THE EFFECT OF MILD COPPER DEFICIENCY ON FEAR EXTINCTION AND MOTOR FUNCTIONING

by

Stephen Lawrence Patrick Lippi

A Thesis
Submitted to the
Graduate Faculty
of
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The Requirements for the Degree
of
Master of Arts
Psychology

Committee:

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Fairfax, VA

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Bachelors of Science
George Mason University, 2013

Director: Jane Flinn, Professor
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DEDICATION

This thesis is dedicated to my parents Stephen Edward Lippi and Jamie Michelle Lane for their love and support while I have been in college and as I continue in the field of academia. Also, to my closest friend, Anthony Amaya, who has been there for me every step of the way from high school to graduate school.

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I would like to thank the many friends and family that have accompanied me on my ride through graduate school and through the completion of this thesis project. My lab members are the greatest people to work beside and have helped me with handling, equipment, and teaching me how to do things such as plot graphs in Sigma Plot and GraphPad, how to perfuse rats, perform the Golgi-Cox staining protocol, run fear extinction, and lent me much moral support all along the way. Gretchen Knaack shared her thesis data allowing us to compare our results with that of the standard“7012” diet group. Caroline Neely, statistics extraordinaire, is deeply in my debt due to her countless hours of help in regards to statistical analysis and Dr. Jane Flinn, my adviser, for her patience and understanding when I would continually email to meet up and discuss my project. I would also like to thank my committee members, Dr. Jane Flinn, Dr. Patricia Wanschura and Dr. Jennifer Brielmaier for guidance and feedback along the way to the completion of this project.

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ABSTRACT

THE EFFECT OF MILD COPPER DEFICIENCY ON FEAR EXTINCTION AND MOTOR FUNCTIONING

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Biometals play a large role within the human body where a deviation from normal levels can result in behavioral abnormalities. Copper (Cu) is a transition metal with physiological importance and has been shown to have a remediating effect on the behavioral impairments caused by excess Zinc (Zn). In order to examine the effects of a Cu deficient diet directly, diets mildly deficient in Cu with identical Zn levels were developed and resulting behavioral effects were examined. Forty-five male Sprague-Dawley rats were tested for cued fear learning, fear extinction, and motor functioning in order to assess the effect of dietary metal content on behavior. Using specially formulated diets, a mild Cu deficiency was examined directly, as opposed to a Cu deficiency attained through excess levels of Zn. Animals were given a Cu deficient diet (4ppm Cu) while still in prenatal development, a Cu deficient diet (4ppm) after birth (postnatal), and a Cu control diet (16ppm) after birth (postnatal) and were raised for a period of 4 months. Throughout the fear learning and extinction paradigm, there were no significant

differences between the dietary conditions except on the second day of fear extinction recall where a marginally significant difference was detected with the prenatal Cu deficient group exhibiting higher percent freezing than the postnatal Cu deficient and control groups. However, in analysis of the accelerating rotarod task, significant differences between the diets were found on days 2 and 3 as well as when the performance was averaged across all three days of testing and across the nine total trials. The postnatal Cu deficient group consistently exhibited lower latencies to fall. Significant differences were noted in the weights between the dietary groups. At weaning (PND 21), the prenatal Cu deficient weighed significantly more than the postnatal Cu deficient, and at 4 months of age, the postnatal Cu control group weighed significantly more than the pre- and postnatal Cu deficient groups. These results suggest that a diet mildly deficient in Cu may lead to motor abnormalities and impairment and not necessarily lead to significant differences in fear learning. These data show the important role that dietary metal content plays in behavior and that using appropriate control and experimental diets can have a significant effect on rodent behavioral experiments.

