THE EFFECTS OF EXCESS ZINC AND COPPER ON ANXIETY AND DEPRESSION IN A RAT MODEL

by

Erin H. Beech

A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Arts
Psychology

Committee:

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Date: April 26, 2012

Spring Semester 2012
George Mason University
Fairfax, VA

# The Effects of Excess Zinc and Copper on Anxiety and Depression in a Rat Model

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

By

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Bachelor of Science
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## DEDICATION

This is dedicated to John, who has been my support throughout the process, as well as to Mom, Amy, Dad, and Brenda, as well as all of my siblings and cats.

## ACKNOWLEDGEMENTS

I would like to thank Dr. Jane Flinn, Dr. Robert Smith, and Dr. Patrick McKnight for their dedication and guidance throughout the thesis process. I would also like to thank my lab mates: Casey Ea, Chance Sizemore, Caitlin Groeber, Gretchen Knaack, Katie Taylor, Peter Kakalec, Stephanie Howell, Erica Holliday, Lorenzo Bozzelli and Gina Fernandez for their invaluable help and support in the preparation of this project.

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## ABSTRACT

THE EFFECTS OF EXCESS ZINC AND COPPER ON ANXIETY AND DEPRESSION IN A RAT MODEL

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Zinc deficiency is associated with both anxiety and depression in animal models. Zinc supplementation reduces depression-like behaviors in the forced swim test and increases grooming behavior and line crossing in the open field test. Zinc is a toxin, however, and excess zinc has been associated with increased anxiety-like behaviors, including a reduced ability to extinguish fear and greater thigmotaxicity in the Morris water maze. The addition of copper was reported to remediate the negative effects of zinc supplementation in this case. Many of the studies reporting the anti-depressant and anti-anxiety effects of zinc supplementation employed a single, acute zinc dose, or treatment over a short period of time. This study employed a chronic, pre- and post-natal zinc supplementation paradigm to determine if there would be a difference between zinc, zinc plus copper, or lab water fed rats, on behavioral measures of anxiety and depression in rats. It was hypothesized that the zinc-supplemented rats would display a higher amount

of anxiety- and depression-like behaviors than controls, and that the zinc + plus copper animals would show fewer anxiety- and depression-like behaviors than the zinc-supplemented rats, similar to controls. However, the results showed that there was no difference in depression-like behaviors between the groups on the forced swim test. There was also no difference in anxiety-like behaviors between the groups on the elevated plus maze or on day one of the open field test. On day two of the open field test, there was a significant interaction between water group and time. The zinc rats spent more time in the center at the end of the trial than at the beginning, while the zinc + copper animals and the lab animals spent less time in the center at the end of the trial than at the beginning. The multiple, conflicting results in this field of research may be due to the complex mechanisms behind the relationship between zinc homeostasis and affective disorders, but are more likely due to differences in methodology and high variability. Although zinc supplementation seems to be a promising treatment, it should seek to restore zinc homeostasis without leading to a state of excess zinc.

# 1. INTRODUCTION

Zinc is a transition metal that is needed for brain development and many brain functions. Throughout the body, zinc is essential to both the structure and function of many proteins (For review see Frederickson, Won Suh, Silva, Frederickson, & Thompson, 2000). Zinc is also responsible for the catalytic activity of many enzymes, especially those involved in metabolism and hormone regulation. Cellularly, zinc is critical for proliferation, differentiation, and apoptosis. (Evans, Overton, Alshingiti & Levenson, 2004; Terhune & Sandstead, 1972).

In the brain, in addition to these roles, zinc is secreted by neurons, and is essential for synaptogenesis, neurogenesis, neuronal growth, and neurotransmission (Maret & Sandstead, 2006). Zinc-containing neurons contain high concentrations of zinc in its ionic form ($Zn^{2+}$) in their synaptic vesicles, and are found nearly exclusively in the forebrain, specifically the cerebral cortex, amygdalar nuclei, and hippocampus. Their efferent fibers project to areas of the cortex, the amygdala, striatum, septum, and medial hypothalamus (Frederickson et al., 2000). Zinc-containing neurons are found in several areas of importance: the infralimbic cortex, the perirhinal cortex, a group of pyramidal neurons in the subiculum, the hylus, the CA1 and CA3 areas of the hippocampus, and the amygdala. These areas contain large numbers of zinc-containing neurons with widespread

projections (Frederickson et al., 2000). The synapses most enriched with Zn2+ exist in the CA3 region of the hippocampus (Vogt, Mellor, Tong, & Nicoll, 2000).

The high levels of zinc in the hippocampus, along with co-localization of zinc and Glutamate in neurons, imply that zinc plays a role in cognition, specifically learning, including spatial learning. Synaptically-released Zn2+ interacts with ion channels, receptors, and transporters. In this way, zinc modulates synaptic transmission and plasticity. Zinc inhibits NMDA receptors, which play a crucial role in long-term potentiation, through the multiple Zn2+ modulatory binding sites on the receptor (Peters, Koh, & Choi, 1987). Also, Zn2+ has been shown to flow from the synapses of ZnT3 glutaminergic synapses into nearby GABAergic synapses, exciting AMPA receptors and inhibiting release of GABA, which is implicated in induction of long-term potentiation (Kodirov et al., 2006).

Zinc-containing neurons are abundant in areas associated with anxiety and depression, including cortical regions, the hippocampus, amygdala, lateral septum, and the paraventricular nucleus of the hypothalamus (Whittle, Lubec, & Singewald, 2009). Zinc modulates long-term potentiation in the amygdala by regulation of GABAergic inhibition (Kodirov et al., 2006). There are significantly lower plasma zinc levels in depressed versus non-depressed individuals (McLoughlin & Hodge, 1990). In fact, serum zinc levels are negatively correlated with the severity of symptoms in depression (Tassabehji, Corniola, Alshingiti, & Levenson, 2008). The forced swim test has been used to gauge the efficacy of anti-depressants, and has recently been used to examine the usefulness of zinc as an anti-depressant (Nowak et al., 2003; Szewczyk et al., 2010). In

the forced swim test, zinc deficiency leads to a longer duration of immobility, indicative of depression. Zinc supplementation has been shown to reverse the effects of zinc deprivation in the forced swim test, alleviate depression in rat models, and, when given along with an antidepressant, increase its efficacy (Nowak et al., 2003; Szewczyk et al., 2010). This is indicated by less time spent immobile in the task.

The relationship between zinc deficiency and anxiety is less well understood, but zinc-deficient rats are more anxious than controls on behavioral measures of anxiety such as the open field (Takeda, Tamano, Kan, Itoh, & Oku, 2007). In one study, after two-week zinc deprivation, rats spent less time grooming and crossed into the open field less, as well as spending less time in the open arms of the elevated plus maze. This suggests that these rats are more anxious than controls (Takeda et al., 2007). Takeda et al. (2007) attribute this increase in anxiety behaviors to an increased concentration of free calcium in neurons of the hippocampus, caused by zinc deficiency. Another study observed that zinc-deficient animals exhibit anxiety-like behaviors in the light-dark box test, with significantly fewer passes into the light side of the box than controls, as well as less time spent in the light side of the box (Tassabehji et al., 2008). Considering the high co-morbidity of depression and anxiety in humans, it is not surprising that anxiety-like behaviors are being seen as a result of zinc deficiency.

Because of the low response rate, as well as unwanted side effects of existing anti-depressant drugs, new kinds of treatments are being examined for depression. The link between zinc deficiency and depression has led to the consideration of zinc supplementation as a possible anti-depressant treatment. In order for zinc

supplementation to be used safely, the effects of excess zinc on anxiety and depression need to be investigated. Elevated zinc levels have been associated with deficits in spatial memory and a reduced ability to extinguish fear in rats (Railey, Micheli, Wanschura, & Flinn, 2010). Additionally, excess zinc has been linked to copper deficiency, which is thought to be the cause of the memory deficits associated with excess zinc (Magee & Matrone, 1960). Copper supplementation has been shown to remediate these effects (Railey et al., 2010).

