rates

than lab controls. There was a trend for zinc and copper animals to have higher freezing rates than controls, but it was not significant. Researchers found that in Morris Water Maze, zinc-treated animals had significantly longer escape latencies on days 2 through 5, and zinc and copper treated animals were not significantly different than controls or zinc-treated but had shorter escape latencies. Zinc-treated animals also spent significantly less time searching in the correct quadrant of the pool, while zinc and copper animals were not significantly different, but spent more time in the correct quadrant than zinc-treated. Thus, these results focus on hippocampus levels of zinc and indicate that supplementation of zinc results in cognitive deficits, even in non-AD models. Further, adding copper to zinc supplementation can help alleviate some of the cognitive deficits.

It is necessary to investigate if the same deficits are prevalent in Alzheimer's mice models as well. Unfortunately, not many studies have featured both metals supplemented to AD mice models, and one known study uses the Tg2576 mouse, with only the APP mutation (Railey, Groeber, Flinn, 2011). Female mice were given either lab water, zinc carbonate or zinc carbonate plus copper carbonate post-natally and tested for spatial memory using Morris Water Maze at 14 months of age. Mice were tested using four 60-second trials for six days and then one day with an Atlantis probe trial. Zinc-treated animals had significantly longer latencies than lab water mice on days four through six. Zinc and copper animals had a trend of shorter escape latencies than zinc animals, but was not significant, nor did they have a significantly longer latency period compared to controls. Tg zinc-water had significantly longer latency compared to zinc and copper and

lab water groups for days one through six and days four through six. Wildtype mice did not display water treatment differences (Railey, Groeber, Flinn, 2011).

Treatment of zinc and copper helped alleviate some of the spatial deficits brought about by zinc-only supplementation for both AD-transgenic and non-transgenic animals. This indicates that providing copper to zinc supplements can be beneficial to lessen the behavioral effects evidenced by the studies above. Providing zinc with copper supplementation may prevent those animals from being significantly different compared to controls but may still trend towards decline compared to zinc-only supplementation. It is important to note that in the study above, the mice were older than the ones that will be tested for this project, and only had one AD mutation, which may affect results.

The research above indicates the contradicting evidence for the role of zinc and copper in AD patients, and the overall lack of research regarding behavioral tests outside of spatial memory. Additionally, genotypic research is not as conflicted as metal treatment, but this study can solidify findings already present. Thus, this project aimed to clarify the behavioral effects of zinc and zinc and copper on spatial memory while providing dimension to other behavioral tests including activities of daily living and circadian activities. Current research often emphasizes nesting and there are limited studies that include burrowing, which allowed this study to explore nuances in the findings between the two ADL tests. Mice will be tested at 3.5 months. Given the previously cited research, the hypotheses for this study regarding water treatment are as follows: 1) zinc-only treatment mice will have significantly worse scoring in Morris Water Maze, including latency, thigmotoxicity, and Atlantis probe trial compared to

controls, but not compared to zinc and copper treatment groups; 2) zinc-only treatment mice will have significantly worse scores in nesting, burrowing, and circadian activities compared to controls but not the zinc and copper treatment group; 3) zinc and copper treatment group will trend towards the zinc-only treatment group, but not be significantly different than them or controls in any behavioral test. Furthermore, since both AD and wildtype (WT) mice are being tested, the genotypic theses for this study are: 1) dual transgenic mice will perform significantly worse than WT controls in every behavioral test, and 2) under the possibility that the dual transgenic pathology overshadows the water treatment effects, there will be water treatment differences in the WT groups, but not dual transgenic groups.

METHODS & MATERIALS

Animals

Mice were bred through a pairing of the J20 hAPP mouse (Tg(PDGFB-APPSwIND)20Lms/2Mmjax) and the rTg4510 tau mouse (Tg(Camk2a-tTA)1Mmay Tg(tet0-MAPT*P301L)#Kha/J) ordered through Jackson Laboratory and MMRRC. Animal genotype was confirmed through analysis of tail snips by Transnetyx, Inc, and then animals were housed in separate cages based on sex and genotype. Mice that had both APP and tau mutations are referred to as dual Tg whereas mice with neither mutation are referred to as wildtype (WT) or controls.

Housing

Each cage (Animal Care Systems) was equipped with an igloo with an attached running wheel. The colony maintained a 12-hour light/dark cycle. Mice were handled weekly to acclimate to human touch for behavioral testing. Animals were provided food and water ad libitum. All procedures were approved by the George Mason University Institutional Animal Care and Use Committee.

Testing Water

At eight weeks, the mice were taken off lixit water and placed on bottled water according to their assigned type (zinc, zinc with copper, lab). One-third of WT mice

received each water type and one-third of dual Tg mice received each water type. Preparation of zinc water (Linkous et al, 2009; Railey et al., 2010; Railey et al., 2011) and zinc and copper water (Railey et al, 2011) was completed on site and according to previous studies. Water was provided to the animals via one plastic water bottle attached to the cage and shaken during routine animal handling to ensure proper dispersion of the metal ions throughout. Water samples were taken prior to the start of the study and tested for metal content using inductively coupled plasmaoptical emission spectroscopy and ion chromatography at the United States Geological Survey (USGS), Reston, VA.

Zinc water was prepared by first acquiring 10 liters of lixit water from the facility. Ten milliliters of a 10,000ppm solution of zinc dissolved in 5% nitric acid were then added. This water was buffered using three grams of sodium carbonate ($NaCO_3$) in a one-liter distilled water solution to bring it to a pH of between 6.8 and 7.2. On average, about 80 mL of the buffer was needed to bring the concentration to the desired acidic range.

Zinc and copper water was prepared as zinc carbonate with the addition of 2.5 mL of copper carbonate. On average, about 150 mL of the buffer was needed to bring the concentration to the desired acidic range.

Preparation was completed on site for both types of metal waters as well as the buffer solution. The zinc, copper, and sodium carbonate were provided by SPEXcertiprep.

Behavioral Tests and Measurements

Mice were taken out of their home cages and placed in individual cages on testing days. Animals were tested at 3.5 months of age in the following order: Morris Water Maze, nesting, burrowing, and circadian activities.

Morris Water Maze.

The Morris Water Maze is a circular pool with a hidden platform (~1 cm below surface) beneath opaque water and is used to assess spatial memory. Four distinct cues were placed around pool to aid in recognition. Testing occurred over a seven-day period. Each day had three trials of 60 seconds and 45 seconds inter-trial waiting period. On the even number days (2, 4, 6), the platform was fully submerged for the third trial to conduct an Atlantis probe trial. The seventh day featured a visual platform to ensure animals did not have visual deficits. Latency to find platform, thigmotaxis (time spent around edges of pool), number of platform crosses on probe trials, and time in target quadrant were measured.

Nesting.

Individual cages were set-up for each animal that contained 2.5 grams of shredded printer paper spread evenly over corncob bedding. Mice were left undisturbed for 18 hours after all cages were photographed. Photographs were taken at 2 hours and at 18 hours. Two blind raters with no knowledge of the experiment scored the pictures on a scale of 1-5 with 5 indicating a well-made nest with no stray papers and 1 indicating no attempt at organizing the bedding into a nest. Scores were averaged.

Burrowing.

Individual cages were set up with PVC pipes containing 300 grams of pea-gravel (small rocks) with one end closed off. Cohort 1 only had 225 grams of pea-gravel due to lack of material. Measurements of the amount of pea-gravel removed were measured at 2 hours and 18 hours. After weighing the PVC tube at the two-hour mark, the tube was placed carefully back in the same location to try to avoid disturbing pea-gravel already displaced.

Circadian Activities.

Mice were placed in individual running-wheel cages for seven days with access to food and water and a layer of bedding. The running wheel was monitored, and data collected by ClockLab software which monitors wheel rotation. The first and last day's data was discarded to avoid experimenter interference. The software totals the number of wheel rotations for each hour and averages it across the five-day experimental period.

Statistical Analysis

Morris Water Maze.

A total of six 2 (genotype: dual Tg, WT) x 3 (water type: zinc-only, zinc and copper, lab) x 6 (days) repeated measures ANOVA was conducted on the dependent variables mentioned above. Those variables are latency to platform, number of platforms crosses on hidden platform day, time in target quadrant, and thigmotaxis. Time in target quadrant and thigmotaxis were analyzed for both probe and non-probe trials whereas

latency to platform was only analyzed for non-probe trials and number of platform crosses was only analyzed for probe trials. Independent variables included genotype, water type, sex, and day of testing.

Nesting.

A 2 (genotype: dual Tg, WT) x 3 (water type: zinc-only, zinc and copper, lab) factorial ANOVA was performed using the dependent variable of the averaged score provided by the two nesting assessors for the mouse. The test was conducted for both hour intervals. Independent variables are genotype, water type, and sex.

Burrowing.

A 2 (genotype: dual Tg, WT) x 3 (water type: zinc-only, zinc and copper, lab) factorial ANOVA was performed using the dependent variable of percentage of pea gravel lost. The test was conducted for both measurement checks. Independent variables are genotype, water type, and sex.

Circadian Activities.

Two 2 (genotype: dual Tg, WT) x 3 (water type: zinc-only, zinc and copper, lab) x 12 (time: active hours 8pm-8am; non-active hours 8am-8pm) repeated measures ANOVA test was performed using the dependent variables of total activity. The time when the mice start running, stop running, and overall activity were observed and analyzed. The same test was conducted for non-active hours. Independent variables are

genotype and water type but does not include days because the days' wheel rotations are averaged.

RESULTS

In total, there were 82 mice used in this study. Numbers were kept as evenly as possible with consideration to breeding conditions. There were 40 dual Tg mice and 42 WT mice; there were 39 male mice and 43 female mice. Further, there were 27 mice on zinc water, 27 mice on zinc & copper water, and 28 mice on lixit water.

Table 1. Breakdown of mice categorized by genotype, water type, and sex. The table below lists the overall number of mice in each of the three independent variables that were evaluated for this study.

		Zinc	**Zinc & Copper**	**Lixit**	**TOTAL**
Dual Tg	**Female**	7	8	8	23
	Male	6	5	5	16
WT	**Female**	6	7	7	20
	Male	8	7	8	23
TOTAL		27	27	28	**82**

While there were 82 total mice used in the study, there were varied complications with data collection that fewer mice were used for tests. In Morris Water Maze, there

were errors in saving data for cohort 1 (n = 9), so it is only represented for latency in non-probe trials. Additionally, there were two mice that did not record any data. Further, in the circadian activity test, seven cages did not sufficiently record wheel rotations, and one mouse died in the middle of testing, so that test only featured 74 mice tested. There were no complications for nesting or burrowing, so those tests used all 82 mice.

Morris Water Maze

At 3.5 months, the latency to platform had a main effect of genotype, $F(1, 68) = 245.766$, $p < .001$ in which WT mice swam significantly faster than dual Tg mice. There was no main effect of water, $F(2, 68) = 1.175$, $p = .315$ nor sex, $F(1, 68) = .039$, $p = .843$. There was a significant main effect of day, $F(4.545, 309.080) = 23.22$, $p < .001$, and two interactions involving the variable. First, there was an interaction between day and genotype, $F(4.545, 309.080) = 7.312$, $p < .001$, in which WT mice had significant learning improvement in the first days whereas the dual Tg mice did not have significance unless comparing the last day to the first three days. Additionally, there was an interaction between day and water, $F(9.091, 309.080) = 2.698$, $p = .005$ in which zinc and copper mice learned faster by showing improvement on day two compared to the lixit and zinc mice that do not show learning until day four.

Table 2. Cumulative average of latency for genotype and water type. The table below lists the overall average time it took the mice to reach the platform averaging the times across the six days that featured non-Atlantis platforms (excluding visual platform).

Genotype	**Water**	**Average Time to Platform**
WT	Zinc	22.27
	Zinc & Copper	18.80
	Lixit	20.95
Dual Tg	Zinc	48.68
	Zinc & Copper	46.33
	Lixit	49.75

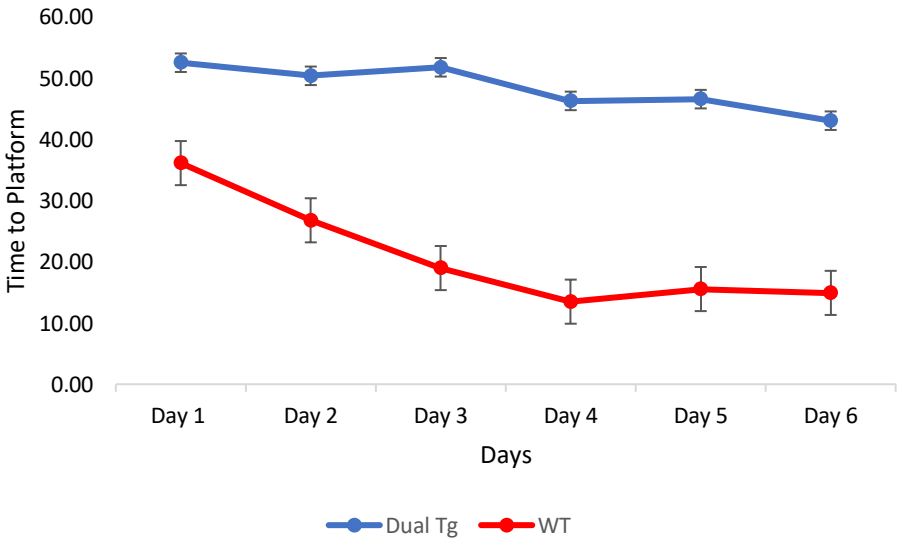

Figure 1. Latency differences in genotypes. This figure demonstrates the disparate difference in genotypes in which WT mice (M = 20.671) swam significantly faster than

dual Tg mice (*M* = 48.253) starting on the first day, and that pattern continued for all six days.