Keywords: Copper, Zinc, Fear Extinction, Nutrition, deficiency, biometals

1. INTRODUCTION

Copper (Cu) is an essential trace mineral and is an essential element for all developing mammals (Gybina & Prohaska, 2003) and for normal brain development (Prohaska & Gybina, 2005). Copper's role in the nervous system is critical, particularly in the electron transfer associated with oxidative enzymes (Zatta & Frank, 2006), and its unique role in oxidation-reduction reactions (Gaier, Eipper & Mains, 2012). Copper is also included in a large number of cuproenzymes which depend on it for catalytic activity (Johnson, 2005; Gaier, Eipper & Mains, 2012). Copper homeostasis is essential, because a deficiency or excess levels of Cu can both lead to pathological events; for example, a Cu deficiency restricts activity of cuproenzymes and can alter biochemical and physiological phenotypes (Prohaska & Brokate, 2001). A number of neurological diseases involve the direct or indirect involvement of Cu, including Alzheimer's disease (AD), Huntington's disease, Parkinson's disease, Menkes' disease, and Wilson's disease (Desai & Kaler, 2008). Copper transport genes can undergo mutations, which can lead to either copper deficiency or excess copper within the human body (Menkes' disease and Wilson's disease respectively) (Desai & Kaler, 2008). Copper's importance has been seen through extensive use of animal studies, which generally focus on examining the biochemical effects of Cu deficiency as well as through some behavioral assays. An

excess of Cu can be problematic as seen in Wilson's disease (Desai & Kaler, 2008) but in this thesis, the focus is on the effects of Cu deficiency.

Much of the research which has been conducted under the umbrella of examining deficiencies in Cu have been mainly biochemical, not behavioral, with animals only being raised for a month or so on a diet that is severely deficient, with biochemical assays subsequently used to examine markers for apoptosis, levels of cytochrome c, and mitochondria (Gybina & Prohaska, 2003). Previous research looking at the biochemical alterations caused by diets deficient in Cu have shown cytochrome c levels increasing, which correlate with the degree of perinatal copper deficiency (Gybina & Prohaska, 2003), differences in the levels of dopamine β-monooxygenase protein, with higher levels being found in Cu-deficient rats (Prohaska & Brokate, 2008), and lowering of brain norepinephrine (NE) concentrations (Prohaska & Smith, 1982). Rats are typically the subjects of such dietary manipulation experiments and a plethora of Cu deficiency research has been obtained. Perinatal Cu deficiency in rats has also been shown to include lower iron concentration in the rat brain (Prohaska & Gybina, 2005) as Cu is involved in Fe transport (Askwith & Kaplan, 1998). A large part of the research conducted on Cu deficiency deals with understanding the nutritional and dietary effects of Cu and formulating special diets in order to test the effects of Cu deficiency, not just knowing the Cu levels in a standard bag of rodent chow.

Nutrients help in regulating brain development through fetal and early postnatal life. Although the brain is plastic, nutritional insult can modify the brain's plasticity, affecting myelination and development of critical brain structures including the

cerebellum and the hippocampus (Georgieff, 2007). In regards to Cu, one region of the rat brain which has a large percentage (μg Cu/g Dry tissue) is the cerebellum (Johnson, 2005) and in gestational Cu deficiency the developing cerebellum can be at risk for long-term effects on balance, coordination, and motor functioning (Penland & Prohaska, 2004). In order to understand the role and importance that Cu can have and its effects on behavior, a diet that was Cu depleted, yet still able to support lactation and gestation, was created in order to provide a diet that was low in Cu but could also sustain rats for 4 months. In order to develop a diet that involves the manipulation of biometals, the diet must be formulated correctly with consideration of the levels of more than one metal so that the potential for variation in research results is reduced, rather than increased. (Mickelson, B., 2009, 2013). The standard lab "7012" diet contained 63ppm Zn and 23ppm Cu and contained other nutritive factors including phytates and fiber which could impact metal absorption (Lönnerdal, 2000); because of this, a control diet that matched the experimental diet was needed. The diets used in the present study were identical except for differences in the levels of Cu (4ppm Cu in the deficient and 16ppm in the control).

Diets to be administered to laboratory animals can be classified as either purified or natural. Purified diets use refined ingredients and can be manipulated to contain very high or low levels of specific macro or micronutrients, whereas natural ingredient diets are made from agricultural feedstuffs that are minimally processed (Mickelson, B., 2009, 2013). In the initial development of the diet, Cu was the element of interest and the levels of Zn needed to be balanced in order to prevent unintended variability in results, since an