Excess zinc has been implicated in neuronal death, and many diseases in which neuronal death plays a part. Besides its many essential functions, Zn2+ can be a toxin, inducing death of neurons and glia (Sensi, Paoletti, Bush, & Sekler, 2009). In cerebral ischemia, in CA1 pyramidal neurons of the hippocampus, a rise in Zn2+ precedes a rise in Ca2+, which causes these neurons to lose membrane permeability and induces apoptosis. Accumulation of Zn2+ in the mitochondria also leads to death signaling in ischaemia, due to a loss of mitochondrial membrane potential, which creates reactive oxygen species, leading to death (Medvedeva, Lin, Shuttleworth, & Weiss, 2009).

Although zinc toxicity is high, and unlikely to occur in normal circumstances, consumption of very high concentrations in drinks (2500mg/L) has been linked to poisoning, causing nausea, vomiting, cramping, and diarrhea (Maret & Sandstead, 2006). Recently, Nations et al. (2008) published a study concluding that chronic exposure to the high levels of zinc present in several denture creams can lead to serious neurological illness, including cognitive impairment, possibly due to an induced copper deficiency.

This has led to removal of zinc from denture cream by many of the major pharmaceutical companies.

Beyond the diffuse effects of zinc toxicity, excess zinc has been linked to deficits in spatial memory and impairments in extinction of fear in normal rats (Flinn et al., 2005; Railey et al., 2010). It also causes memory loss in serious, chronic disorders, such as Alzheimer's disease (Railey, Groeber, & Flinn, 2011). In Alzheimer's disease, Zn2+ induces rapid aggregation of amyloid-β, speeding up plaque accumulation in transgenic mouse models. Synaptic Zn2+ increases attachment of amyloid-β to the NR2B NMDA receptor subunit, which induces exitotoxicity (Bush et al., 1994). Zinc's role in Alzheimer's disease may be due to Zinc dyshomeostasis caused by an increase in blood-brain barrier permeability (Takeda & Tamano, 2009).

Because many people do not obtain an adequate amount of zinc from their diets alone, many people take nutritional supplements containing zinc among many other vitamins and minerals, particularly in the United States (Maret & Sandstead, 2006). Studies attempting to evaluate the positive and negative effects of zinc supplementation have had inconsistent results. Results have shown that zinc supplementation during pregnancy has a positive effect on children's growth, as well as reducing morbidity (Bhutta, Black, & Brown, 1999; Brown, Peerson, & Allen, 1998). On the other hand, Hamadani, Fuchs, Osendarp, Huda, & Grantham-McGregor (2002) were surprised to find that the children of women given zinc supplements throughout pregnancy had lower scores on tests of mental development. Because of the link between zinc deficiency and depression and anxiety, zinc supplements are also currently being used as anti-

depressants. This widespread use of zinc supplements may be problematic, due to zincs' potential to cause adverse effects along with positive ones, and a possible risk of over-supplementation. When determining the proper administration and dosage of such supplements, it is essential that we know the point when zinc supplementation is causing adverse effects that outweigh the benefits.

Some studies report that zinc supplementation does not increase depression or anxiety-like behaviors in rats (Nowak et al., 2003; Szewcyk et al., 2009). However, these studies either use an acute, single dose of zinc, or a 14-day chronic zinc paradigm. When considering whether zinc supplementation is safe for long-term use, a 14-day administration may not be sufficient to be considered chronic. A 2010 study by Railey et al. raised rats both pre- and post-natally on zinc, and tested the rats after four months of supplementation. They examined the effect of zinc supplementation on spatial memory and fear response in rats. In addition to finding that excess zinc leads to deficits in spatial memory, Railey et al. also saw that zinc-animals had higher freezing rates during retention and extinction compared to control animals. The zinc-supplemented rats exhibited anxiety-like behaviors and had a decreased ability to extinguish fear.

A study using a similar chronic zinc supplementation paradigm using the Morris Water Maze found that rats receiving excess zinc had significant deficits in reference and working memory compared to controls (Flinn et al., 2005). Additionally, the zinc supplemented rats showed significantly greater thigmotaxicity, a measure of anxiety on the Morris Water Maze. Anxiety disorders, including post-traumatic stress disorder, are related to a decreased ability to extinguish fear (Myers & Davis, 2007). A transgenic

mouse model of impaired fear conditioning, the 129S1/ScImJ mouse strain, has been used to investigate this phenomenon. The 129 mouse shows a drastically reduced ability to extinguish a learned fear response compared to a normal mouse (Bolivar, Pooler, & Flaherty, 2001). Whittle, Hauschild, Lubec, Holmes, & Singewald (2010) fed these mice a zinc-restricted diet, and results showed that the zinc-restricted diet completely reversed the deficits typically seen in extinction learning in the 129 mouse. Again, this shows the relationship of excess zinc and increased anxiety-like behaviors. This has researchers questioning the use of zinc supplementation as a treatment for mood disorders, or at the least, the amount of zinc that can be given without harmful side effects.

It is thought that the detrimental effects of excess zinc may be due to the interaction of zinc and copper; chronic zinc toxicity has been shown to induce copper deficiency (Bhandari et al., 2002). Zinc interferes with the metabolism of copper by decreasing the amount that can be used by the body and increasing the amount that is excreted (Magee & Matrone, 1960). It could be this deficiency in copper that is actually causing deficits in learning and memory. Nations et al. (2008) believe that the cognitive impairment seen as a result of chronic zinc exposure via denture cream is a result of copper deficiency induced by excess zinc, rather than a result of the excess zinc itself. Railey et al. (2010), in addition to having a group of zinc-supplemented rats, also had a group that received copper supplements in addition to zinc. The copper had a remediating effect, causing the rats to perform at levels much closer to the controls.

In another paper, Railey et al. (2011) looked at spatial memory in a transgenic mouse model of Alzheimer's disease, supplementing the drinking water with zinc and

copper. The zinc + copper group performed as well as controls on the Morris water maze, while the zinc group performed more poorly. Again, this suggests that the cognitive deficits seen associated with zinc supplementation are due to copper deficiency induced by excess zinc.

This study better establishes the link between excess zinc and copper and anxiety- and depression-like behaviors in a rat model. Rats underwent a pre- and post-natal chronic supplementation paradigm, receiving either lab water, water supplemented with zinc, or water supplemented with both zinc and copper. At four months, depression and anxiety were examined using common behavioral measures: the forced swim test, the elevated plus maze, and the open field. It was hypothesized that zinc-supplemented animals would exhibit more depression- and anxiety-like behaviors than rats receiving tap water or water supplemented with both zinc and copper.

# 2. METHODS

## *2.1 Subjects*

Fifty-eight male Sprague-Dawley rats were raised pre- and post-natally on either zinc carbonate (10 ppm $ZnCO_3$) (n=22), zinc carbonate plus copper (10 ppm $ZnCO_3$ + 0.25 ppm Cu) (n=18), or lab water (n=18) in two cohorts. Water samples were sent to the Unites States Geological Survey (USGS) and analyzed to ensure correct metal content and pH. The rats were housed four to a cage, on a 12-hour light/dark cycle, with ad libitum access to food and water. The rats were tested at four months of age.

## *2.2 Open Field Test*

Testing was carried out in four Plexiglas open field chambers (42 x 42 x 30 cm). Animals habituated to the room for 15 minutes prior to testing. Each rat was placed in the center of the box and observed for fifteen minutes. A camera mounted above the apparatus recorded each trial. Total distance traveled, duration of time spent in the center, and distance traveled in the center, were measured using Videotrack software. Each box was cleaned with disinfectant between.

## *2.3 Elevated Plus Maze*

This behavioral test occurred one week following the open field test. The maze consisted of two open arms (50.2 x 10.8 cm) and two opposing closed arms (50.2 x 10.8 cm) with 40 cm high black Plexiglas walls. The maze was elevated 86 cm from the floor, and light was kept at 5 lux. Animals habituated to the room for 15 minutes prior to

testing. Each rat was placed in the center of the maze, facing an open arm. Each trial was five minutes, and an overhead camera recorded activity. Videos were hand scored, with the experimenter blind to which group each rat belonged, measuring total time spent in the open arms and number of entries into the open arms.