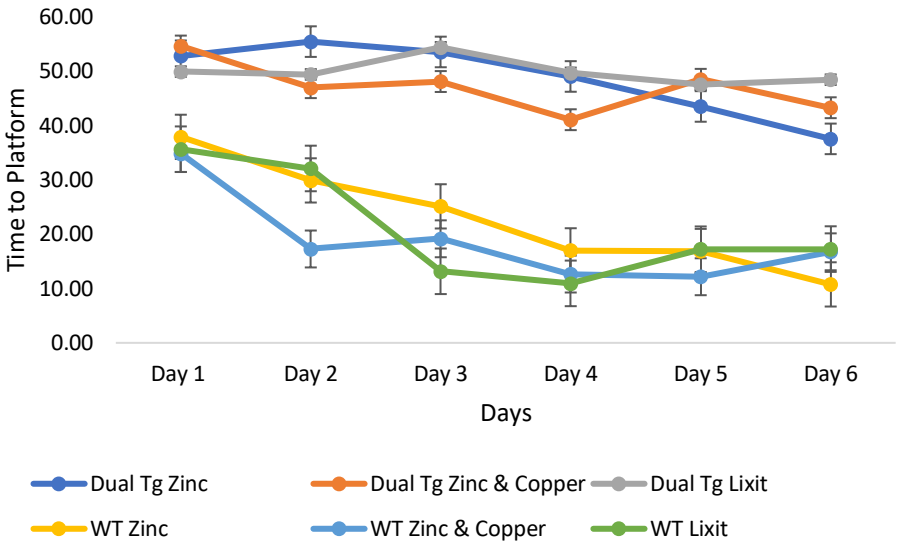

Figure 2. Latency differences between genotypes and water type. The figure demonstrates how WT mice found the platform quicker than dual Tg mice regardless of water type.

While there was a main effect of genotype for latency, the same effect was not observed for percentage of time spent in the target quadrant, $F(1, 59) = 2.060$, $p = .157$. Similarly, there was no main effect of water, $F(2, 59) = .979$, $p = .382$ nor sex, $F(1, 59) = .180$, $p = .673$. There were no significant interactions. There was also only a main effect of day, $F(4.456, 262.924) = 2.725$, $p = .025$, but no interaction effect involving day indicating an overall improvement over time, but no specific categorization resulted in that improvement.

Table 3. Cumulative average percent time spent in target quadrant. The table below details the average percent mice spent in the target quadrant over the course of the six days across all trials.

Genotype	Water	Average Percent in Target Quadrant
WT	Zinc	22.29
	Zinc & Copper	20.37
	Lixit	22.36
Dual Tg	Zinc	22.29
	Zinc & Copper	20.04
	Lixit	22.04

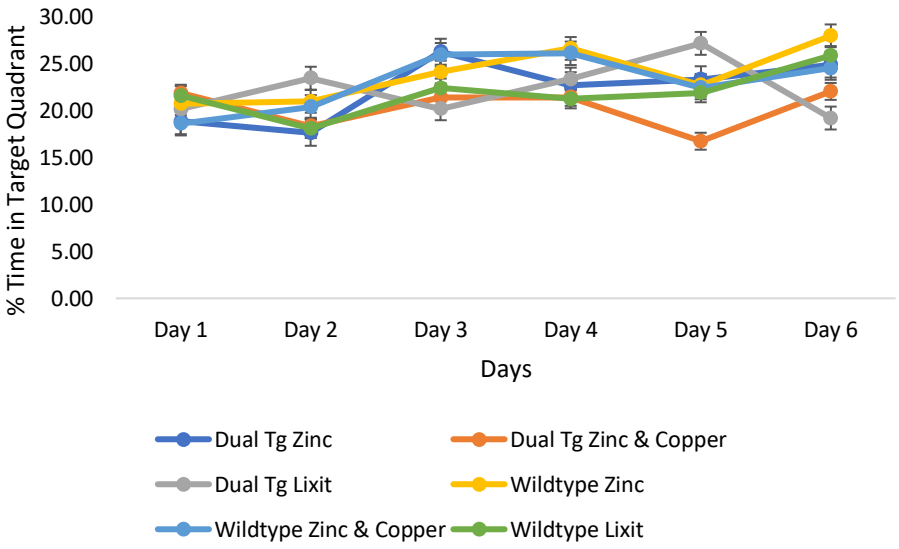

Figure 3. Percentage in target quadrant examining genotypes and water type. It is evident all mice regardless of genotype or water spent approximately the same amount of time in the target quadrant.

The last variable measured for non-probe trials was thigmotaxis, which is the percent of time the mice spent along the edges. As before, there was a significant effect of day, $F(3.993, 235.596) = 33.310$, $p < .001$, as well as a significant interaction between day and all three independent variables: genotype, $F(3.993, 235.596) = 7.692$, $p < .001$, water type, $F(7.986, 235.596) = 2.065$, $p = .040$, and sex, $F(3.993, 235.596) = 2.728$, $p = .030$. The WT mice demonstrated a rapid improvement in thigmotaxis compared to the dual Tg mice. The zinc and copper mice also demonstrated faster improvement compared to the other two water groups whereas the male mice had a faster improvement of time spent along the walls compared to females. There was a main effect of genotype in which dual transgenic mice spent significantly more time along the edges, $F(1, 59) = 173.99$, $p < .001$. There was not a main effect of water, $F(2. 59) = .857$, $p = .430$. In contrast to the other variables, there was a trend for female mice to spend significantly more time along the edges, $F(1, 59) = 3.143$, $p = .081$. There was also an interaction between sex and genotype, $F(1, 59) = 9.100$, $p = .004$ in which dual transgenic males spent less time along the edges compared to the females of the same genotype, $F(1, 59) = 12.116$, $p = .001$. Further, male dual transgenic mice spent significantly less time along the edges compared to male wildtype mice, $F(1, 59) = 55.743$, $p < .001$, and the same pattern was observed among female mice, $F(1, 59) = 122.572$, $p < .001$.

Table 4. Cumulative average percent time spent along edges. The table below displays the average percent time spent along the edges across the six days, separated by genotype, water type, and sex.

Genotype	Water	Thigmotaxis Percentage
WT	Zinc	*Male*: 23.97 *Female*: 17.36 *Total*: 20.66
	Zinc & Copper	*Male*: 15.49 *Female*: 16.48 *Total*: 15.99
	Lixit	*Male*: 20.22 *Female*: 13.64 *Total*: 16.93
Dual Tg	Zinc	*Male*: 52.68 *Female*: 71.94 *Total*: 62.31
	Zinc & Copper	*Male*: 47.45 *Female*: 66.99 *Total*: 57.22
	Lixit	*Male*: 59.34 *Female*: 67.52 *Total*: 63.43

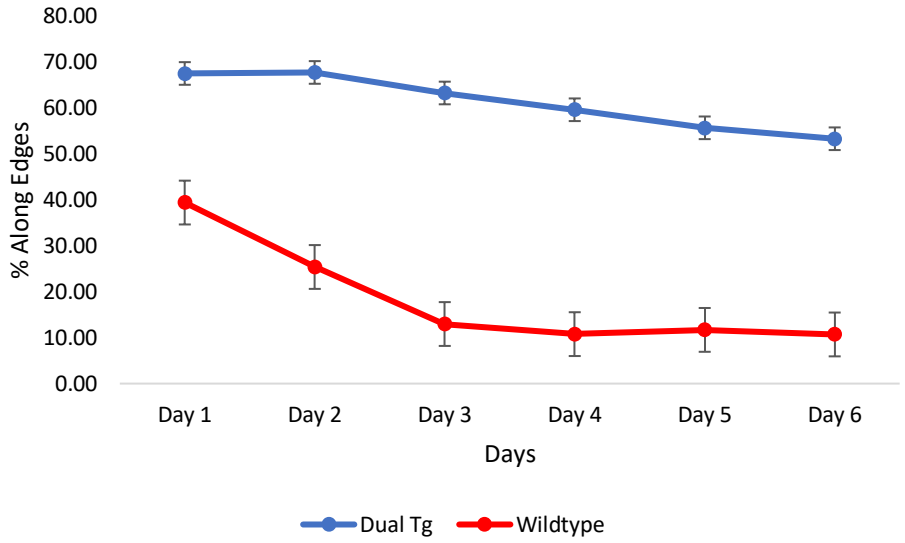

Figure 4. Thigmotaxis percentage between genotypes. WT mice (*M* = 17.86) spent significantly less time among the edges of the pool compared to dual Tg mice (*M* = 60.99) all days.

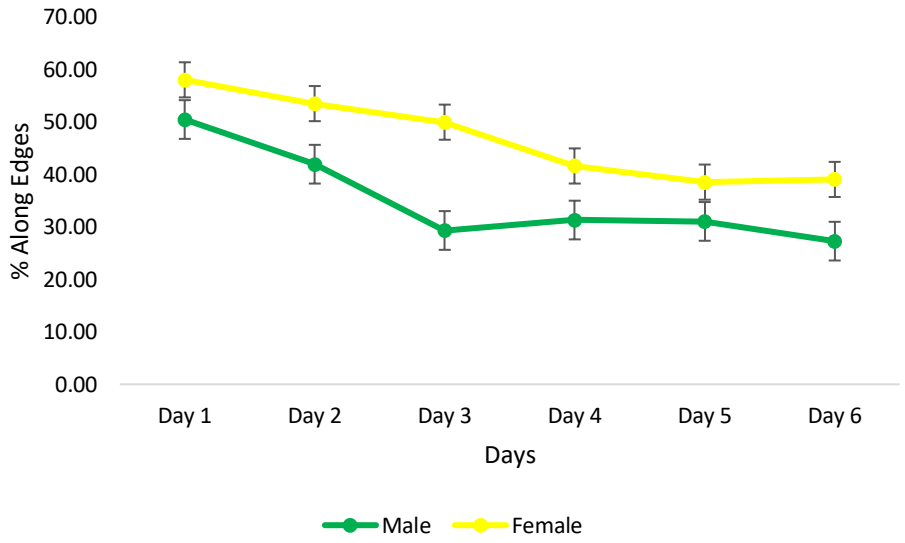

Figure 5. Thigmotaxis percentage between sex. The final figure depicting main effects for thigmotaxis displays the difference between the male and female mice. Female mice (*M* = 42.32) spent significantly more time along the edges compared to male mice (*M* = 36.52) which may indicate that female mice were more anxious compared to male mice.

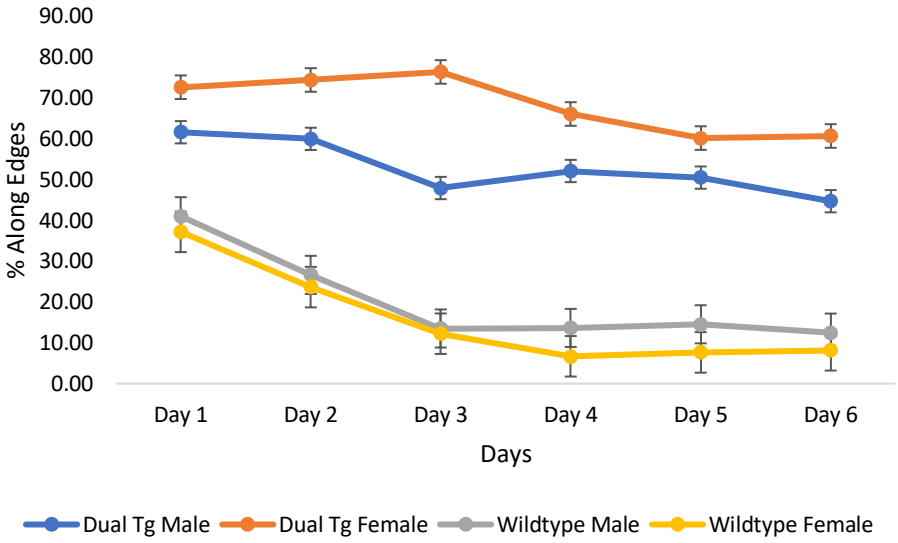

Figure 6. Thigmotaxis percentage examining sex and genotype. Regardless of sex, dual transgenic mice spent more time along the edges, but dual transgenic females (*M* = 68.82) spent more time along the edges compared to dual Tg males (*M* = 53.16) and WT females (*M* = 15.83). Further, there was not a significant sex difference between the wildtype mice as there was for the dual transgenic mice.

Instead of latency, the number of platform crosses is observed for probe trials. There was a main effect of day, $F(2.824, 166.632) = 6.862$, $p < .001$, as well as an interaction between day and genotype, $F(2.824, 166.632) = 4.950$, $p = .003$. The WT mice achieved significant improvement between day 2 and day 7, whereas the dual Tg mice never had significant improvement in the number of platform crosses. There was a main effect of genotype in which WT mice had more platform crosses than dual transgenic mice, $F(1, 59) = 118.29$, $p < .001$. There was not a main effect of sex, $F(1, 59) = .000$, $p = .986$, but there was a main effect of water type, in which zinc and copper treated mice had significantly more platform crosses compared to both zinc and lixit water mice, $F(2, 59) = 3.670$, $p = .031$. While there was no significant interaction effect,

there was a trending interaction between genotype and sex, $F(1, 59) = 3.580$, $p = .063$, which was largely driven by the genotypic effect. This is evident given that male dual transgenic mice had less platform crosses compared to male wildtype mice, $F(1, 59) = 43.464$, $p < .001$, and the same pattern was observed for the female mice, $F(1, 59) = 76.070$, $p < .001$.