increase in Zn can result in a decrease in Cu levels. The amount of fiber also affects how the metals are absorbed; in particular, dietary fiber can inhibit zinc retention (Sandstead, et al., 1997). Questions that needed to be considered in regards to this diet included whether the levels necessary to support gestation and lactation (and overall rodent health) were satisfied and also whether the levels of Cu and Zn in the diet were controlled. Refined ingredients were used, in contrast to more natural ingredients such as corn, wheat, grain, etc. since the Cu level could be better controlled (per discussion with Dr. Herfel, nutritionist at Harlan Laboratories, Inc.). Another important aspect to consider in a diet is the ratio of metals. When the human body fails to regulate the amount of key trace metals, abnormal levels and ratios of trace metals can develop and the ratio of Cu to Zn may be clinically more important than the concentration of either of the metals alone (Osredkar & Sustar, 2011). An important example of the importance of noting the ratio of metals rather than strictly just the levels is the enzyme Cu/Zn superoxide dismutase (SOD1) which is important for the detoxification of superoxides (Osredkar & Sustar, 2011). The ratio of Cu to Zn helps this enzyme function properly rather than the absolute amount of Cu or Zn alone (Harris, 2001; Groff, Gropper & Hunt, 1995). The ratio of metals can play a role in the efficiency of enzymes and deserves to be noted in the production of a diet alongside that of the levels of biometals alone. In developing the diet and in planning an experiment that would look at metal manipulation, the interaction of metals is important to consider so that there will not be unforeseen results based on the interactions between the metals.

Zn, Cu, and also Iron (Fe) exhibit important interactions within the body and excessive intake of one metal may lead to an overt deficiency in another element (Barone, Ebesh, Harper & Wapnir, 1998). An interesting interplay occurs between Zn and Cu within the body, whereby excess Zn prevents adequate Cu absorption through the wall of the intestines and high Zn levels can lead to a Cu deficiency (Maret & Sandstead, 2006); in addition metallothionein levels can rise leading to a reduction of the absorption of copper (Bertinato, J. & L'Abbé, M.R., 2004). For this experiment, Zn levels were controlled and balanced between the diets created so that only the levels of Cu differed between the experimental diets and the control diet. This was done so that the manipulations of Cu were intended to only affect Cu, since these levels were the only ones that changed. However, because the Zn levels were held constant, the Zn/Cu ratio does differ between the experimental and control diets with the deficient and control diets having ratios of 10 and 2.5 respectively. The diets used in the present study were formulated in partnership with Teklad and Dr. Tina Herfel.

Of particular interest to researchers, besides biochemical assays and determining the biochemical ramifications of diet manipulation of metals, is what role the alteration of metals can have on behavior. Little research has been done behaviorally, compared to chemically, on measures related to deficiencies in Cu. Previous research conducted has shown that excessive Zn levels led to impairments in the extinction of learned fear (Railey, Micheli, Wanschura & Flinn, 2010). Excessive Zn levels also led to higher latencies to find a hidden platform in the Morris Water Maze task and fewer platform crossings in both rats (Railey et al., 2010) and transgenic mice (Railey, Groeber & Flinn,

2010). In pre- and post-natal Zn supplemented rats, the addition of Cu has been seen to bring freezing levels closer to control animals in fear extinction (Railey et al., 2010) due to the fact that an increase in Zn levels compared to Cu can induce Cu deficiency which has been established (Maret & Sandstead, 2006). Other behavioral studies have used the elevated zero maze in looking at anxiety-like behavior in Cu supplementation in peptidyglycine α-amidating monooxygenase (PAM) +/- mice and Cu depletion in wildtype mice. PAM is a Cu-dependent enzyme important in peptide hormone processing and PAM +/- mice exhibit an increase in anxiety-like behavior and decreased peptide amidation. Anxiety-like behavior was sensitive to Cu supplementation in PAM +/- mice and in wildtype mice these results were seen in Cu depletion (Bousquet-Moore, Eipper, Prohaska & Mains, 2009). The accelerating rotarod procedure was used to assess motor functioning following recovery from perinatal copper deficiency; even after five months of Cu repletion, a persistent impairment in motor function compared to control animals was seen (Penland & Prohaska, 2004). The results of the the Penland & Prohaska (2004) study showing deficits even after repletion of Cu following perinatal copper deficiency spurred our idea of examining motor functioning based on a diet that is deficient in Cu. In our study, Cu deficiency is examined directly rather than by Zn supplementation.

The main behavioral task employed in the current thesis is the Pavlovian fear conditioning task which requires an animal to associate a conditioned stimulus (CS) which was previously neutral (neutral stimulus, NS; a tone) with an aversive stimulus (unconditioned stimulus, US; a shock). After tone-shock pairings have occurred, the animal will display a conditioned response (CR) indicated by freezing when the CS

(tone) is played alone without the shock. Freezing during the period of time before the tone-shock pairings occur on conditioning day is also something to look at to note if the rats exhibit any unusual freezing behavior to being placed in a new environment. After fear conditioning, fear extinction will be examined whereby the animal will hear the CS presented alone without the shock.