### *2.4 Forced Swim Test*

This test occurred one week following the elevated plus maze testing. Animals habituated to the room for 15 minutes prior to testing. Four Plexiglas cylinders (18 cm tall, 8 cm diameter) were filled to 12 cm with water and kept at 25° C. for fifteen minutes. Two cylinders were used at a time, separated by a black, cardboard divider. A video camera was placed in front of the cylinders. At all times the experimenter observed the trials on closed-circuit television from behind a curtain. Each animal was placed into a cylinder for a 15-minute trial. At the end of the trial, the animals dried under a heat lamp and were returned to their home cages.

Twenty-four hours later, the rats were again placed in the cylinders for a five-minute period. This data was recorded on VHS tape, and hand scored by an experimenter blind to group assignment. Both latency to immobility and total duration of immobility were measured. Immobility was defined as passively floating

# 3. RESULTS

## *3.1 Open Field*

A mixed ANOVA was used to determine if the three water groups spent significantly different amounts of time in the center of the open field across days, as well as across the trial on each day. Each day's fifteen-minute trial was divided into three five-minute segments, and analyzed for group differences across these time points. There was a significant change in center duration between day one and day two of testing in the open field overall ($F(1,55) = 10.99$, $p = .002$), but not a significant difference between water groups ($F(2,55) = 0.73$, $p = .49$) or a significant interaction ($F(2,55) = 0.85$, $p = .43$) overall (Fig. 1). The ratio between time spent in the center and total time in the test was similarly significantly different between days ($F(1,55) = 10.99$, $p = .002$), and not significant between groups ($F(2,55) = 0.73$, $p = .49$), and there was not a significant interaction ($F(2,55) = 0.85$, $p = .43$)(Fig. 2).

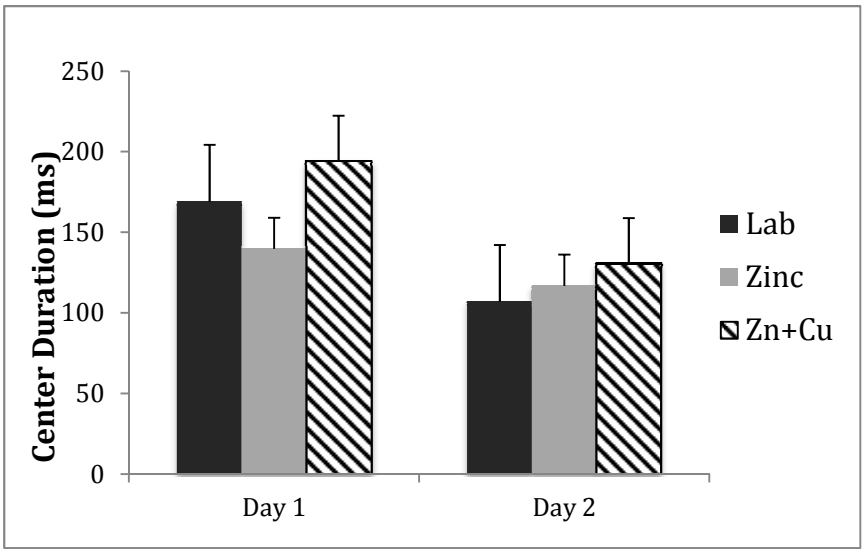


***Figure 1*. Open field center duration. Center time decreased on the second day for all three groups, but the groups were not statistically different from one another.**

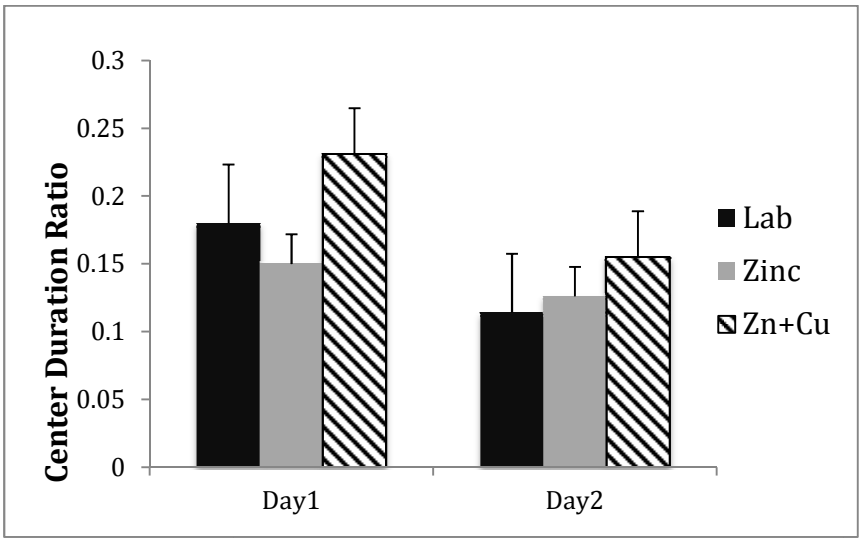


***Figure 2.* Ratio between center duration and total time in open field test. The ratio decreased on day two for all three groups, but the groups were not significantly different.**

Between day one and day two, there was a significantly difference in the total distance traveled during the task ($F(1, 55) = 11.06$, $p = .002$). While there was no significant different in total distance traveled between water groups ($F(2,55) = 2.11$, $p = .131$), differences in distance traveled can influence the time spent in the center. To address this, the ratio between distance traveled in the center and total distance traveled was calculated. The ratio was significantly different between day one and day two ($F(1,55) = 11.06$, $p = .002$), but not between groups ($F(2,55) = 2.11$, $p = .131$)(Fig. 3). Despite the fact that total distance traveled decreased on day two, when you account for this change by looking at the ratio, the distance traveled in the center still decreases.

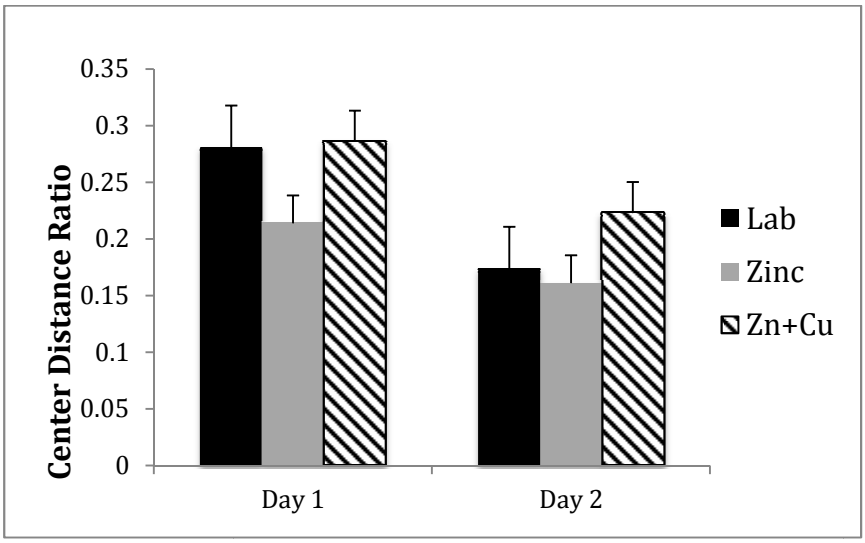


***Figure 3.*** **Ratio between distance traveled in the center and total distance traveled. The ratio decreased on day two for all three groups, but the groups were not significantly different.**

Within the trial on day one, center duration was significantly different across the three five-minute segments overall ($F(2,55) = 16.43$, $p = .000$), but there was not a significant difference in center duration between the water groups across the trial ($F(2,55) = 1.08$, $p = .37$) or a significant interaction ($F(2,55) = 0.77$, $p = .55$)(Fig. 4). Although the Zn group spent the least amount of time in the center and the Zn+Cu group spent the most, the difference was not significant.