Table 5. Number of platform crosses during probe trials. The table features the average number of platform crosses during probe trials on days 2, 4, 6, and 7. Day 7 features the 24-hour probe trial in which the first trial of the day is a probe trial where the mice have not swum the test since the day prior.

Genotype	**Water**	**Number of Platform Crosses**
WT	Zinc	3.39
	Zinc & Copper	4.15
	Lixit	3.18
Dual Tg	Zinc	.54
	Zinc & Copper	1.15
	Lixit	.63

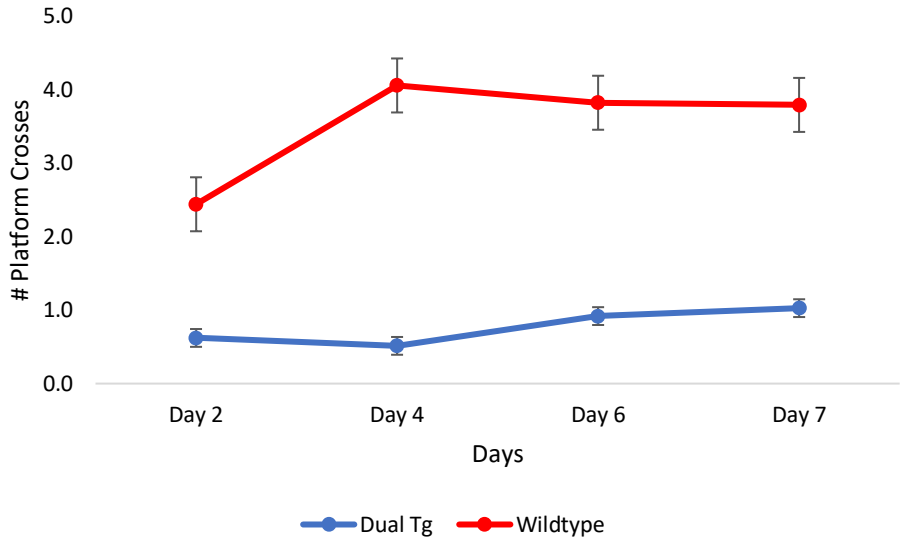

Figure 7. Number of platform crosses during probe trials comparing genotypes. The figure above features the number of times the mice crossed the platform during probe trials comparing the two genotypes. The dual transgenic mice (*M* = .677) crossed the platform significantly less for all probe trials compared to the WT mice (*M* = 3.57).

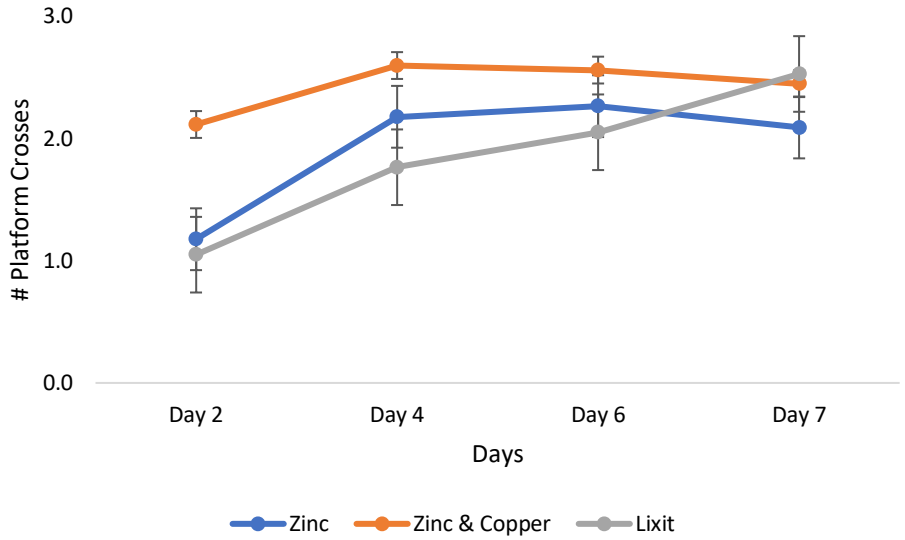

Figure 8. Number of platform crosses during probe trials between water types. The figure above features the number of times the mice crossed the platform during probe

trials comparing the three water types. Mice on zinc & copper (*M* = 2.65) had significantly more platform crosses than both zinc (*M* = 1.96) and lixit water (*M* = 1.90) mice. Further, the figure demonstrates that while the zinc & copper mice had more platform crosses the first two days, but by the remaining days, all water types were similar.

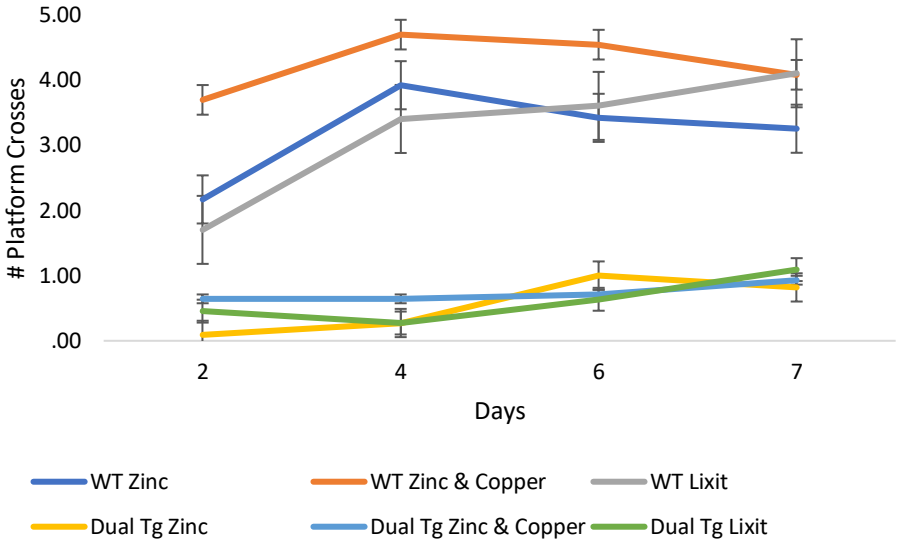

Figure 9. Number of platform crosses during probe trials examining genotypes and water types. WT mice had more platform crosses regardless of water type, but the WT mice on zinc & copper water (*M* = 4.15) crossed significantly more than WT mice on zinc water (*M* = 3.39) and WT mice on lixit water (*M* = 3.18). The same effect was not observed with the dual Tg mice.

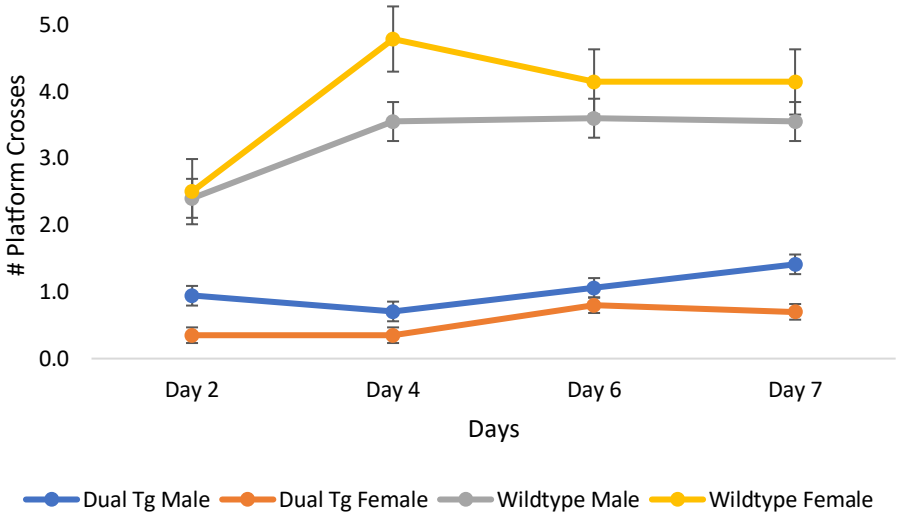

Figure 10. Number of platform crosses during probe trials examining genotype and sex. WT mice had more platform crosses regardless of sex, but female WT mice (*M* = 3.81) had more platform crosses compared to male WT mice (*M* = 3.33). The dual transgenic mice displayed similar behavior regardless of sex, though the males (*M* = 1.01) had a slightly higher average each day compared to the females (*M* = .53).

Percentage of time spent in the target quadrant was also measured for the probe trials. Unsurprisingly, dual Tg mice spent significantly less time in the target quadrant compared to WT mice, $F(1, 59) = 7.067$, $p = .010$. There was not a main effect of water type, $F(2, 59) = .030$, $p = .971$, nor sex, $F(1, 59) = .301$, $p = .585$. There were no interaction effects, as well as no main effect of day, $F(2.855, 168.447) = 1.374$, $p = .253$, indicating an overall small change in the amount of time spent in the target quadrant across days.

Table 6. Cumulative average percent time spent in the target quadrant during probe trials. The table features the average percentage time spent in the target quadrant during probe trials on days 2, 4, 6, and 7. Day 7 features the 24-hour probe trial in which the first trial of the day is a probe trial where the mice have not swum the test since the day prior.

Genotype	Water	Percent Time Spent in Target Quadrant
WT	Zinc	23.72
	Zinc & Copper	20.95
	Lixit	23.19
Dual Tg	Zinc	23.72
	Zinc & Copper	20.95
	Lixit	23.19

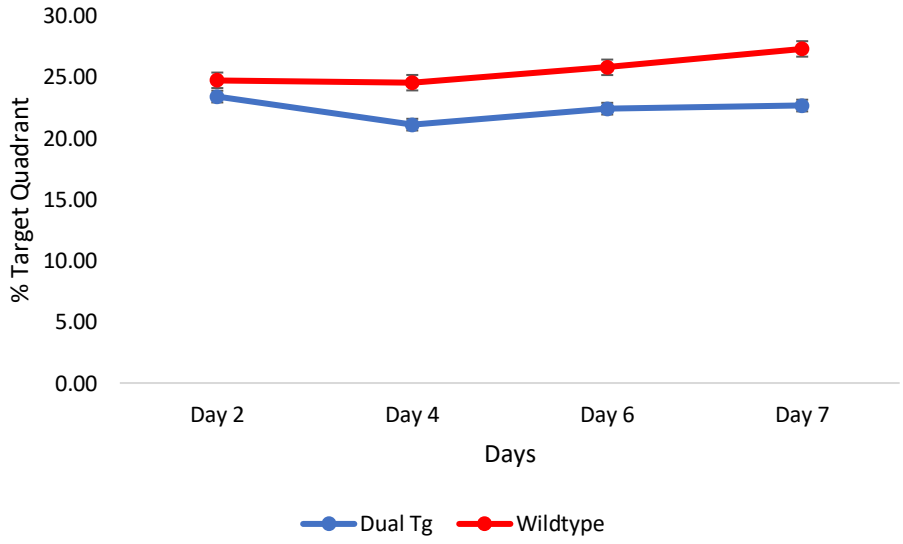

Figure 11. Percentage in target quadrant during probe trials comparing genotypes. The figure above demonstrates the percent time spent in the target quadrant for each

genotype. Wildtype mice spent significantly more time in the target quadrant (M = 25.63) compared to dual Tg mice (M = 22.62).

The last variable observed for probe trials was thigmotaxis. First, there was a main effect of day, $F(2.919, 172.218) = 34.692$, $p < .001$, as well as an interaction between genotype and day, $F(2.919, 172.218) = 14.116$, $p < .001$, in which dual Tg mice had significant improvement between measuring the last day to all others whereas WT mice was only significant between the first and last day. There was also an interaction effect between water type and day, $F(5.838, 172.218) = 2.307$, $p = .038$ in which zinc mice had the quickest significant improvement compared to the other two water groups. Both dual transgenic and wildtype mice spent less time along the edges with the progression of days, though dual transgenic mice spent more time along the edges, leading to the main effect of genotype in which wildtype mice spent significantly less time along the edges of the pool compared to dual Tg mice, $F(1, 59) = 193.995$, $p < .001$. Though there was no main effect of water, $F(2, 59) = .732$, $p = .485$, the interaction indicated that each water type spent less time along the edges with each day. There was a main effect of sex, $F(1, 59) = 4.824$, $p = .032$. This effect indicates male mice spent significantly less time along the edges compared to female mice. There was also an interaction between genotype and sex, $F(1, 59) = 12.641$, $p = .001$, that was driven by the genotypic effect and the dual transgenic mice. Male dual transgenic mice spent significantly more time along the edges compared to male wildtype mice, $F(1, 59) = 57.941$, $p < .001$, and the same effect was observed among the female mice, $F(1, 59) =$

142.638, $p < .001$. The dual transgenic male mice also spent significantly less time along the edges compared to female dual transgenic mice, $F(1, 59) = 17.473$, $p < .001$, a difference that was not seen in the WT mice.

Table 7. Cumulative average percent time spent along edges during probe trials. The table features the average time spent along the edges of the pool during probe trials on days 2, 4, 6, and 7. Day 7 features the 24-hour probe trial in which the first trial of the day is a probe trial where the mice have not ran the test since the day prior.