To acquire the extinction of a conditioned fear, active learning occurs and requires N-methyl-D-aspartate (NMDA) receptors (Burgos-Robles, Vidal-Gonzalez, Santini & Quirk, 2007). These NMDA receptors, particularly in the basolateral amygdala complex (BLA), are important for the formation of the association between the UCS and the CS (Schauz & Koch, 2000). Copper is relevant to NMDA receptor functioning because in cultured hippocampal neurons, Cu 2+ is an antagonist at NMDA receptors. This antagonism makes fear extinction a useful behavioral task that can allow the effects of Cu to be observed (Vlachová et al., 1996; Weiser & Wienrich, 1996). Cu 2+ also plays an inhibitory role on $GABA_A$ receptors, specifically those containing the α5 subunit (Collinson, Kuenzi, Jarolimek, Maubach, Cothliff, Sur et al., 2002; McGee, Houston & Brickley, 2013). Decreased GABA inhibition, particularly in the hippocampus, could be a reason for enhanced performance in a water maze task and comparable performance to wildtypes in an elevated plus maze in mice lacking the α5 subunit of the $GABA_A$ receptor, suggesting that this inhibition can have an impact on learning and memory processes (Collinson et al., 2002).

The purpose of these experiments was to examine the nutritional effects that a diet low in Cu can have on fear conditioning and fear extinction, and on motor coordination

as assessed through the accelerating rotarod task. Nutritional effects were assessed by raising three groups of rats for 4 months prenatally on 4ppm Cu, postnatally on 4ppm Cu, and postnatally on 16ppm Cu. A diet specifically altered in only Cu was developed due to the fact that previous behavioral studies have not looked at Cu directly; rather through excess Zn causing deficiencies in Cu. Also, there are few behavioral studies in the nutritional literature examining fear extinction and motor impairment for rats that are beyond 1 month of age. Using the formulated diet allowed comparison of how a diet with excess Zn relates to a diet that is solely restricted/differs with just Cu. The following was hypothesized:

1. There will be significant differences in fear extinction between the three groups (4ppm Cu prenatal & postnatal and 16ppm Cu control postnatal), with the 4ppm Cu prenatal group performing the worst and the 16ppm Cu postnatal group performing the best.

2. Animals raised on 4ppm Cu prenatally will perform significantly worse on the accelerating rotarod task compared to the postnatal 4ppm Cu and 16ppm groups.

3. Average weights between groups will be significantly different with the 4ppm Cu prenatal animals weighing the least and those on the 16ppm Cu control diet weighing the most.

2.1 METHODS: SUBJECTS

Forty-five male Sprague-Dawley rats were raised pre- and post-natally on diets deficient in Cu (4ppm) (n=15 prenatally, n=15 postnatally) and a control diet (16ppm Cu) (n=15 postnatally).

2.2 METHODS: ANIMAL BREEDING, HOUSING & GUIDELINES

Breeding & Housing:

Fifteen Sprague Dawley female rats and 5 Sprague Dawley males were ordered from Harlan Sprague-Dawley (Indianapolis, IN) for breeding purposes. Once at George Mason, the breeder rats acclimated for one week before being paired. One male Sprague-Dawley rat was paired with 3 female rats for a period of 1 week. At the end of the week, the male was removed and the females were monitored for signs of pregnancy and around 1 week before birth, the moms were separated into individual cages. Male Sprague Dawley rats were housed separately when not being paired with females in groups of 2. After the mom gave birth, the pups were sexed, the females sacrificed, and 3 males were saved per litter. Three Sprague Dawley females were paired with a Sprague Dawley male for pilot work and the 12 remaining Sprague Dawley females were paired with males in order to establish 2 groups: 1 Postnatal Copper Deficient group (n = 18), and 1 Postnatal Copper Control group (n = 18). Timed pregnant females were ordered so that precision in administering the prenatal Cu deficient diet could be obtained and be as accurate as possible (diet to be administered on day 7 of gestation): 6 timed pregnant females were ordered and saving an n of 3 pups per mom, the size of the prenatal group was n=

18. Previous studies have used an n of 12 animals and have received statistically significant results (Railey et al., 2010). After the mom gave birth to pups and after females and extra males were sacrificed, the pups were weaned at postnatal day 21. Post-weaning, the pups were housed with same-sex littermates for the duration of the experiment until 4 months of age when behavioral testing occurred. Females that had given birth were then placed into cages with the other breeding moms that were assigned in their group.