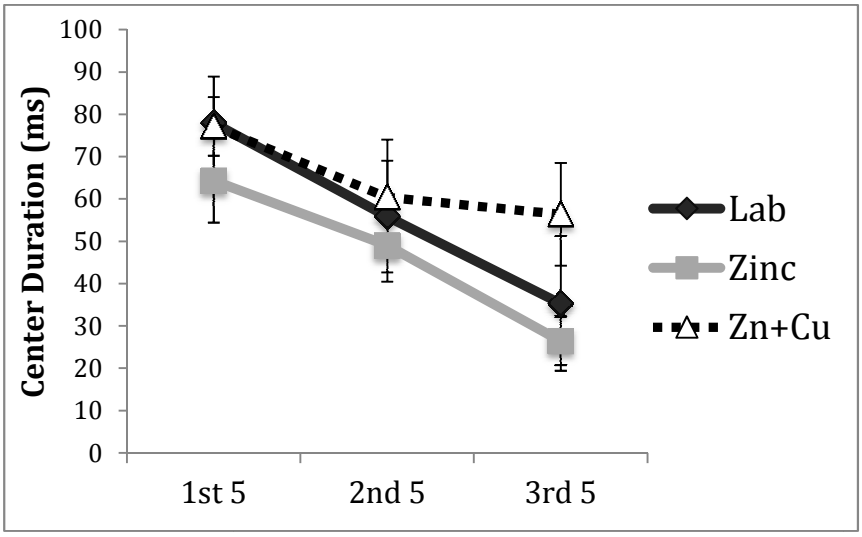


***Figure 4.*** **Day one center duration. There were no significant differences between water groups across the trial.**

A mixed ANOVA with a Greenhouse-Geisser correction was used to determine if any group differences existed across the trial on the second day of open field testing. Center duration was significantly different across the trial, when split into three five-minute segments ($F(2,55) = 12.15$, $p = .000$), and there was a significant interaction

between water group and time ($F(4,55) = 3.81$, $p = .01$) (Fig. 5). However, there was no significant difference between the water groups overall ($F(2,55) = 0.32$, $p = .73$), and multiple comparisons were not significant.

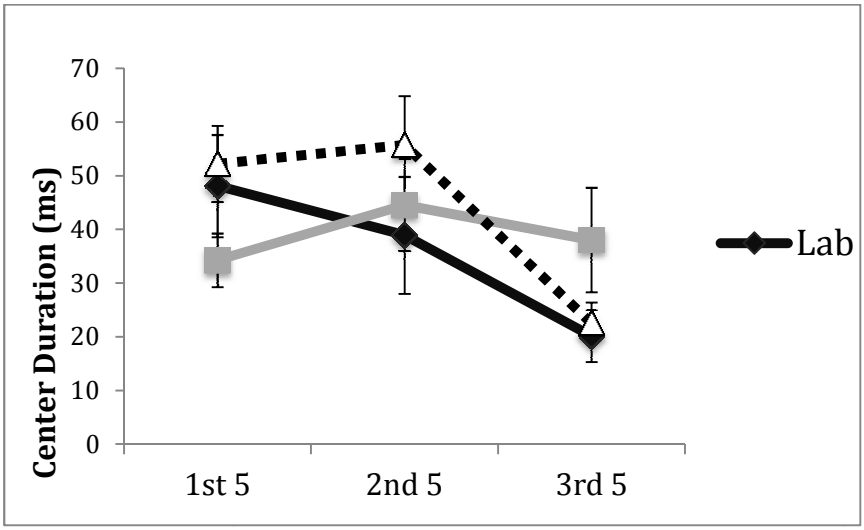


***Figure 5.*** **Day two center duration. Center duration was significantly different between water groups across the trial.**

Non-significant univariate ANOVAs make the significant MANOVA interaction more difficult to interpret. The zinc animals spent the least time in the center in the first five minutes ($M = 34.25$). Lab animals ($M = 48.08$) and zinc + copper animals ($M = 52.19$) spent more time in the center. During the second five minute period, zinc animals spent slightly more time in the center ($M = 44.54$), as well as zinc + copper animals ($M = 55.73$), while lab animals spent slightly less time in the center ($M = 38.89$). During the third five minutes, the zinc animals spent more time in the center ($M = 38.04$), while the zinc + copper animals ($M = 22.55$) and lab animals ($M = 20.11$) spent less time in the center.

*3.2 Elevated Plus Maze*

Using MANOVA, no significant differences were found between water groups on the anxiety-like behaviors in the elevated plus maze. There was not a significant difference between water groups on anxiety-like behaviors (V = 0.04, $F(4, 104) = 0.47$, $p$=.76). Univariate ANOVAs showed non-significant effects of water group on both number of entries into the open arms (Fig. 6), $F(2, 52) = 0.26$, $p$=.77, and total duration of time spent in the open arms (Fig. 7), $F(2, 52) = 0.01$, $p$=.99 The first cohort was run on the elevated plus maze a second time, a week after the first run, due to computer problems. The values used for the first cohort reflect this second attempt. This could be a potential confoud, but when only the second cohort is analyzed, results are similar (V = 0.10, $F(4, 44) = 0.59$, $p$=.67).

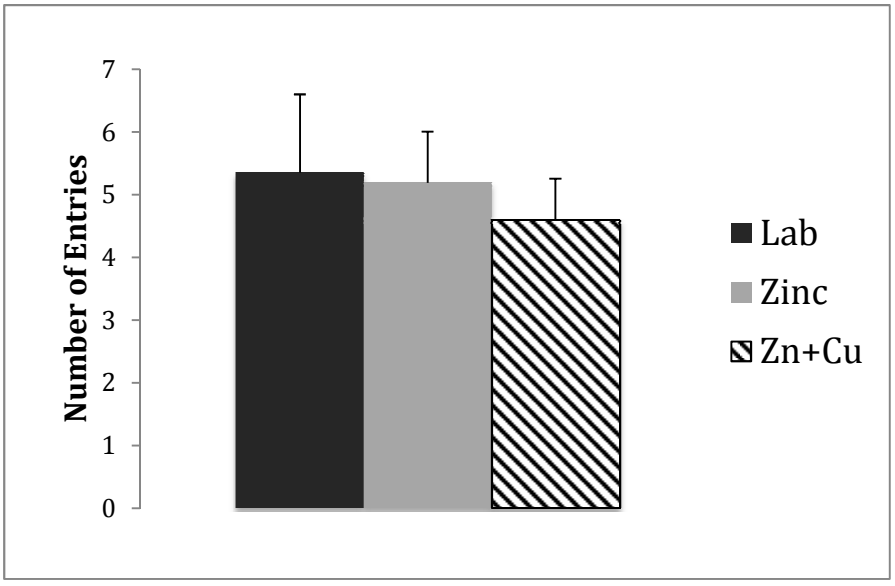


***Figure 6.*** **Number of open arm entries in the elevated plus maze. Number of open arm entries was not significantly different between the water groups.**

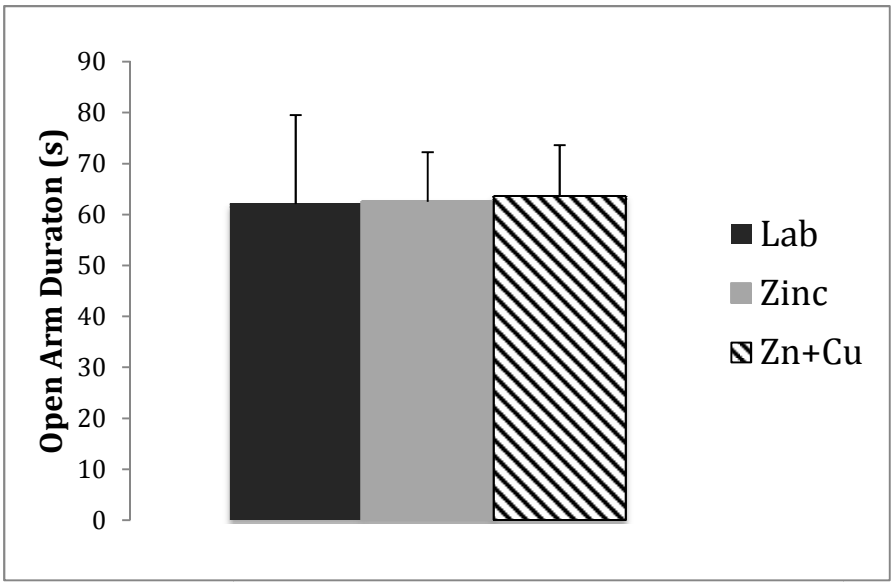


***Figure 7.*** **Open arm duration in the elevated plus maze. Open arm duration was not significantly different between the water groups.**

*3.3 Forced Swim Test*

No significant differences were found between water groups on the forced swim test on measures of depression like behaviors, V = 0.01, $F(4, 100) = 0.17$, $p = .95$. Univariate ANOVAs showed non-significant effects of water group for both latency to immobility (Fig. 8), $F(2, 50) = 0.25$, $p = .78$, and total duration of immobility (Fig. 9), $F(2, 50) = 0.25$, $p = .78$.