Genotype	**Water**	**Thigmotaxis Percentage**
WT	Zinc	*Male*: 24.15 *Female*: 12.81 *Total*: 18.48
	Zinc & Copper	*Male*: 17.13 *Female*: 14.52 *Total*: 15.83
	Lixit	*Male*: 13.48 *Female*: 13.27 *Total*: 13.37
Dual Tg	Zinc	*Male*: 48.83 *Female*: 80.78 *Total*: 64.80
	Zinc & Copper	*Male*: 45.35 *Female*: 71.86 *Total*: 58.61
	Lixit	*Male*: 68.63 *Female*: 70.07 *Total*: 69.35

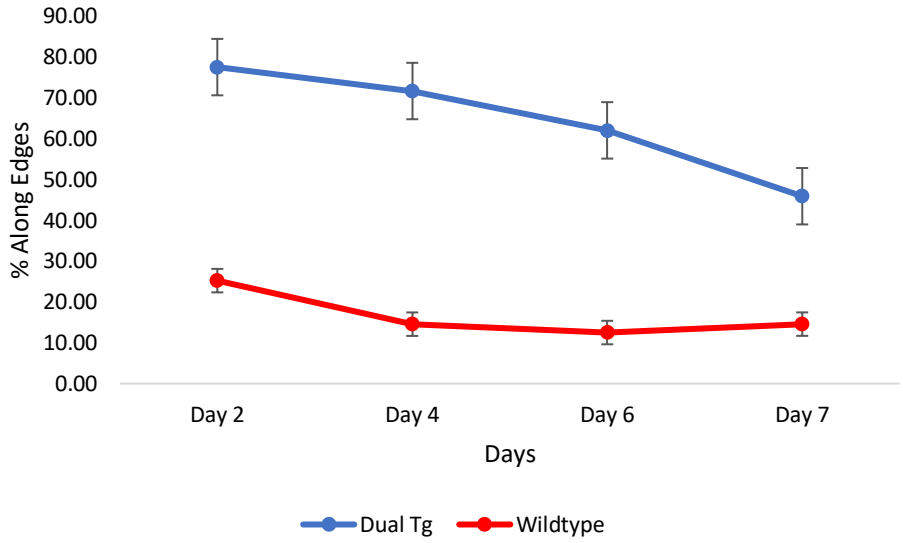

Figure 12. Thigmotaxis percentage between genotypes during probe trials. The figure displays the genotypic differences during the probe trials across days 2, 4, 6, and 7. WT mice (*M* = 15.89) swam along the walls for a significantly shorter period than dual Tg mice (*M* = 64.25) for all days.

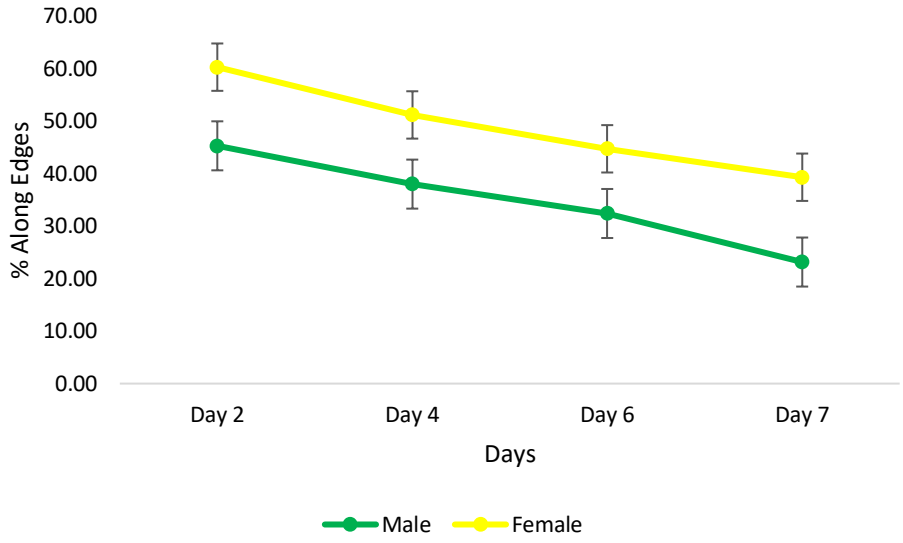

Figure 13. Thigmotaxis percentage between sexes during probe trails. Females (*M* = 43.89) swam along the walls significantly more than male mice (*M* = 36.26) for all probe trials.

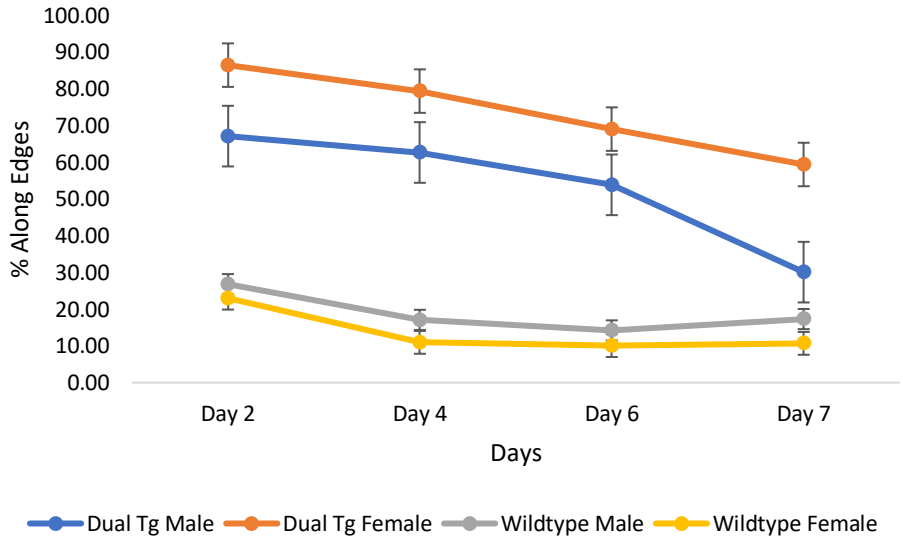

Figure 14. Thigmotaxis percentage examining genotype and sex during probe trials. Dual transgenic mice spent more time along the edges compared to wildtype mice regardless of sex, but dual transgenic female (*M* = 74.24) mice spent significantly more time along

edges compared to dual transgenic males (M = 54.27) and the WT female mice (M = 13.54).

Nesting

At 2 hours, there were no main effects for any of the three independent variables: genotype, $F(1, 70) = 3.613$, $p = .061$, water type $F(2, 70) = 1.138$, $p = .326$, nor sex, $F(1, 70) = 2.596$, $p = .112$. There was an interaction between genotype and sex, $F(1,70) = 4.039$, $p = .048$ in which female dual Tg mice scored significantly less than female WT mice, $F(1, 70) = 8.113$, $p = .006$, and male dual Tg mice scored significantly more than female dual Tg mice $F(1, 70) = 6.291$, $p = .014$. There was no overall interaction between genotype and water type, $F(2, 70) = 1.162$, $p = .319$, though zinc and copper dual Tg mice scored significantly less than zinc and copper WT mice $F(1, 70) = 4.891$, $p = .030$.

Table 8. Average nesting scores at 2 hours. Average nesting score at two hours. Scores are based on the following 1-5 scale: 1: shredded paper remains untouched, 2: some attempt to build a nest visible but majority untouched, 3: nest construction is partially completed but some paper is still scattered, 4: the nest is almost completed except for a few stray pieces, 5: all of the shredded paper was used to construct the nest.

WT Zinc	Dual Tg Zinc	WT Zinc & Copper	Dual Tg Zinc & Copper	WT Lixit	Dual Tg Lixit
2.714 (*SD* = .871)	2.192 (*SD* = .990)	2.929 (*SD* = 1.016)	1.857 (*SD* = 1.184)	2.786 (*SD* = 1.354)	2.250 (*SD* = 1.166)

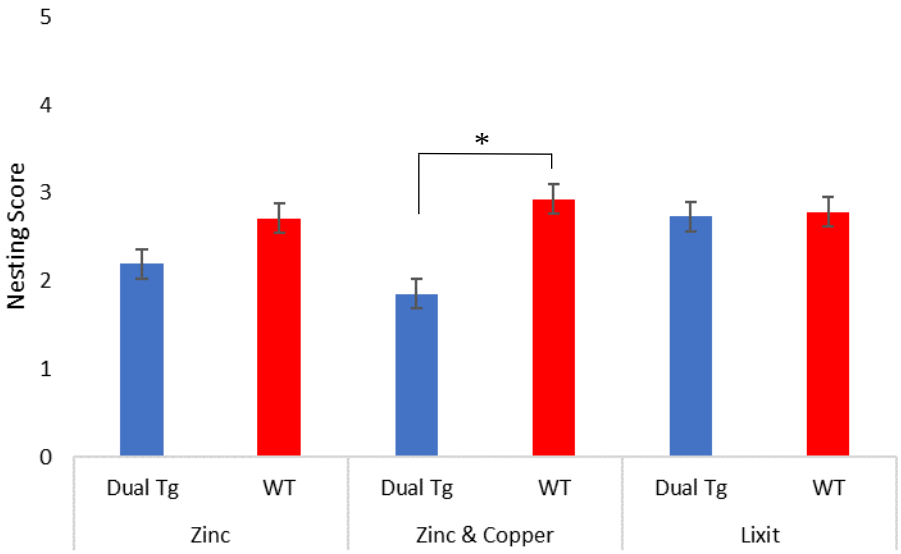

Figure 15. Nesting scores at 2 hours. Average nesting score collected at two hours. Scale explained above. An asterisk (*) denotes significance at $p < .05$. There is only one significant result in which the dual Tg mice on zinc and copper water scored significantly less than the WT mice on the same water.

In contrast to the two-hour interval, there were two main effects at 18 hours: genotype and sex. Dual Tg mice scored significantly less than WT mice, $F(1, 70) = 27.391$, $p < .001$, and male mice scored significantly higher than female mice, $F(1, 70) = 5.123$, $p = .027$. There was no main effect of water, $F(2, 70) = .363$, $p = .697$. There was an interaction effect between genotype and sex in which female dual Tg mice scored significantly less than male dual Tg mice, $F(1, 70) = 9.529$, $p = .003$, and female WT mice, $F(1, 70) = 29.263$, $p < .001$. Male dual Tg mice also scored less than male WT mice, $F(1, 70) = 4.370$, $p = .040$.

Table 9. Average nesting score at 18 hours. Average nesting score at two hours. Scores are based on the following 1-5 scale: 1: shredded paper remains untouched, 2: some attempt to build a nest visible but majority untouched, 3: nest construction is partially completed but some paper is still scattered, 4: the nest is almost completed except for a few stray pieces, 5: all of the shredded paper was used to construct the nest.

WT Zinc	Dual Tg Zinc	WT Zinc & Copper	Dual Tg Zinc & Copper	WT Lixit	Dual Tg Lixit
4.179 (*SD* = .953)	3.039 (*SD* = 1.361)	4.750 (*SD* = .380)	2.714 (*SD* = 1.326)	4.143 (*SD* = 1.099)	3.192 (*SD* = 1.451)

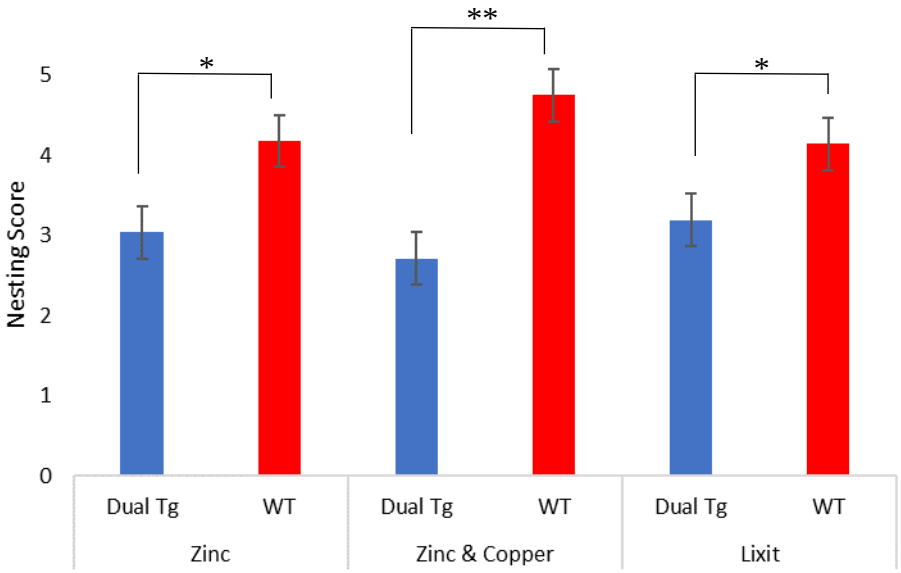

Figure 16. Nesting scores at 18 hours. Average nesting score collected at eighteen hours. Scale explained above. An asterisk (*) denotes significance at $p < .05$ and a double asterisk (**) denotes significance at $p < .001$ level. All dual Tg mice scored significantly less than their WT mice counterparts on the same water type. There was also an overall main effect of genotype.

Table 10. Average nesting score at 18 hours examining genotype and sex. Average nesting scores at 18 hours to include the sex differences.