General Colony Guidelines:

The colony is run on a 12:12 light dark schedule. All cages were lined with Tek-FRESH bedding and cleaned by SoBran, Inc. once per week. All personnel involved with the animals wore clean gloves, a lab coat, long pants, and closed toed shoes. Loud noises and strong odors were avoided.

Food/Water:

Food and water was offered *ad libitum*. With the exception of the timed-pregnant females which were given the prenatal copper deficient diet on prenatal day seven, all breeder animals were fed the lab's standard Teklad lab animal diet "7012" obtained from Harlan Industries. In the postnatal deficient and postnatal control groups, once the pups were born, the diet was switched to either 4ppm Cu (postnatal group) or 16ppm Cu (control group) that was created alongside nutritionists at Teklad diets at Harlan.

New diets were created which deviated from the standard 7012 diet currently used in the lab. Both diets are identical in content except that the amount of Cu differs between them (deficient diet = 4ppm Cu, control diet = 16ppm Cu). The level of Zn remained the

same across the experimental diets (40ppm). The "7012" diet could not be used as an accurate control diet due to additional Cu and Zn content (23 ppm and 63 ppm respectively) among other ingredients such as phytates which could have an impact on metal absorption (Lönnerdal, 2000). Also, due to the fact that Zn can influence the amount of Cu in the body, having an experimental and control diet which solely differed in Cu was key in order to prevent any unforeseen biological interactions.

The groups used in the study are shown in Table 1.

Table 1: *Experimental groups*

Copper Deficient (Prenatal)	**Copper Deficient (Postnatal)**	**Copper Control (Postnatal)**
Lab water + 4ppm Cu	Lab water + 4ppm Cu	Lab water + 16ppm Cu
n = 15	n = 15	n = 15

2.3 METHODS: BEHAVIORAL ASSESSMENTS

General:

Rats were taken from home cages and placed into individual cages on testing days. The cages and cart were labeled with the animals' ID in order to avoid any unforeseen errors. The rats had 10 minutes to habituate in a separate room other than the Fear Conditioning room before testing began. During the 4 days of experimental testing, animals were only removed from their cages when they were being run through the apparatuses. After the day's testing, the animals were placed back into their home cages. Bedding was changed in transport cages daily while between days 1 and 2 of Fear Conditioning/Fear Extinction new cages were used.

Fear Conditioning, Fear Extinction & Extinction Recall

Behavioral testing began at 4 months. Conditioning occurred in two identical clear Plexiglas (26cm long x 26cm wide x 18cm high) fear conditioning chambers inside sound attenuating boxes (Coulbourn Instruments). FreezeScan software (Clever Sys, Inc.) monitored freezing behavior and administered the learning paradigm. The animals underwent delay fear conditioning which involved them receiving the unconditioned stimulus (US), a foot shock (2 seconds, 0.5 mA), at the end of the conditioned stimulus (CS) tone (85 dB, 2kHz, 20 seconds). The animals acclimated to the test chamber for 180s, during which no stimulus was presented. After the habituation period, the animals received a 20-second CS that coterminated with a 2s US. Animals received 2 additional tone/shock parings at 240s and 300s before being returned to the home cage (a total of 3 trials were done on conditioning day per animal). In between trials, the fear conditioning boxes were cleaned with acetic acid (diluted vinegar solution). Twenty-four hours after the animals experienced initial fear conditioning, the animals then underwent fear extinction. The fear conditioning chambers were changed by adding black strips and an odorant of diluted vanilla extract to disguise the boxes from the initial training day. Other changes were made such as altering the lighting in the room, and adding black plexiglas to the chamber floor covered with bedding. Animals habituated to the chambers for the first 180 seconds with no stimuli. At the end of the acclimation period, eighteen 20-second tones were administered without a shock, one minute apart (18 trials per animal). In between trials on fear extinction days, 70% alcohol was used to clean the fear boxes.

Forty-eight hours after initial training (24 hours after extinction trials) the rats were placed back into the altered chambers in order to test extinction recall. Eighteen 20-second tones were presented, one minute apart (18 trials per animal). Another day of fear extinction recall was also performed (48 hours after initial extinction trial). The behavior being analyzed during extinction and extinction recall is the animals' percent freezing to the played tone; recorded through use of the FreezeScan software (Clever Sys, Inc.)