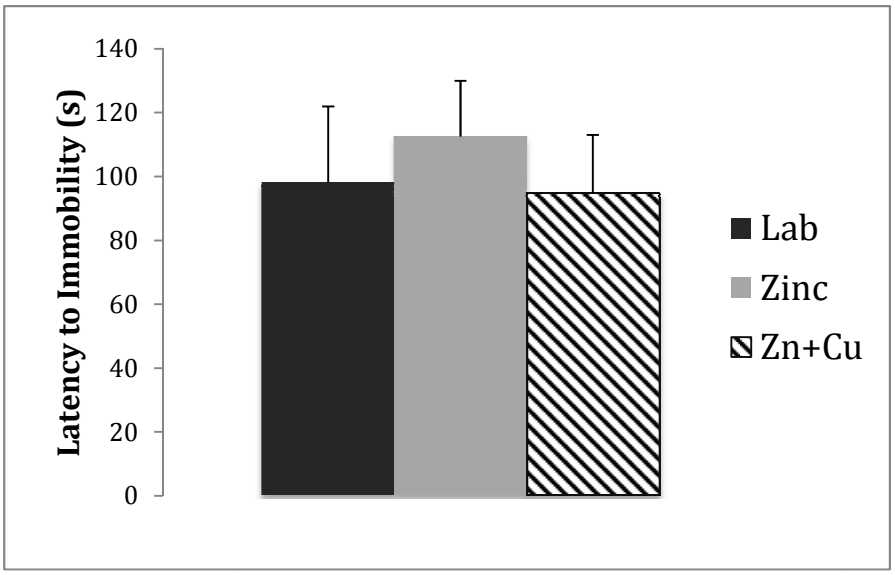


***Figure 8.*** **Latency to immobility on the forced swim test. Water group did not have a significant effect on latency to immobility.**

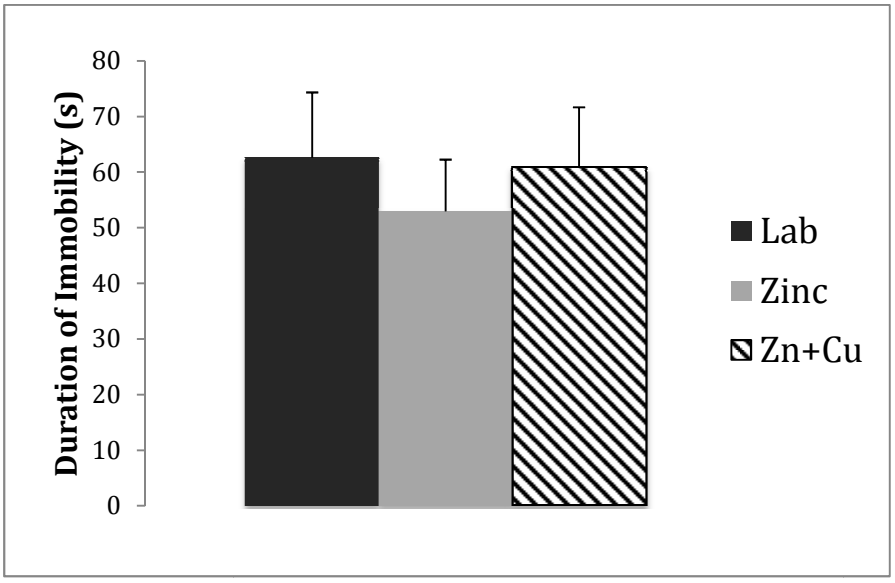


***Figure 9.*** **Total duration of immobility in the forced swim test. Water group did not have a significant effect on duration of immobility.**

# 4. DISCUSSION

Research on the impact of zinc supplementation and deficiency on anxiety and depression has produced conflicting results. Studies finding that zinc supplementation decreases anxiety- and depression-like behavior often employ short, acute supplementation paradigms (Nowak et al., 2003; Partyka et al., 2011; Szewcyk et al., 2009). On the other hand, studies using chronic, pre- and post-natal supplementation paradigms have found excess zinc to lead to impairments in spatial memory and a decreased ability to extinguish fear (Flinn et al., 2005; Railey et al., 2010).

This study employed the same paradigm, yet found no significant differences between water groups on depression-like behaviors on the forced swim test. These results conflict with many studies that have found zinc supplementation to significantly decrease depression-like behaviors on the forced swim test (Nowak et al., 2003; Szewczyk et al., 2010). These studies used either a single, acute dose of zinc, or a short treatment. It is possible that the chronic nature of the paradigm used in this study led to an excess of zinc that negated the previously seen antidepressant effects of zinc. This amount of zinc did not, however, cause an increase in depression-like behavior compared to controls. In the future, a study using several different doses of zinc could determine if this is the case.

Water group did not have a significant effect on anxiety-like behavior on the elevated plus maze. Takeda et al. (2007) found that a 2-week zinc deprivation

significantly decreased time spent in the open arms of the elevated plus maze in rats, but, to the researchers' knowledge, no studies have looked at the effects of chronic zinc supplementation on anxiety-like behavior in the elevated plus maze. Railey et al. (2010) used the same chronic zinc supplementation paradigm, and found that zinc-supplemented rats had a significantly reduced ability to extinguish fear. In accordance with those results, it was expected that the zinc-supplemented rats would show more anxiety-like behaviors than control rats and rats receiving copper in addition to zinc. No differences were found between water groups; possibly because the zinc supplementation was in excess of the amount that would be beneficial, as seen in Takeda et al. (2007), but was not enough to see the anxiolytic effects observed by Railey et al. (2005).

Takeda et al. (2012) found that 4-weeks of a zinc-deficient diet significantly reduced grooming behavior in the open field, and Takeda et al. (2007) found decreased grooming and line crossing in the open field after 1-2 weeks of a zinc deficient diet. The present study appears to be the first to examine the effects of zinc supplementation on anxiety-like behaviors in the open field, as opposed to zinc deprivation, as well as the first to look at center duration as the primary measure of anxiety. The literature associates zinc deprivation with an increase in anxiety behaviors (Takeda et al., 2007; Takeda et al., 2008; Takeda et al., 2012)

During open field testing, no differences were found between the water groups on day one or day two of testing as a whole, but all of the water groups spent less time in the center of the open field on day two than on day one. It is unusual that a mixed ANOVA

found that all three water groups spent less time in the open field as time went on. Typically, as animals habituate, they explore their environment more.

On day 2, center duration was significantly different between the groups over time, when dividing the trial into three 5-minute segments. The zinc rats spent slightly more time in the center during the second 5-minute period than the first and third. The Zn+Cu group also spent slightly more time in the center during the second 5-minute period, but center duration dropped by a greater amount during the third 5-minute period. The Zinc group started out with the lowest center duration, but ended with the highest, while the Zn+Cu and lab groups started higher and dropped off (Fig. 3). However, post-hoc tests did not show significant differences between the groups, suggesting that the significant interaction between group and time may have been due to the strong effect of time and high variability, and not true group differences.

During a pilot study of this experiment, with only a lab water group and a zinc group, results were seen that were consistent with the hypothesis (Beech, Ea, Holliday, Sizemore, & Flinn, 2011). No differences in center duration were found on day one of open field testing, but on day two, the zinc-supplemented rats spent significantly less time in the center than lab water animals. Compared to the present study, the center duration for the zinc animals was similar in the pilot study, but the center duration for the lab animals shot up on day two in the pilot study, while in the present study it decreased similar to the zinc animals. In the current study, all of the animals exhibited a greater amount of anxiety-like behaviors on the second day of open field testing. This is discordant with the open field literature, and what is known about habituation.

Differences between the pilot study and the current experiment could be due to differences in handling. During the pilot study, the animals were handled regularly. During the current experiment, the animals were not handled regularly in order to increase baseline anxiety.