Dual Tg Male	Dual Tg Female	WT Male	WT Female
3.625 (*SD* = 1.372)	2.542 (*SD* = 1.188)	4.348 (*SD* = .959)	4.368 (*SD* = .831)

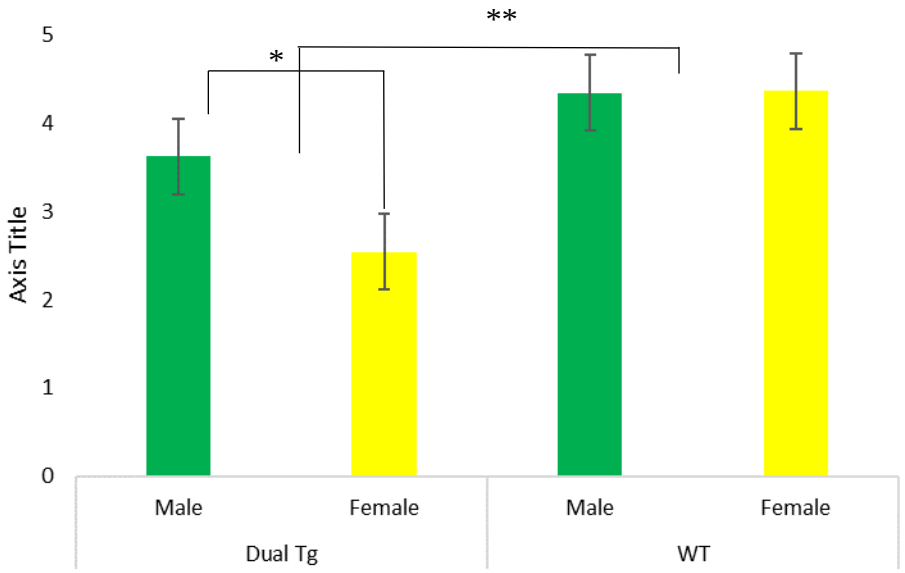

Figure 17. Nesting scores at 18 hours examining genotype and sex. Average nesting score collected at eighteen hours factoring sex. There was a significant main effect of both genotype and sex, though the sex main effect was largely driven by the dual Tg mice in which the males scored significantly more than the female mice. An asterisk (*) denotes significance at $p < .05$ and a double asterisk (**) denotes significance at $p < .001$ level.

Burrowing

As a result of not having enough pea gravel for the first cohort (225 g compared to 300 g for all other cohorts) the values are provided in percentages, demonstrating the percentage of how much pea gravel the mice successfully burrowed from the PVC pipe.

Unlike for nesting, there was a main effect of genotype at two hours. Dual Tg mice burrowed significantly less than WT mice, $F(1, 70) = 13.940$, $p < .001$, but there was no main effect of water, $F(2, 70) = .416$, $p = .662$, nor sex, $F(1, 70) = .497$, $p = .483$. While the dual Tg mice for all water types burrowed significantly less than the WT mice for the same water type, only the zinc, $F(1, 70) = 4.987$, $p = .029$, and zinc & copper, $F(1, 70) = 6.118$, $p = .016$ were statistically significant. Lixit mice were not, $p = .082$.

Table 11. Percentage of pea gravel burrowed at 2 hours. Values for the exact percentages of pea gravel burrowed displayed below.

WT Zinc	Dual Tg Zinc	WT Zinc & Copper	Dual Tg Zinc & Copper	WT Lixit	Dual Tg Lixit
48.069 (*SD* = 34.316)	24.778 (*SD* = 22.125)	51.250 (*SD* = 38.930)	24.113 (*SD* = 21.567)	56.117 (*SD* = 34.142)	34.409 (*SD* = 26.231)

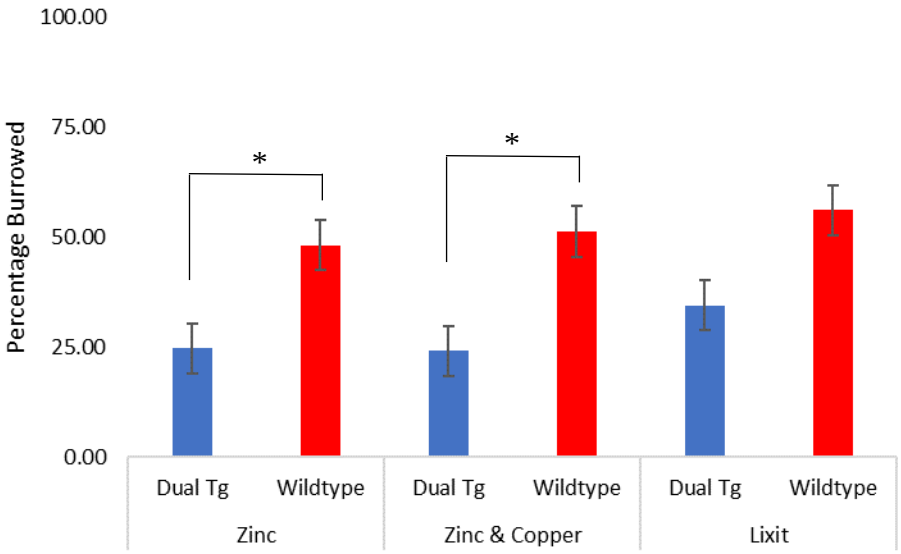

Figure 18. Percentage of pea gravel burrowed at 2 hours. Figure displays the percentage of pea gravel burrowed at two hours. There was a genotypic main effect, as well as an interaction between zinc and zinc & copper mice in which dual Tg mice burrowed significantly less than their WT companions in the same water group. An asterisk (*) denotes significance at the $p < .05$ level.

At 18 hours, there was a main effect of both genotype and sex. Dual Tg mice burrowed significantly less than WT mice, $F(1, 70) = 25.511$, $p < .001$, and male mice burrowed significantly more than female mice, $F(1, 70) = 4.402$, $p = .040$. There was no main effect of water type, $F(2, 70) = 1.844$, $p = .166$. Similar to the two hour interval measurement, the zinc and zinc & copper dual Tg mice burrowed significantly less than their WT mice water companions, $F(1, 70) = 14.567$, $p < .001$ and $F(1, 70) = 12.656$, $p = .001$, respectively, but the lixit dual Tg mice did not burrow significantly less, $p = .167$. Further, male dual Tg mice burrowed significantly less than their WT companions, $F(1,$

70) = 14.217, $p < .001$, and the same pattern was observed for the female mice $F(1, 70) = 11.314$, $p = .001$.

Table 12. Percentage of pea gravel burrowed at 18 hours. The table below displays the percentage values of pea gravel burrowed at 18 hours.

WT Zinc	Dual Tg Zinc	WT Zinc & Copper	Dual Tg Zinc & Copper	WT Lixit	Dual Tg Lixit
94.605 (*SD* = 5.384)	61.381 (*SD* = 26.478)	83.423 (*SD* = 19.622)	50.230 (*SD* = 17.507)	77.062 (*SD* = 31.966)	63.072 (*SD* = 23.789)

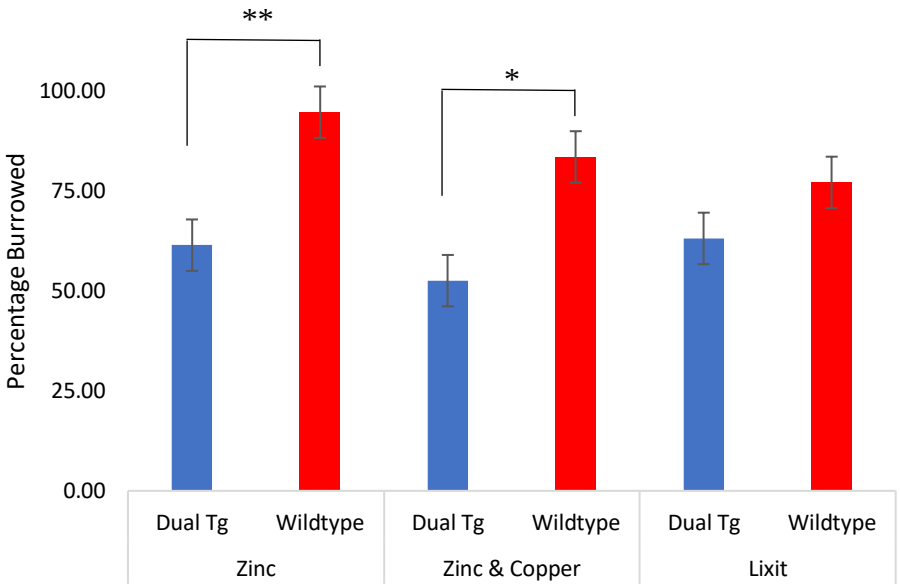

Figure 19. Percentage of pea gravel burrowed at 18 hours. The figure displays the genotypic main effect in which dual Tg mice burrowed significantly less than WT mice. It also displays the interaction effect in which zinc and zinc & copper dual Tg mice burrowed significantly less than the mice on the same water type. An asterisk (*) denotes significance at $p < .05$ and a double asterisk (**) denotes significance at $p < .001$ level.

Table 13. Percentage of pea gravel burrowed at 18 hours examining genotype and sex. The table below features the percentage of pea gravel burrowed with the sex differences.

Dual Tg Male	Dual Tg Female	WT Male	WT Female
64.124 (*SD* = 22.951)	55.296 (*SD* = 23.916)	90.648 (*SD* = 15.865)	78.231 (*SD* = 27.854)

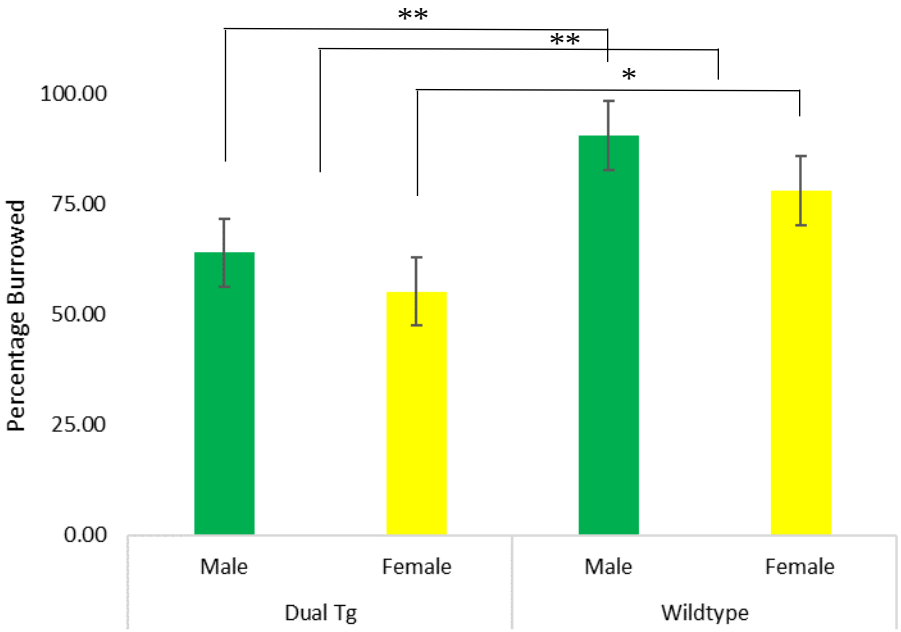

Figure 20. Percentage of pea gravel burrowed at 18 hours examining genotype and sex. This chart visually displays the genotypic and sex main effects. Dual Tg mice burrowed significantly less than WT mice, and male mice burrowed significantly more than female mice. This effect was observed between the genotypes. An asterisk (*) denotes significance at $p < .05$ and a double asterisk (**) denotes significance at $p < .001$ level.

Circadian Activities

A 2(genotype) x 3(water type) x 12(time) repeated measures ANOVA was conducted for both the 12-hour active (8pm-8am) and non-active hour (8am-8pm) time periods. During active hours, there was a trend for dual Tg mice to run more than WT

mice, though it was not significant, $F(1, 63) = 3.198$, $p = .079$. There were also no significant main effects for water type, $F(2, 63) = 2.012$, $p = .142$, nor sex $F(1, 70) = .757$, $p = .387$. There was a trending interaction between genotype and sex, $F(1, 70) = 3.613$, $p = .062$, driven by male dual Tg mice that ran more than male WT mice, $F(1, 63) = 6.465$, $p = .013$. There was also a trend for male WT mice to run less than female WT mice, $F(1, 63) = 3.765$, $p = .057$.

Further, while the entire twelve hour period was not significant, interaction effects reveal dual Tg mice ran significantly more than WT mice from 3am to 8am, $F(1, 63) = 13.201$, $p < .001$. There were no consecutive hours of significance for water type nor sex.

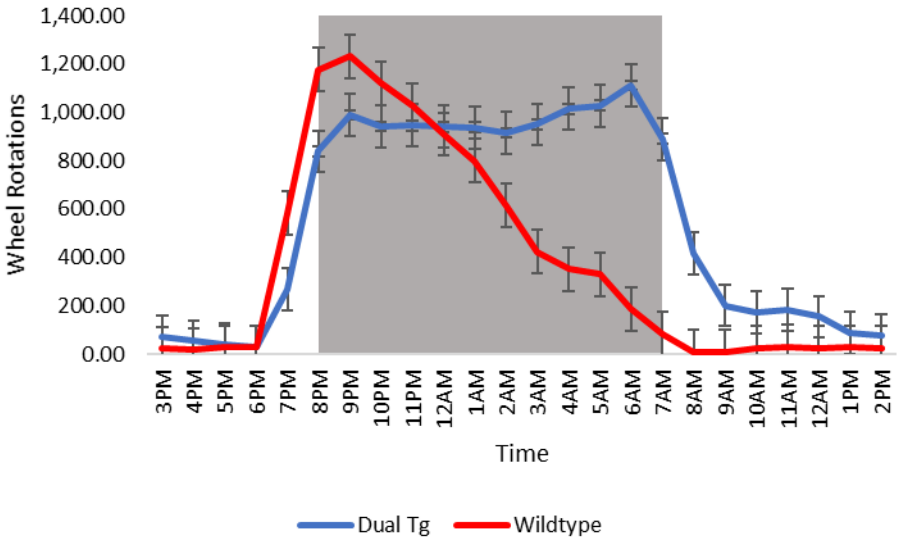

Figure 21. Circadian activities genotypic effects from 8pm-8am. While there was no overall significance, the figure does display that the dual Tg mice ran more than WT mice in the latter half of active hours, which may be what resulted in the ultimate trend towards dual Tg running more than WT mice.