One potential explanation for these unusual findings, and the different results between the pilot study and actual experiment, may be high variability between different suppliers of animals (Palm, Havermark, Meyerson, Nylander, & Roman, 2011). The pilot study utilized Sprague-Dawley rats from Charles River, while the present study ordered Sprague-Dawley rats from Harlan. Variability between suppliers may result from both genetic factors or differences in laboratory conditions. Palm et al. (2011) found significant differences between Wistar rats from different suppliers on measures of general activity, exploration, risk-assessment behavior, and shelter-seeking behavior. Notably, though, this study found no differences between the groups on anxiety-like behaviors.

The increase in anxiety-like behaviors over time and across days in the open field task may be due to the open field apparatus used for this experiment. The open field boxes have clear, Plexiglas bottoms, but sit on a wood table. The lights sit underneath the table, so the open field is not receiving light from underneath, only ambient light from above. As a result, the open field is not much brighter than the sides, and the main incentive for the animals to avoid it is because it is more open. Other studies looking at anxiety-like behavior involving zinc have used overhead lighting (Takeda et al., 2007; Takeda et al., 2012).

There are many potential explanations for the lack of effect zinc supplementation has on depression-like behaviors and anxiety-like behaviors on the first day of open field testing. Other recent studies have found results inconsistent with the general findings of the field. Cope, Morris, Scrimgeour, VanLandingham, & Levenson (2011) fed rats either a zinc deficient (5 ppm), zinc adequate (30 ppm) or zinc supplemented (180 ppm) diet for four weeks before inducing traumatic brain injury (TBI). Zinc-supplemented rats showed reduced depression-like behaviors using the two-bottle choice paradigm for saccharin preference, a model of anhedonia. There was not a statistical difference between zinc-supplemented, zinc-adequate, or zinc-deficient rats on anxiety-like behaviors on the elevated plus maze. There was a trend towards more time spent in open arms and more entries into the open arms for the zinc-supplemented animals. While this paradigm is for a shorter period of time, it is still chronic, and zinc supplementation was found to decrease depression-like behaviors, but not anxiety-like behaviors.

Similar, seemingly contradictory results have been found in studies looking at the role of zinc in Alzheimer's disease. Zn2+ plays an important role in Alzheimer's, as a major component of its characteristic amyloid plaques. Zinc dyshomeostasis contributes to the cognitive decline seen in Alzheimer's (Sensi et al., 2011). In Alzheimer's, Zn2+ contributes to aggregation of Aβ into amyloid plaques. Some studies have found zinc supplementation to exacerbate spatial memory deficits seen in transgenic mouse models of Alzheimer's (Railey et al., 2010; Flinn et al., 2005), while others have found that zinc supplementation improves cognitive function (Corona et al., 2010).

Corona et al. (2010) treated 3xTg-AD mice, a model of Alzheimer's disease, with 30 ppm ZnSO4 beginning at one month until 11-13 months. Cognitive deficits were seen in the 3xTg-AD mice, as measured by performance on the Morris water maze and novel object recognition. 3xTg-AD mice receiving zinc supplementation did not show cognitive deficits. In this case, chronic zinc supplementation prevented the cognitive decline seen in non-supplemented transgenic mice.

The many discrepancies in findings throughout zinc research suggest that the effects of zinc supplementation may be dose dependent; rather than correcting a deficiency or excess, treatments need to return zinc levels to their state of homeostasis, which is more complex. Both zinc deficiency and zinc toxicity are dangerous, each being associated with a range of health problems. Free zinc concentration of the mammalian brain is normally around 1-10 nM; if levels are too low, cell death can occur, while levels in excess of this amount can result in convulsions (Frederickson, Koh, & Bush, 2005). Just as nutrition experts are still determining the recommended daily allowance of zinc, and Alzheimer's researchers are seeking a way to correct zinc homeostasis, rather than a deficiency or excess, the use of zinc supplementation as a treatment for anxiety and depression must be approached in the same way.

In anxiety and depression, changes in the brain bioavailability of zinc may occur, causing redistribution instead of an overall change in amount. For example, Takeda et al. (2007) found that total zinc concentration in the hippocampus was not decreased after 4-weeks of a zinc deficient diet, but extracellular zinc concentration in the hippocampus was significantly decreased. The zinc deficient rats spent significantly less time in the

open arms of the elevated plus maze, indicating greater anxiety. Also, chronic administration of citalopram or imipramine increases zinc levels in the hippocampus while decreasing it in the cortex, cerebellum, and basal forebrain (Nowak & Schlegel-Zawadzka, 1999).

The neurobiological mechanisms underlying the role of zinc in mood disorders such as anxiety and depression are not well understood. The lack of a straightforward, linear relationship between zinc supplementation and symptoms of anxiety and depression is not surprising considering the complex interactions between zinc and several neurotransmitter systems. In the brain, zinc modulates glutamatergic NMDA receptors. One study found that low doses of an NMDA antagonist in addition to zinc significantly reduced total immobility time in the forced swim test (Szewczyk, et al., 2009). An AMPA-receptor potentiator also reduced total immobility time. The same study also found that the antidepressant effects of zinc in the forced swim test were negated by administration of NMDA. These results suggest the involvement of both NMDA and AMPA receptor in the antidepressant effects of zinc.

Anxiety and depression are typically accompanied by stress. It is not surprising that these mood disorders are associated with changes in the hypothalamic-pituitary-adreno-cortical (HPA) axis. Many patients of depression have higher levels of cortisol, as well as characteristic flattening of its normal diurnal rhythm (Linthorst & Reul, 2008). Having an inappropriate or inefficient stress response leads to a genetic phenotype that can leave one vulnerable to mental illness. Hypercortisolemia, excess levels of circulating cortisol, is seen in depression (de Kloet, Joels, & Holsboer, 2005).

Takeda et al. (2007) found that serum corticosterone concentration was significantly increased in rats after 2-weeks of zinc deprivation. In addition, line crossing and grooming behavior were decreased in the open field test, as well as less time spent in the open arms of the elevated plus maze. These findings suggest a link between the anxiety-like behaviors and elevated corticosterone levels seen in zinc deficient rats (Takeda et al., 2007).

Expression of brain-derived neurotrophic factor (BDNF) is decreased in affective disorders, and use of certain antidepressants is accompanied by an increase in BDNF expression in the hippocampus (Sowa-Kucma et al., 2008; Szewczyk et al., 2008). In one study, one to five weeks of zinc supplementation at a low dose (1.8 mg/kg) increased BDNF mRNA expression in the hippocampus (Sowa-Kucma et al., 2008). Many antidepressants increase BDNF activity, which may be associated with reduced NMDA glutamate receptor function (Szewczyk et al., 2008). Because zinc is an NMDA receptor antagonist, its antidepressant effects may be associated with elevated BDNF expression. These zinc-mediated elevations in BDNF expression may be attributed to the interactions of zinc with the serotonergic system (Szewczyk, et al., 2008).

The relationship between zinc and affective disorders is complex, involving several anatomical regions of the brain, neurotransmitter systems, and other factors. While it is clear that zinc deficiency is associated with anxiety and depression, the goal of supplementation should be to achieve zinc homeostasis, to avoid the negative effects associated with chronic, excess zinc.

Many studies have found zinc supplementation to decrease depression-like behaviors in animal models (Nowak et al., 2003; Sowa-Kucma et al., 2008), as well as increasing the efficacy of traditional antidepressants (Szewczyk et al., 2002; Szewczyk et al., 2009). This study found no significant differences in depression-like behaviors on the forced swim test between rats supplemented with zinc, zinc plus copper, or controls receiving lab water. The chronic, pre- and post-natal supplementation paradigm may have created conditions of excess zinc in the zinc-supplemented rats that negated the possible anti-depressant effects of zinc supplementation.

It was hypothesized that, similar to the impaired ability to extinguish fear and increased thigmotaxicity seen in the Morris water maze seen by Railey et al. (2005), chronic zinc supplementation would lead to increased depression-like behaviors in zinc-supplemented rats that would be remediated by copper. Although similar, depression and anxiety utilize distinct brain circuits, which may explain why this study did not see a similar effect.

There was a significant interaction between water group and time on day two. However, post hoc tests were not significant, suggesting that this interaction effect may be due to high variability between subjects and the strong effect of time, and not due to group differences. While previous studies have not examined the effects of zinc supplementation in the open field, researchers have found reduced grooming behavior and line-crossing in zinc-deprived rats (Takeda et al., 2007; Takeda et al., 2008; Takeda, et al., 2012). In contrast, this study did not find significant group differences.

It was hypothesized that the chronic zinc supplementation would induce anxiety-like behaviors in the zinc-supplemented rats, as seen in Railey, et al. (2010). The discrepancies in results may be due to a difference in subjects; Railey et al. used transgenic Alzheimer's mice, while this study used Sprague-Dawley rats. The Sprague-Dawley rats used as breeders were obtained from a different supplier than from the pilot study, which can produce variability (Beech, et al., 2011). Also, the pathways associated with the generalized anxiety captured by the open field test, and the cued fear tested by fear extinction are not identical (File, Lippa, Beer, & Lippa, 2004). It is possible that a higher amount of zinc, or a longer period of supplementation, may have negated these effects. These results should still be interpreted with caution.

The results of this study add to a growing pool of knowledge about the relationship of zinc and affective disorders. This relationship is complex, involving zinc's modulatory role in the glutamatergic system on both NMDA and AMPA receptors (Szewczyk, et al., 2009). Recent research has also revealed an association between zinc, depression and corticosterone levels (Takeda, et al., 2007), as well as brain-derived neurotrophic factor (BDNF) (Sowa-Kucma et al., 2008; Szewczyk et al., 2008). It is clear that zinc deficiency is related to depression and anxiety, in both animal models and humans. Many research studies have found zinc supplementation to be an effective treatment for these affective disorders. However, zinc is a toxin, and zinc supplementation treatments should restore zinc homeostasis, and not lead to a state of excess brain zinc. Understanding the multiple mechanisms associated with the

relationship between zinc and affective disorders may be the key to developing effective treatments that restore zinc homeostasis in depression or anxiety patients.

# APPENDIX

## *A. Expanded Methods*

### 1. Drinking Water

The water was mixed and stored in 10-liter carboys. The carboys were color-coded: lab water was labeled with white tape, zinc water was labeled by yellow tape, and zinc plus copper water was labeled with blue tape. The water was be obtained from the tap water faucet in the chemical lab (Room 2035 in David King Hall). To flush the water pipes and achieve a homogenous sample, the faucet was be turned on and allowed to run for 15 minutes before the carboys were filled. Once 15 minutes passed, each carboy was rinsed thoroughly to prevent contamination from the previous water samples. After each carboy was rinsed, they were filled to the black line at the top of the container with tap water. At this point, the lab water carboys are done.

The carboys labeled with yellow tape for zinc had a zinc carbonate ($ZnCO3$) solution added at a concentration of 10 parts per million. To achieve this concentration, 10 mL of the zinc carbonate solution was pipetted into each of the zinc carboys. For the zinc plus copper containers, 10 mL of zinc carbonate was added, as well as 0.25 parts per million of Copper (0.25 mL). Then the carboys were thoroughly shaken to disperse the solution throughout each container.

To control for a confound of pH, samples of each carboy were taken, and a pH meter was used to determine the pH of each sample, including the lab water samples. A

pH above seven is considered basic, and a pH below seven is considered acidic. The lab water generally had a pH of seven. The addition of zinc makes the water more acidic, averaging a pH of four. In general, a pH of four required the addition of around 7.0 mL of pH buffer (sodium carbonate) to make the solution neutral. After sodium carbonate was added, the carboys were shaken again to distribute the buffer throughout the container. The carboys then sat for ten minutes to allow the buffer to neutralize the acidity. After ten minutes passed, the carboys were shaken again to evenly distribute the zinc throughout the container. Another sample was taken from the carboys that received the buffer to ensure that the pH is seven.

Once each of the carboys reached a neutral pH, samples were taken from each carboy, in small plastic containers properly labeled with the water type, carboy number, and date. These samples were sent to USGS for analysis to ensure that the metal concentrations are correct. A record of all water made was kept in a notebook, containing the date, which carboys were filled, the initial pH, how much buffer is added, and the final pH.

**2. Open Field Testing**

*Preparation*

In the testing room, both sets of floor lights were turned on; the room lights remained off. The four, white, square boxes were placed on the table within the tape outline. The insides of the boxes were cleaned with ethanol. A counter-balanced, randomized animal list was be created in excel and printed. In the colony, the cart was labeled with appropriate animal labels on each cage. The appropriate animal was then

placed in each of the cages. The cart was then wheeled down into the room, where the animals habituated for 15 minutes.

*Computer Set-up*

The following steps were taken to set up the computer for testing:

1. Double click on VideoTrack Tracking to open the program
2. Go to File>Open>Local Disk C>VideoTrack Data>Open cntr4sprague.vte
3. Go to parameters>experiment parameters>experiment duration>set minutes to 15.
4. Set integration period to 300 seconds.
5. Go to edit>animal list creation wizard
6. Under locations: double click on each one
7. Session count: number of animals divided by four
8. Create list>save in desired location
9. Minimize program and go into the location and open the list
10. Enter the animal IDs, hit save, and exit
11. Go back into Videotrack program
12. Parameters>animals>click OK
13. Double click on video to look at OF boxes, right clock and edit. Move boxes to ensure outer box matches the white box and inner box looks like it is in the center. DO NOT change the size of the squares!
14. Click tiles on top and go to experiment>execute. During testing do not minimize the program! Dots on bottom left should be green.

*Testing*

Two people are required for testing to ensure that all four rats are placed in the boxes simultaneously. After the animals are placed in the boxes, the play button on the computer in the adjacent room was immediately pressed to start recording. The computer was monitored to ensure that the rats are okay, and that the tracking software was working correctly. Each trial was 15 minutes long, with four rats being run at a time (one in each box). Between each trial, the boxes were thoroughly cleaned with ethanol and paper towels, and then rinsed with water and dried off to get rid of the ethanol smell.

**3. Elevated Plus Maze Testing**

*Preparation*

Both sets of floor lights were placed in the corners of the room; the room lights remained off. The maze was then set up and thoroughly cleaned with ethanol. A randomized and counter-balanced animal list was made prior to testing, as well as ensuring that the camera is properly set up to record. In the colony, labels were made and placed on the appropriate cage of the metal cart. Each animal was placed in its appropriately labeled cage on the cart, and the cart was wheeled down into the testing room. The animals then habituated for fifteen minutes.

*Computer Set-up*

The following steps were taken to set up the computer for testing:

1. Double click on Videotrack tracking
2. Click on edit> animal list creation wizard
3. Under session count, enter your total number of animals

4. Animals per session count: double click cage one under locations
5. Click create list and choose where you want to save
6. Close the window, minimize the program, and open the txt file you saved your list as (has to be .txt)
7. Type in subject numbers and save, close out, and re-open Videotrack
8. Click parameters>experiment parameters and ensure that trial length is set to 5 minutes and hit OK.
9. Go to parameters>animals and import your animal file and hit OK.
10. To run, hit experiment>execute and tell the program where to save the files. At this point, the dots in the bottom left should be green. Once an animal is placed in the maze, they may turn red, because you haven't programmed the program to "track" anything. Again, if you minimize the program, data will STOP RECORDING and will not resume.
11. When done, hit experiment>stop and your data will be generated.

*Testing*

At the end of habituation, the correct animal was placed directly in the center of the maze, facing an open arm, and the experimenter promptly left the room and hit the play button on the computer program. Each animal was be observed from the computer in the adjacent room to make sure the rat didn't jump out of the apparatus or anything else unexpected. Each rat was in the maze for 5 minutes.

**4. Forced Swim Testing**

*Preparation*

In the Morris water maze room, the black cardboard divider was placed on the table. A camera was set-up to view the cylinders from the side. The camera was hooked up to a small black and white Sony television and a VCR. The curtains were pulled around the room to obscure the rats' view. Then all four clear cylinders were rinsed and filled to 25cm (to the bottom of the white tape) with 25° C tap water from the hose. Usually, filling them up to about an inch short with the cold-water faucet on, and then adding the last bit from the warm water faucet will achieve this temperature. The long, glass thermometer was used to take the water temperature, and hot or cold water was added or subtracted until the desired temperature was reached. Two of the cylinders were then be placed into the cardboard divider, one on each side. The television was then checked and the experimenter ensured that the camera was aligned correctly and in focus, as well as making sure there was a stack of dry towels within reach.