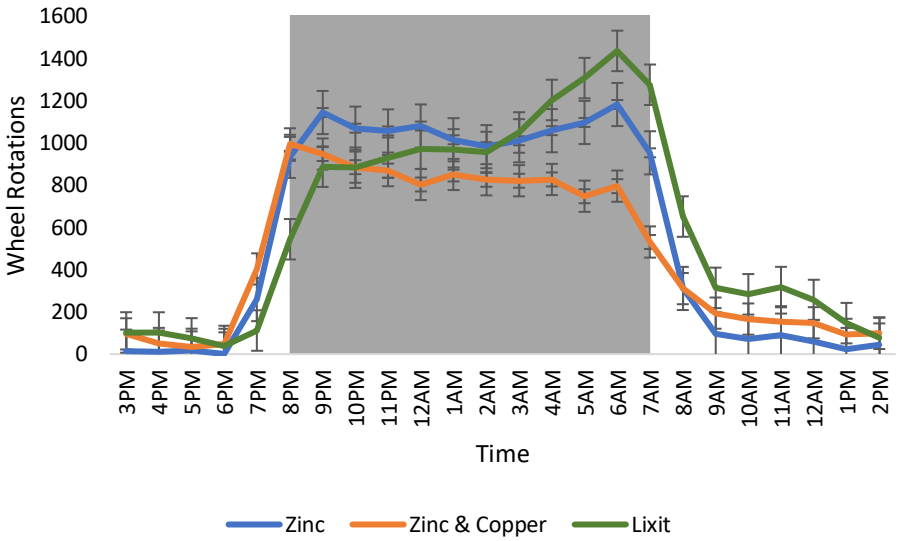

Figure 22. Dual transgenic water effects from 8pm-8am. This figure demonstrates that neither metal supplementation changed the overall pattern for dual transgenic mice in which they essentially plateaued in their activity, though lixit water mice had a spike in activity around 3 am. However, despite this activity, zinc supplemented mice (M = 1087.75) still had the most activity during active hours compared to lixit (M = 999.10) and zinc with copper (M = 841.96) mice, though it was not significant, p = .535.

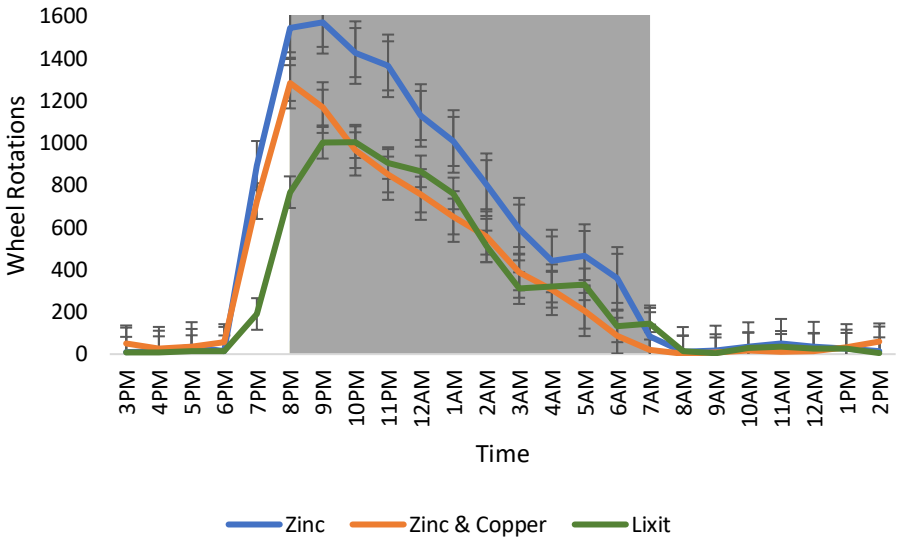

Figure 23. Wildtype water effects from 8pm-8am. This figure demonstrates that metal supplementation did not change the overall pattern for wildtype mice in which they consistently declined in activity throughout the entire 12-hour period. Zinc mice (M = 999.55) had the most activity compared to both zinc with copper (M = 603.21) and lixit (M = 602.97) mice, though it was not significant, p = .108.

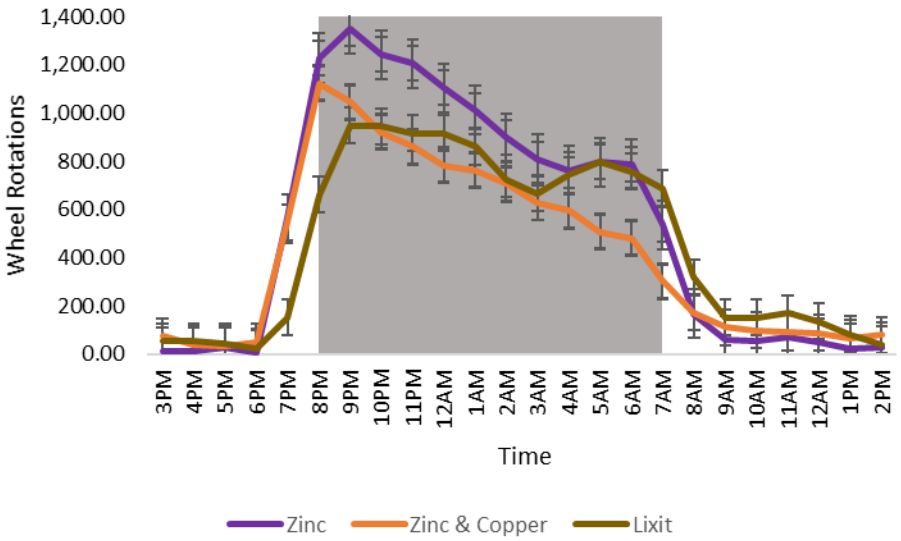

Figure 24. Circadian activities water type effects from 8pm-8am. This figure displays the activity of the mice on the different water types. The mice followed roughly the same pattern for the entirety of the twelve hours.

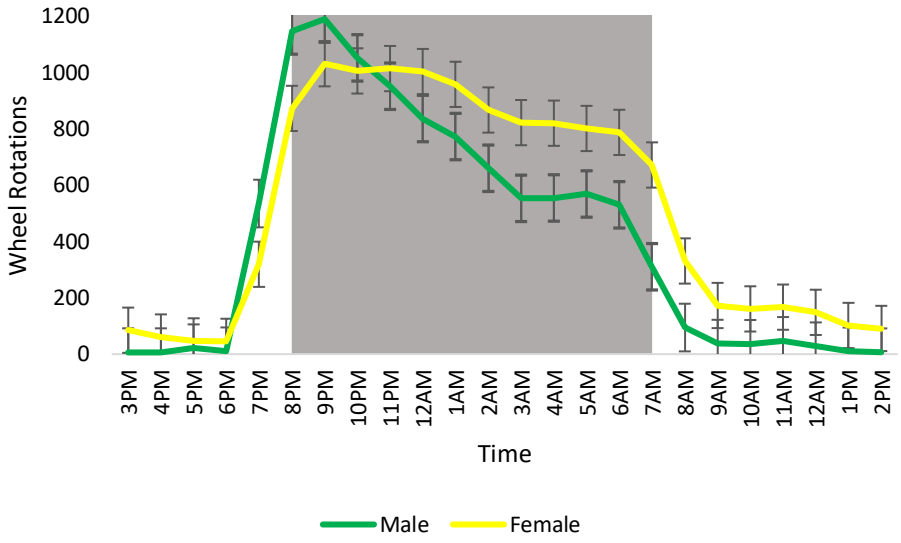

Figure 25. Circadian activities sex effects from 8pm-8am. While there was no overall significance, the chart demonstrates how female mice ran more than male mice for the majority of active hours.

The same 2x3x12 repeated measures ANOVA was conducted for non-active hours. There were no main effects, but there was a trend for both genotype and sex. Dual Tg mice trended towards running more than WT mice, $F(1, 63) = 3.808$, $p = .055$, while male mice trended towards running less than female mice, $F(1, 63) = 3.789$, $p = .056$. While there was no main effect of water, $F(2, 63) = .130$, $p = .878$, there was a trending interaction between genotype and water type, $F(2, 63) = 2.265$, $p = .112$ in which lixit dual Tg mice ran significantly more than lixit WT mice, $F(1, 63) = 7.577$, $p = .008$. Further, there was a trending interaction between genotype and sex, $F(1, 63) = 2.913$, $p = .093$, in which female dual Tg mice ran significantly more than female WT mice, $F(1, 63) = 7.063$, $p = .010$, and male dual Tg mice, $F(1, 63) = 6.808$, $p = .011$.

While there was no overall main effect, the genotypic trend was largely driven from 8am-12pm where dual Tg mice ran more than WT mice, $F(1, 63) = 6.709$, $p = .012$. There were no significant consecutive periods for water type, $p = .224$, but female mice ran significantly more than male mice from 12pm to 4pm, $F(1, 63) = 4.446$, $p = .039$.

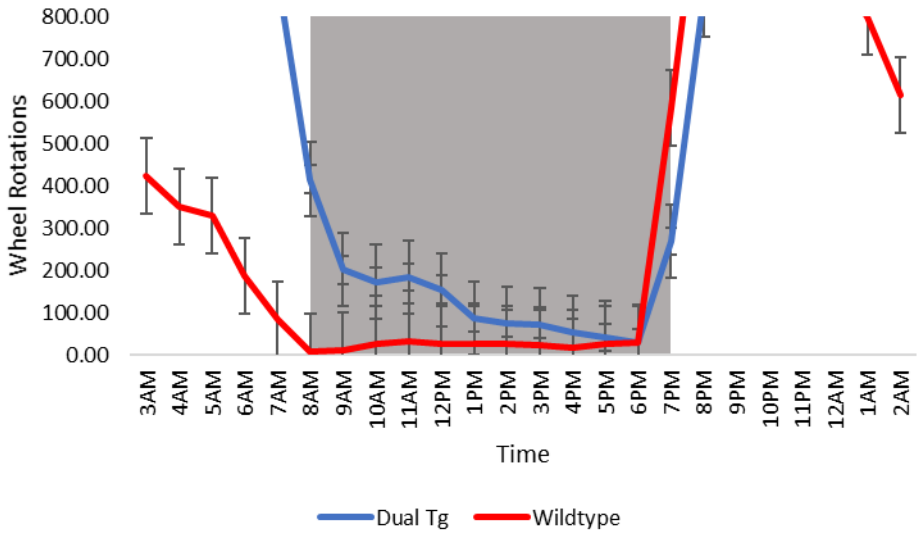

Figure 26. Circadian activities genotypic effects from 8am-8pm. While there was no significant main effect, the figure displays how dual Tg mice ran more than WT mice throughout almost the entirety of the entire 12 hours.

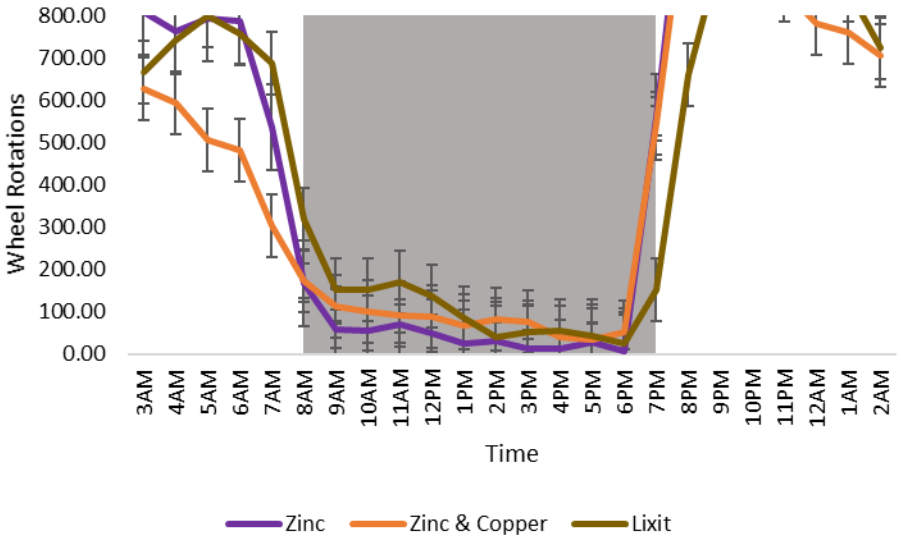

Figure 27. Circadian activities water type effects from 8am-8pm. The figure displays the differences during non-active hours for the different water types. They all followed roughly the same pattern, explaining why there is no significant main effect of water type. The zinc water mice ran less than the other groups while the lixit water mice ran the most, but the difference was non-significant.

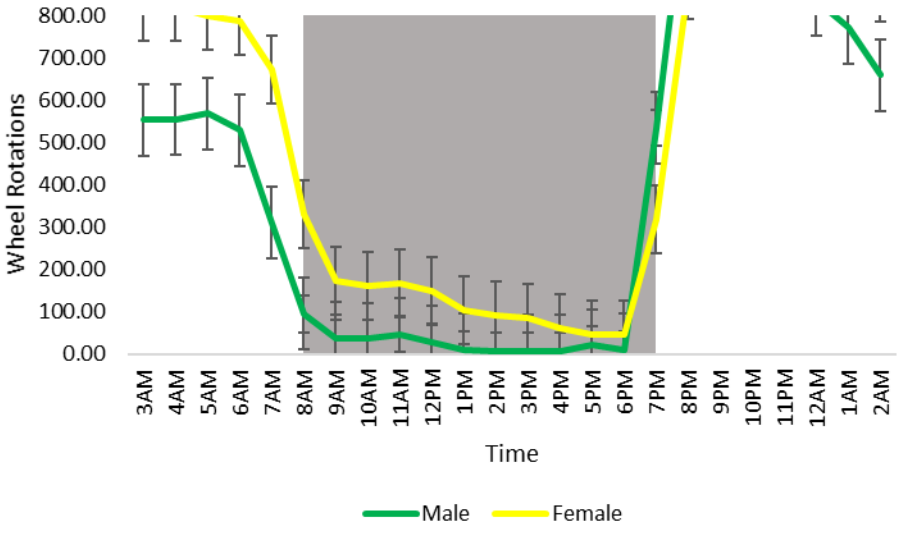

Figure 28. Circadian activities sex effects from 8am-8pm. While there was no significant main effect of sex, the female mice ran more than male mice for all of non-active hours explaining how there was a trend towards significance.