In the colony, two cages were prepared for drying under the heat lamp and placed on a cart. A heat lamp was positioned so it is pointing at the two cages. A list of animals was created in a spreadsheet, with two rats being randomly selected to run at a time. Labels were created on post-it notes to place on the cages.

*Testing Day 1*

The animal cart was labeled with animal subject numbers. The proper animals were removed from their home cages, and placed into the correctly labeled habituation cage. These cages were rolled down and left outside of the room. The experimenter then left the hallway, closed the door, and allowed the animals to habituate for 15 minutes. After 15 minutes, the water-filled cylinders were labeled with the proper animal numbers,

and the animals were transferred from the habituation cages into the cylinders. Two people did this to ensure that each rat is placed in the cylinder at the same time. The experimenter then started the timer, quickly closed the curtain, and sat quietly outside of it, observing the rats on the TV monitor. No data is recorded on the first day; it is a habituation trial. If at any time either animal appeared to be in distress, sinking, or drowning, or managed to jump out of the container, it was be immediately removed from the water and taken out of the experiment.

After the 15 minutes passed, both experimenters carefully removed the animals from the water, dried them off with the towels and placed them in the drying cages. Another timer was be set to ensure that the animals are in the drying cages for 15 minutes. To prevent confusion, the experimenter made sure that the animal that is supposed to go into the left-hand cylinder (cylinder 1), was always in the left of the two cages, and vice versa. Also, the experimenter made sure to transfer the number label for that animal every time it is moved to a new location. Once the next set of animals habituated, the process started again. After every pair, the mesh scooper was used to remove feces from the cylinder. About every three runs, the water was replaced. Once the animals dried off under the heat lamp for 15 minutes, they were returned to the animal cart. Once all of the animals had been habituated, run, dried off, and returned to their home cages, the experimenters cleaned and prepared for Day 2, which included emptying the cylinders, rinsing them out, and laying out the towels to dry.

*Testing Day 2*

Preparation was the same as Day 1, except that a blank VHS tape was labeled and placed in the VCR. Each animal still needs to habituate and dry off for 15 minutes, but was only placed in the water for 5 minutes. The experimenters double-checked to make sure the camera is recording when the animals are being run. Again, the animals were observed from behind the curtain to ensure their safety, and the experimenter made sure all containers were labeled for the correct rat, and that the rat from the left cage was always being placed into the left cylinder, then back into the left cage. A label was placed on each cylinder for which pair is being run (pair 1, pair 2, etc) in view of the camera, and the specific animal numbers were below view of the camera, so that when the data was analyzed, the experimenter was unaware of which group the animal belongs to, but can go back and match the data to the correct animal at a later time.

### *B. Expanded Results*

#### 1. Open Field Results by Cohort

While cohort one and cohort two did not spend significantly different amounts of time in the center of the open field ($F(1, 56) = 2.12$, $p=.15$), some interesting information is gained by analyzing the cohorts separately. When only looking at cohort one, there is an overall difference in center duration between day one and day two ($F(1, 27) = 7.12$, $p=.013$) and a significant difference in center duration between the water groups ($F(2, 27) = 4.29$, $p=.024$), but not a significant interaction ($F(2, 27) = 0.01$, $p=.994$) (Fig. 10). LSD post-hoc comparisons indicate that the lab water group ($M = 86.76$) spent significantly less time in the center than the zinc plus copper group ($M = 161.38$), $p=.011$, as well as the zinc water group ($M = 99.49$), $p=.031$.

When analyzing cohort two alone, there is an overall difference in center duration between day one and day two ($F(1, 25) = 4.41$, $p=.046$), but not a significant different between the water groups ($F(2, 25) = 0.58$, $p=.57$) or a significant interaction ($F(2, 25) = 1.81$, $p=.185$) (Fig. 11). None of the multiple comparisons are significant.

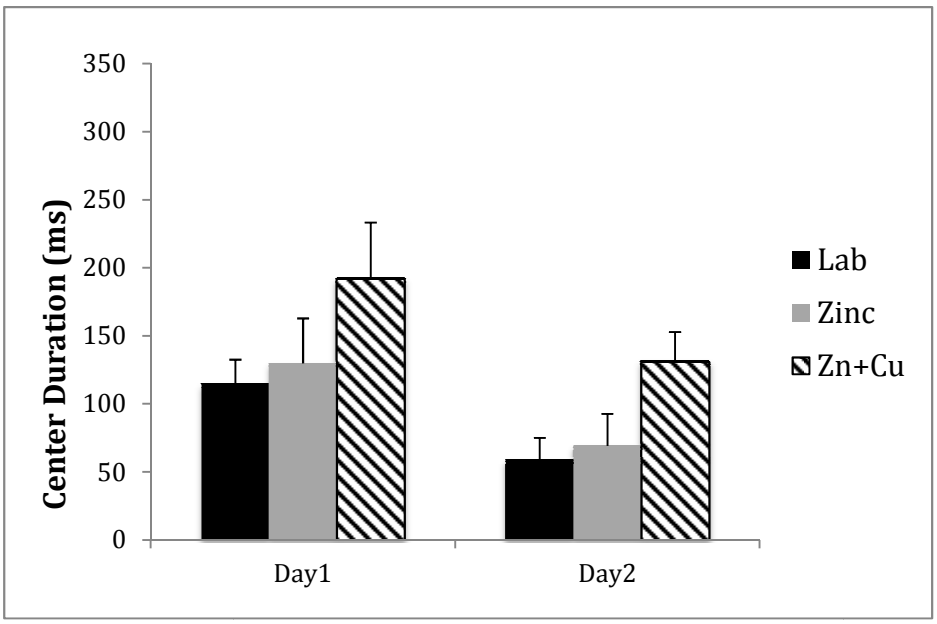


***Figure 10.*** **Open field center duration for cohort one. Center time decreased on the second day for all three groups, and the groups were statistically different from one another.**

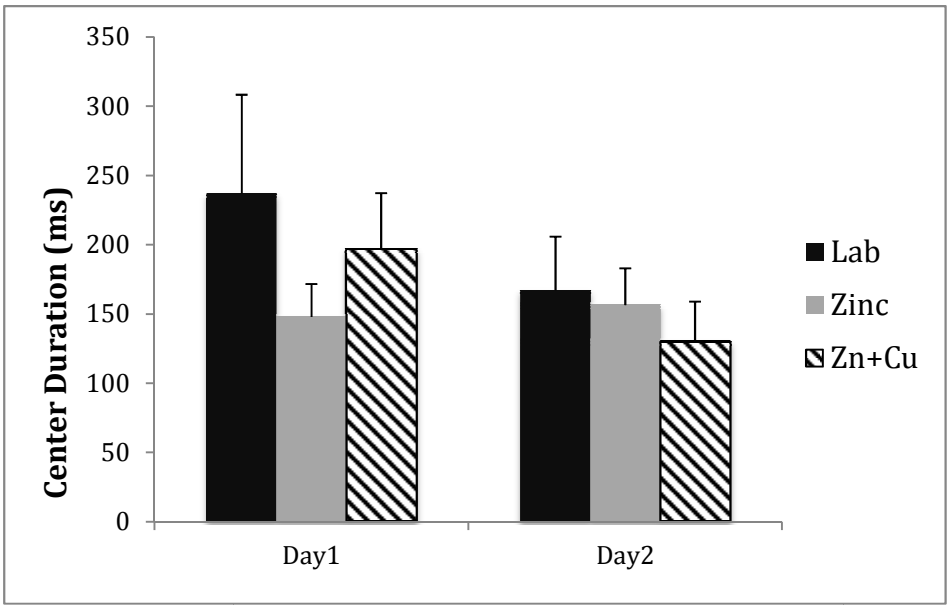


***Figure 11.*** **Open field center duration for cohort two. Center time decreased on the second day for all three groups, but the groups were not statistically different from one another.**

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## CURRICULUM VITAE

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