DISCUSSION

The purpose of this study was to investigate the effect on of metal supplementation on the behavior of dual transgenic Alzheimer's disease mice. Lippi et al (2018) found that zinc supplementation resulted in behavioral deficits at seven months, so this study implemented zinc with copper supplementation to potentially compensate for the negative effects. While the original plan for this study was to observe behavior at both 3.5 and 7 months, only 3.5 months was analyzed because of the lab being shut down for COVID-19.

There were three different behaviors observed: spatial memory, activities of daily living, and circadian activities. However, the simplest and most consistent finding related specifically to the genotypes of the mice. In all four tests, the wildtype mice performed significantly better than the dual transgenics. Biologically, this is unsurprising given that the rTg4510 tau mouse has been shown to develop phospho-tau at two months and mature tangles by the age of four months while the J20 APP mouse has been observed to have an initial plaque development at six months of age (Jankowsky & Zheng, 2017). Coincided with the evidence that tau pathology propagates the development of amyloid plaques (Pooler et al, 2015), the AD mice used in this study most likely had evidence of both tau tangles and amyloid plaques during testing. Histological analyses for these mice are currently being conducted.

These findings indicate stronger genotypic differences than previous literature has indicated. Halagappa et al (2017) and Samaey et al (2019) both did not find latency differences between the genotypes whereas this study did. Similarly, both Wu et al (2018) and Sterniczuk et al (2010) indicated that transgenic mice would have less activity during active hours, but the mice in this study had more activity during this time. The nesting behavioral test reflected similar results as Samaey et al (2019), and this study found increased activity during non-active hours in circadian activites that was indicated in previous research (Sterniczuk, Dyck, LaFerla, & Antle, 2010). Overall, these parallels highlight that this study found stronger genotypic differences than previous findings have found for young mice, but they do not contradict current research. It would be expected that when the mice are tested later, the differences between the two genotypes would only further diverge.

While the genotypic differences were strongly evident in the young mice, the metal differences were infrequent. There was only one finding that indicated a main effect of water type between all four behavioral tests. The mice on zinc and copper had significantly more platform crosses than both zinc-watered and lixit-watered mice during the probe trials. Further exploration indicated that this effect was largely driven by day two and day four because day six and day seven had similar means across the three water types. It may be that the combination of zinc and copper supplementation has beneficial impacts on short term memory, which is why the effect was only observed for the first two probe trials. After that point, the location of the platform had most likely converted to long-term memory, especially considering that day seven is a 24-hour probe trial set to

test reference memory. Since the hippocampus is the storage center for short-term memory (Kitamura et al, 2017), and one of the last areas to be affected by the plaques and tangles (Braak & Braak, 1991) those two facets could work in conjunction to provide a short-term benefit from the combined metals.

With the one main effect of water, the zinc and copper supplementation resulted in the best results, but most aspects of the Morris test resulted in no main effect of water and only an observation of pattern that the lixit water mice were performing best. This effect was further demonstrated in the behavioral tests that measure activities of daily living. While there was not a main effect of water for either test, within the water treatment group, the dual transgenic mice performed worse compared to the wildtype mice on the same water type. However, in all but one of the four ADL assessments, the dual Tg mice on lixit water did not perform significantly worse than the WT mice on lixit water which indicates the metal supplementation may have had an overall negative effect. In contrast to what was hypothesized, the addition of copper to the zinc supplement did not assist leveling any harmful effects that were suspected with zinc only supplementation; instead, mice on the combined supplementation performed at the same level or worse.

Overall, the metal effects in Morris water maze were not strong, which was similar to the Lippi et al (2018) study that this research was based on. Lippi et al (2018) also did not find any main effects of metal water; however, in contrast to their findings, males did not perform worse in the spatial memory test. Instead, there were no sex differences in the results of the spatial memory test except in thigmotaxis which will be

evaluated further in the discussion. The researchers found that the interaction between sex and water type were further evident at seven months, which may mean the male mice on the metal supplementation will perform worse during future testing at which point they will have been on the treated water for 27 weeks.

While metal effects in MWM and circadian activities were rarely or not observed in dual transgenic mice, metal supplementation had a negative effect on these mice during the tests evaluating activities of daily living. It is difficult to characterize where in the brain these actions originate because of the wide-span variety that distinguishes them, but it may be a combination from areas of the brain responsible for sequence of actions, like the rostrolateral prefrontal cortex (RLPFC), and motor movements, like the cerebellum (Desrochers, Chatham, & Badre, 2015; Jacobs et al, 2018). The cortex and cerebellum are overridden in the early stages of the disease which may be why long-term memory and motor deficits were evident despite therapeutic treatment.

The last element of the MWM test that has not been examined is thigmotaxis, which measures anxiety or fear (Higaki et al, 2018). The results found a main effect of sex depicting that female mice were significantly more anxious than males during both probe and non-probe trials. Furthermore, this effect was exacerbated by both metal supplementation and genotype. For instance, during probe trials, dual transgenic females spent more time along the edges compared to the male dual Tg mice, but this same effect was not observed in wildtype mice. Females have been noted to have more anxious thoughts than males in general (Bahrami & Yousefi, 2011), and untreated AD females had higher mean anxiety scores on the NeuroPsychiatric Inventory compared to untreated

AD males (Mielke, 2018). Thus, these results have strong efficacy to human findings, and indicate the importance of understanding the sex differences prevalent in the disease.

This is further exacerbated because it was found that female mice on either metal supplementation spent significantly more time along the edges compared to the male mice on the same metal; however, this sex difference was not evident for mice on lixit water. Thus, treatments meant to universally assist all patients could confound and aggravate negative symptoms which may be what occurred here. There is evidence that patients with anxiety have higher levels of serum and plasma copper, so adding the metal supplement could be inadvertently increasing anxiety levels. However, the same research also found that patients with anxiety often have low levels of zinc, and in this study, both zinc and zinc and copper treated females ran along the edges more than the males on the same water types (Islam et al, 2013; Russo, 2011). It is possible the copper supplementation interacted negatively with the females already higher anxiety levels and resulted in the current findings. Therefore, while the metal supplementation did not have overarching positive effects on the AD mice, this evidence highlights the importance of understanding how sex differences may impact treatments differently.

Furthermore, thigmotaxis scores were not the only analysis in which females performed worse than males. Female mice had worse nesting scores and burrowed less pea gravel than males at 18 hours, resulting in a sex main effect for both ADL tests. The nesting sex main effect was similar to the Morris water maze thigmotaxis scores in which the difference between the sexes of the dual transgenic mice drove the effect. The female dual Tg mice scored significantly lower nesting scores at 18 hours compared to the male

dual Tg mice, but this same sex difference was not observed in the wildtype mice. There is evidence that stress can result in poorer nests, especially for females considering that nesting is the instinctual behavior meant to provide protection for pups (Lexark, Missig, & Carlezon, 2017; Otabi, Okayama, & Toyoda, 2020). Thus, the anxiety that was evident in the female mice during MWM may have again affected their performance in this test, but the mice were not as affected by any negative effects of the metals in these tests.

In contrast, the sex main effect for burrowing at 18 hours was largely driven by the genotypic effect because the WT mice burrowed more pea gravel than the dual transgenic mice of the same sex. Since burrowing has not been found to have the same correlation to anxiety and performance, it may explain why the sex difference was solely driven by the genotypic differences.

While there was not a main effect of sex in the circadian activity test, female mice had more activity during both active and non-active hours compared to male mice. This was in contrast to both Sterniczuk et al (2010) and Wu et al (2018) who found that females either had a decrease or no difference in activity levels, respectively. However, this result was similar to previous studies that have been conducted in this lab, including the research on which this project was based on (Lippi, Kakalec, Smith, Flinn, 2020). This may be as a result of a protective factor estrogen may provide against circadian activities as Royston et al (2014) found that estradiol increased total activity. It may also be that given that the female mice in this study have been shown to be more anxious than the males, that anxiety is presenting as a low-grade insomnia in this test. Future research could use traditional tests of anxiety like the elevated zero maze in order to confirm the

relationship between anxiety and sex. It has been demonstrated that anxious mice can display sleep disruptions (Tang, Yang, Fishback, Sanford, 2009). While the female mice had more activity, they followed the same pattern of activity, indicating overall, there is not a large difference between the sexes.

The largest difference in circadian activity pattern was between the genotypes. While neither 12-hour period resulted in a main effect, they trended towards dual transgenic mice running more than WT mice. Instead of the slow decline during active hours present in the WT mice, dual Tg mice plateaued for almost the entirety of the 12 hours. This is similar to results found by Lippi et al (2020). In contrast to their findings, the metal supplementation had no effect on either dual transgenic or WT mice. They had found zinc supplementation in dual transgenic mice resulted in less activity than lab water treated mice, but the same observation was not seen for either supplementation in this study. Instead, in this study, there were no significant differences between metals, and mice on zinc water trended towards more activity, whereas zinc supplementation resulted in less activity for dual Tg mice at both 3.5 and 7 months (Lippi et al, 2020). Overall, this study coincides with Lippi et al (2020) in which no effects were found at 3.5 months, and it is necessary to analyze behavior at seven months to know if the similarity continues.

In contrast to Lippi et al (2020) zinc-treated mice in this study had the most activity in both WT and dual Tg mice, though the differences were not significant. Instead, there was a trend for WT zinc supplemented mice to run more compared to the WT mice in other groups. For dual transgenic mice, while zinc-treated mice had the most

activity, the lixit mice had a spike in activity around 3am that resulted in lixit mice almost having the same activity level. Overall, this behavior indicates that the zinc and copper supplementation may not be harmful for circadian cycles considering the combined metal treatment had the best patterned behavior within the genotype. However, given that this was not significant, it is difficult to ascertain for sure, and needs future testing.

Coinciding with this theory, Lippi et al (2020) also found that relationship between zinc and activity strengthened when the mice were tested again at seven months. This may indicate that metal supplementation effects will be more evident once the mice from this study are tested later. It would also be expected that there would be a main effect of genotype for both 12-hour periods given that the difference between the two genotypes will only grow stronger as the mice age. The sex difference may not result in a main effect, but it is expected that female mice would continue to have more activity.

Conclusions

Overall, there were limited behavioral effects of the metal supplementation for the mice at 3.5 months, and many of the effects that were present resulted in negative outcomes. It is important to further investigate the long-term effects of metal supplementation, which is currently being studied by another researcher in the lab in order to determine if these results persist or change. Besides the seven-month testing that will take place for this study, brain histology for these mice will also be examined to observe if the negative effects also correlated to neurological changes in the brain. This is

important to understand the relationship between brain and behavior in therapeutic treatment.

As the results stand currently, it would not be recommended to give AD patients either zinc or zinc and copper supplementation because of the overall negative effect. While the negative effect of zinc was expected, the addition of copper did not positively affect the dual transgenic mice. Instead, the copper supplementation may have correlated poorly with other factors, including anxiety. It would be necessary to further investigate if copper supplementation results in higher anxiety levels and that was the reason for the poor performances in these tests. If so, future research could focus on if copper and zinc supplementation have positive behavioral effects not affected by anxiety, and if the supplementation may be beneficial for males but not females as the findings may represent.

REFERENCES

Bagheri, S., Squitti, R., Haertlé, T., Siotto, M., Saboury, A. A. Role of Copper in the Onset of Alzheimer's Disease Compared to Other Metals. *Frontiers in Aging Neuroscience, 9,* 446. doi: 10.3389/fnagi.2017.00446

Bahrami, F., & Yousefi, N. (2011). Females Are More Anxious Than Males: a Metacognitive Perspective. *Iranian Journal of Psychiatry and Behavioral Science, 5*(2): 83-90.

Bao et al (2010). Zinc decreases C-reactive protein, lipid peroxidation, and inflammatory cytokines in elderly subjects: a potential implication of zinc as an atheroprotective agent. *American Journal of Clinical Nutrition, 91*(6), 1634-1641. 10.3945/ajcn.2009.28836

Braak, H. & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathologica*, *82*, 239-259. doi.org/10.1007/BF00308809

Brewer, G. J. Copper excess, zinc deficiency, and cognition loss in Alzheimer's disease. *Biofactors, 38*(2), 107-113. doi: 10.1002/biof.1005

Bucossi, S. et al (2011). Copper in Alzheimer's disease: a meta-analysis of serum, plasma, and cerebrospinal fluid studies. *Journal of Alzheimer's Disease, 24*(1), 175-185. doi: 10.3233/JAD-2010-101473.

Bush, A. I., & Tanzi, R. E. (2008). Therapeutics for Alzheimer's disease based on the metal hypothesis. *Neurotherapeutics, 5*(3), 421-432. doi: 10.1016/j.nurt.2008.05.001

Constantinidis, J. (1992). Treatment of Alzheimer's disease by zinc compounds. *Drug Development Research, 27*(1). doi.org/10.1002/ddr.430270102

Corona et al (2010). Dietary zinc supplementation of 3xTg-AD mice increases BDNF levels and prevents cognitive deficits as well as mitochondrial dysfunction. *Cell Death and Disease, 1*(e91). e91. doi:10.1038/cddis.201

Craven, K. M., Kochen, W. R., Hernandez, C. M., Flinn, J. M. (2018). Zinc Exacerbates Tau Pathology in a Tau Mouse Model. *Journal of Alzheimer's Disease, 64*, 617-630. DOI 10.3233/JAD-180151

Desrochers, T. M., Chatham, C. H., & Badre, D. (2015). The Necessity of Rostrolateral Prefrontal Cortex for Higher-Level Sequential Behavior. *Neuron, 87*(6), 1357-1368. doi.org/10.1016/j.neuron.2015.08.026

Farrar, J. R., Hoss, W., Herndon, R. M., & Kuzmiak, M. (1985). Characterization of muscarinic cholinergic receptors in the brains of copper-deficient rats. *The Journal of Neuroscience: the official journal of the Society for Neuroscience, 5*(4), 1083–1089. https://doi.org/10.1523/JNEUROSCI.05-04-01083.1985

Filali, M. et al (2012). Cognitive and non-cognitive behaviors in the triple transgenic mouse model of Alzheimer's disease expressing mutated APP, PS1, and Mapt

(3xTg-AD). *Behavioural Brain Research, 234*(2), 334-342. doi.org/10.1016/j.bbr.2012.07.004

Flinn, J. M., Bozzelli, P. L., Adlard, P. A., Railey, A. M. (2014). Spatial memory deficits in a mouse model of late-onset Alzheimer's disease are caused by zinc supplementation and correlate with amyloid-beta levels. *Frontiers in Aging Neuroscience*. doi.org/10.3389/fnagi.2014.00174

Flinn, J. M. et al (2005). Enhanced zinc consumption causes memory deficits and increased brain levels of zinc. *Physiology & Behavior, 83*(5), 793-803. doi.org/10.1016/j.physbeh.2004.10.009

Fu, H. et al (2017). Tau Pathology Induces Excitatory Neuron Loss, Grid Cell Dysfunction, and Spatial Memory Deficits Reminiscent of Early Alzheimer's Disease. *Neuron, 93*(3), 533-541. https://doi.org/10.1016/j.neuron.2016.12.023

Geppert, A., Losy, J., Przedpelska-Ober, E., & Kozubski, W. (2010). CCL3 correlates with the number of mood disturbances and personality changes in patients with Alzheimer's disease. *Psychiatry Research, 176*(2), 261–264. https://doi.org/10.1016/j.psychres.2009.02.007

González, C. et al (1999). Serum zinc, copper, insulin and lipids in Alzheimer's disease epsilon 4 apolipoprotein E allele carriers. *European Journal of Clinical Investigation, 29*(7), 637-642. doi: 10.1046/j.1365-2362.1999.00471.x.

Halagappa, V. K. M. et al (2007). Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of

Alzheimer's disease. *Neurobiology of Disease, 26*(1), 212-220. doi.org/10.1016/j.nbd.2006.12.019

Higaki, A. et al (2018). Recognition of early stage thigmotaxis in Morris water maze test with convolutional neural network. *PLoS One, 13*(5), e0197003. doi: 10.1371/journal.pone.0197003

Islam, M. R. et al (2013). Comparative Analysis of Serum Zinc, Copper, Manganese, Iron, Calcium, and Magnesium Level and Complexity of Interelement Relations in Generalized Anxiety Disorder Patients. *Biological Trace Element Research, 154*: 21-27.

Jacobs, H. I. L. et al (2018). The cerebellum in Alzheimer's disease: evaluating its role in cognitive decline. *Brain, 141*(1), 37-47. doi-org.mutex.gmu.edu/10.1093/brain/awx194

Janus C., et al (2000). Spatial learning in transgenic mice expressing human presenilin 1 (PS1) transgenes. *Neurobiology of Aging, 21*(4), 541–549.

Jankowsky, J. L., & Zheng, H. (2017). Practical considerations for choosing a mouse model of Alzheimer's disease. *Molecular Neurodegeneration, 12*(89). doi.org/10.1186/s13024-017-0231-7

Kametani, F., & Hasegawa, M. (2018). Reconsideration of Amyloid Hypothesis and Htau Hypothesis in Alzheimer's Disease. *Frontiers in Neuroscience, 12*(25). doi:10.3389/fnins.2018.00025

Kitamura, T. et al (2017). Engrams and circuits crucial for systems consolidation of a memory. *Science, 356*(6333), 73-78. DOI: 10.1126/science.aam6808

Lai et al (2012). The efficacy of zinc supplementation in depression: systematic review of randomised controlled trials. *Journal of Affective Disorders, 136*(1-2), e31-e39. doi: 10.1016/j.jad.2011.06.022

Lezark, K. R., Missig, G., Carlezon Jr., W. A. Behavioral methods to study anxiety in rodents. *Dialogues in Clinical Neuroscience, 19*(2): 181-191. doi: 10.31887/DCNS.2017.19.2/wcarlezon

Li, D., Zhang, W., Wang, Z., Zhao, P. (2017). Serum Copper, Zinc, and Iron Levels in Patients with Alzheimer's Disease: A Meta-Analysis of Case-Control Studies. *Frontiers in Aging Neuroscience, 9*. doi.org/10.3389/fnagi.2017.00300

Linkous, D. H., Adlard, P. A., Wanschura, P. B., Conko, K. M., Flinn, J. M. (2009). The Effects of Enhanced Zinc on Spatial Memory and Plaque Formation in Transgenic Mice. *Journal of Alzheimer's Disease, 18*, 565-579. DOI 10.3233/JAD-2009-1162

Lippi, S. L. P., Kakalec, P. A., Smith, M. S., Flinn, J. M. (2020). Wheel-Running Behavior Is Negatively Impacted by Zinc Administration in a Novel Dual Transgenic Mouse Model of AD. *Frontiers in Neuroscience, 14*: 854.

Lippi, S. L. P., Smith, M. L., Flinn, J. M. (2018). A Novel hAPP/htau Mouse Model of Alzheimer's Disease: Inclusion of APP With Tau Exacerbates Behavioral Deficits

and Zinc Administration Heightens Tangle Pathology. *Frontiers in Aging Neuroscience*. DOI:10.3389/fnagi.2018.00382

Lyketsos, C. et al (2011). Neuropsychiatric symptoms in Alzheimer's disease. *Alzheimer's and Dementia, 7*, 532-539.

Martyr, A., & Clare, L. (2012). Executive Function and Activities of Daily Living in Alzheimer's Disease:A Correlational Meta-Analysis. *Dementia and Geriatric Cognitive Disorders, 33*, 189-203. DOI: 10.1159/000338233

Mielke, M. M. (2018). Sex and Gender Differences in Alzheimer's Disease Dementia. *Psychiatric Times, 35*(11): 14-17.

Mo, Z. Y. et al (2009) Low micromolar zinc accelerates the fibrillization of human Tau via bridging of Cys-291 and Cys-322. *The Journal of Biological Chemistry, 284*(50), 34648–34657.

National Institute of Aging. (2016). *Alzheimer's Disease Fact Sheet* [Fact sheet].

Nestor, P. J., Fryer, T. D., Hodges, J. R. (2005). Declarative memory impairments in Alzheimer's disease and semantic dementia. *Neuroimage, 30*: 1010-1020. doi:10.1016/j.neuroimage.2005.10.008

Nuttall, J. R., & Oteiza, P. I. Zinc and the aging brain. *Genes & Nutrition, 9*(1), 379. 10.1007/s12263-013-0379-x

Otabi, H., Okayama, T., Toyoda, A. (2020). Assessment of nest building and social interaction behavior in mice exposed to acute social defeat stress using a three-

dimensional depth camera. *Animal Science Journal, 91*(1): e13447. doi.org/10.1111/asj.13447

Penland, J. G., Milne, D. B., Davis, C. W. (2000) Moderately high zinc intake impairs verbal memory of healthy postmenopausal women on a low copper diet. *Trace elements in man and animals, 10*.

Peter-Derex, L., Yammine, P., Bastuji, H., & Croisile, B. (2015). Sleep and Alzheimer's disease. *Sleep and Alzheimer's disease, 19*(1), *29-38.* https://doi.org/10.1016/j.smrv.2014.03.007

Pfeiffer, C., & Braverman, E. (1982). Zinc, the brain and behavior. *Biological Psychiatry, 17*(4), 513-532.

Pilkington, S. (2013). Causes and consequences of sleep deprivation in hospitalised patients. *Nursing Standard*, *27*(49), 35-42.

Pooler, A. M. et al (2015). Amyloid accelerates tau propagation and toxicity in a model of early Alzheimer's disease. *Acta Neuropathologica Communications*, *3*(14). doi: 10.1186/s40478-015-0199-x

Prohaska, J. R., & Bailey, W. R. (1993). Persistent Regional Changes in Brain Copper, Cuproenzymes and Catecholamines Following Perinatal Copper Deficiency in Mice, *The Journal of Nutrition, 123*(7), 1226–1234. https://doi.org/10.1093/jn/123.7.1226

Railey, A. M., Groeber, C. M., Flinn, J. M. (2011). The Effect of Metals on Spatial Memory in a Transgenic Mouse Model of Alzheimer's Disease. *Journal of Alzheimer's Disease, 24*, 375-381. DOI 10.3233/JAD-2010-101452

Railey, A. M., Micheli, T. L., Wanschura, P. B., Flinn, J. F. (2010). Alterations in fear response and spatial memory in pre- and post-natal zinc supplemented rats: Remediation by copper. *Physiology & Behavior, 100*(2), 95-100. doi.org/10.1016/j.physbeh.2010.01.040

Reiman, E. et al (2016). CAP--advancing the evaluation of preclinical Alzheimer disease treatments. *Nature Reviews. Neurology, 12*(1), 56-61. DOI:10.1038/nrneurol.2015.177

Royston, S. E. (2014). ESR1 and ESR2 Differentially Regulate Daily and Circadian Activity Rhythms in Female Mice. *Endocrinology, 55*(7), 2613-2623.

Russo, A. J. (2011). Decreased Zinc and Increased Copper in Individuals with Anxiety. *Nutrition and Metabolic Insights, 4*: 1-5. doi: 10.4137/NMI.S6349

Samaey, C., Schreurs, A., Stroobants, S., Balschun, D. Early Cognitive and Behavioral Deficits in Mouse Models for Tauopathy and Alzheimer's Disease. *Frontiers in Aging Neuroscience*. doi.org/10.3389/fnagi.2019.00335

Sandusky-Beltran, L. A., Manchester, B. L., & McNay, E. W. (2017). Supplementation with Zinc in Rats Enhances Memory and Reverses an Age-Dependent Increase in Plasma Copper. *Behavioural Brain Research, 30*(333), 179-183. doi: 10.1016/j.bbr.2017.07.007

Schrag, M., Mueller, C., Oyoyo, U., Smith, M. A., Kirsch, W. M. (2011). Iron, zinc and copper in the Alzheimer's disease brain: A quantitative meta-analysis. Some insight on the influence of citation bias on scientific opinion. *Progress in Neurobiology, 94*(3), 296-306. doi.org/10.1016/j.pneurobio.2011.05.001

Sensi, S. L., Granzotto, A., Siotto, M., Squitti, R. (2018). Copper and Zinc Dysregulation in Alzheimer's Disease. *Trends in Pharmacological Sciences, 39*(12), 1049-1063. doi.org/10.1016/j.tips.2018.10.001

Spira A.P. et al (2013). Self-reported Sleep and β-Amyloid Deposition in Community-Dwelling Older Adults. *JAMA Neurology, 70*(12), 1537–1543. doi:10.1001/jamaneurol.2013.4258

Squitti, R. et al (2014). Meta-analysis of serum non-ceruloplasmin copper in Alzheimer's disease. *Journal of Alzheimer's Disease, 38*(4), 809-822. doi: 10.3233/JAD-131247

Sterniczuk, R., Dyck, R. H., LaFerla, F. M., Antle, M. C. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: Part 1. Circadian changes. *Brain Research, 1348*(1), 139-148. https://doi.org/10.1016/j.brainres.2010.05.013

Tang, X., Yang, L., Fishback, N. F., Sanford, L. D. (2009). Differential effects of lorazepam on sleep and activity in C57BL/6J and BALB/cJ strain mice. *Journal of Sleep Research, 18*(3): 365-373. doi-org.mutex.gmu.edu/10.1111/j.1365-2869.2009.00736.x

Ventriglia, M., Bucossi, S., Panetta, V., Squitti, R. (2012). Copper in Alzheimer's disease: a meta-analysis of serum, plasma, and cerebrospinal fluid studies. *Journal of Alzheimer's Disease, 30*(4), 981-984. doi: 10.3233/JAD-2012-120244.

Wang, Z. et al (2015). Serum Iron, Zinc, and Copper Levels in Patients with Alzheimer's Disease: A Replication Study and Meta-Analyses. *Journal of Alzheimer's Disease, 47*(3), 565-581. doi: 10.3233/JAD-143108

CDC. "What Is Alzheimer's Disease?" *Alzheimer's Disease and Related Dementias*, Centers for Disease Control and Prevention, 26 Oct. 2020, https://www.cdc.gov/aging/aginginfo/alzheimers.htm.

Watt, N., Whitehouse, I., & Hooper, N. (2010). The role of zinc in Alzheimer's disease. *International Journal of Alzheimer's Disease, 2011*. doi.org/10.4061/2011/971021

Wu, M., et. al., (2018). Abnormal circadian locomotor rhythms and Per gene expression in six-month-old triple transgenic mice model of Alzheimer's disease. *Neuroscience Letters, 676*(1), 13–18. https://doi.org/10.1016/j.neulet.2018.04.008

BIOGRAPHY

Tatiana Gervase graduated from Princess Anne High School, Virginia Beach, Virginia in 2017. She graduated from George Mason University, Fairfax, Virginia with a Bachelor of Science in Psychology. She also received her Masters of Arts from George Mason University in 2021.