Accelerating Rotarod

After fear conditioning and extinction, motor function was tested through use of the accelerating rotarod procedure. Based on previous research, abnormal motor function has been shown to persist following Cu repletion after giving a perinatally Cu deficient diet to Holtzman rats (Penland & Prohaska, 2004). Also, research on gestational copper deficiency has shown that the developing cerebellum may be at risk with motor functioning, balance and coordination being affected (Georgieff, 2007). Given evidence that the cerebellum and motor coordination are affected by a deficiency in copper, the accelerating rotarod test was used to assess motor functioning. In most studies, the level of copper administered is very low; however, the diet administered to the rats in this protocol contained a level of Cu just above the amount needed for healthy gestation. To ensure this, continual monitoring of health and weight was done. Since copper plays a role in cerebellar development (Penland & Prohaska, 2004), it is worthwhile to investigate the effects of varying levels of copper on motor functioning. Each animal underwent three trials a day on the task across a span of three days, resulting in a total of nine trials per animal. The trial began when the rat was placed on the rod with its head

facing the back of the apparatus and ended when the rat fell, and by weight displacement, disconnected the underlying panel from the magnet which acts as a circuit. Falling and displacing this panel allows the machine to record for how long the rat was on the rod and at what speed the rat fell. The rats underwent three placements on the rod, per day, to note any changes based on learning the task; when the rat fell, it was placed back into the cage for an intertrial interval of ten minutes before being placed on the rod again. Data that was recorded included time (s) on the rod (latency to fall, 300 seconds maximum) and the speed of rotation attained (measured in rpm) which increased from 4 to 40 rpm (1 rpm increase every 8 seconds). It is expected that rats administered the copper depleted diet will display a shorter latency to fall in comparison to those rats administered the copper control diet.

2.4 METHODS: STATISTICS

The hypotheses under question were analyzed using repeated measures analyses of variance (ANOVA) followed by post hoc tests, when necessary, to analyze differences between and within treatment groups. Repeated measures ANOVAs were run for fear extinction and fear extinction recall data to identify any significant differences in percent freezing between the prenatal Cu deficient, postnatal Cu deficient, and postnatal Cu control groups across tones and extinction trials. Accelerating rotarod data was also analyzed using repeated measures ANOVA to determine if there were statistical differences among rats for average time spent on the rod (latency to fall) for all three days of testing between the three groups.

2.5 METHODS: EXPERIMENT COMPLETION & BRAIN COLLECTION

After completion of behavioral tasks, the animals were sacrificed using carbon dioxide (CO_2) in order to produce gradual asphyxiation. Animals' tails and paws were pinched in order to test for loss of consciousness and upon confirmation, were decapitated. Upon decapitation, the brains were extracted and placed on dry ice. Once frozen, the brain was placed in a clearly labeled brain bag, and placed in a -80ºC freezer for future data analysis.

3.1 RESULTS: FEAR CONDITIONING

Experiment 1—Fear Conditioning & Extinction

A 3 (diet) x 3 (minute) repeated measures ANOVA was conducted on freezing rates at 3 time points prior to tone onset (preacquisition) on the training day to note levels of locomotion in a novel environment as well as in response to the tones used in the 3 tone-shock pairings involved in conditioning. The 3 x 3 ANOVA was done in order to see if, when introduced to a new, novel environment, there were any differences in freezing between the dietary groups. A 3 (diet) x 18 (tones) repeated measures ANOVA was conducted on freezing rates to compare between groups on fear extinction, fear extinction recall, and a second day of fear extinction recall.

Freezing Prior to Tone Onset

A 3 (diet) x 3 (minute before tone) repeated measures ANOVA was conducted on freezing rates across the first three minutes in the fear chamber, with the Greenhouse-Geisser correction, revealed a significant main effect of time, $F(1.485,62.379) = 3.879$, $p < .05$. There were no differences between the diet conditions during the first 3 minutes prior to tone onset (see Figure 1).

Fear Conditioning

A 3 (diet) x 3 (tones) repeated measures ANOVA was conducted on freezing rates across three tone-shock pairings administered to the animal on conditioning day, with the Greenhouse-Geisser correction, revealed a significant main effect across tones $F(1.620, 68.045) = 63.059$, $p < .05$. There were no differences between the dietary conditions $F(2,42) = .580$, $p > .05$, which indicated that all of the animals were successfully conditioned.

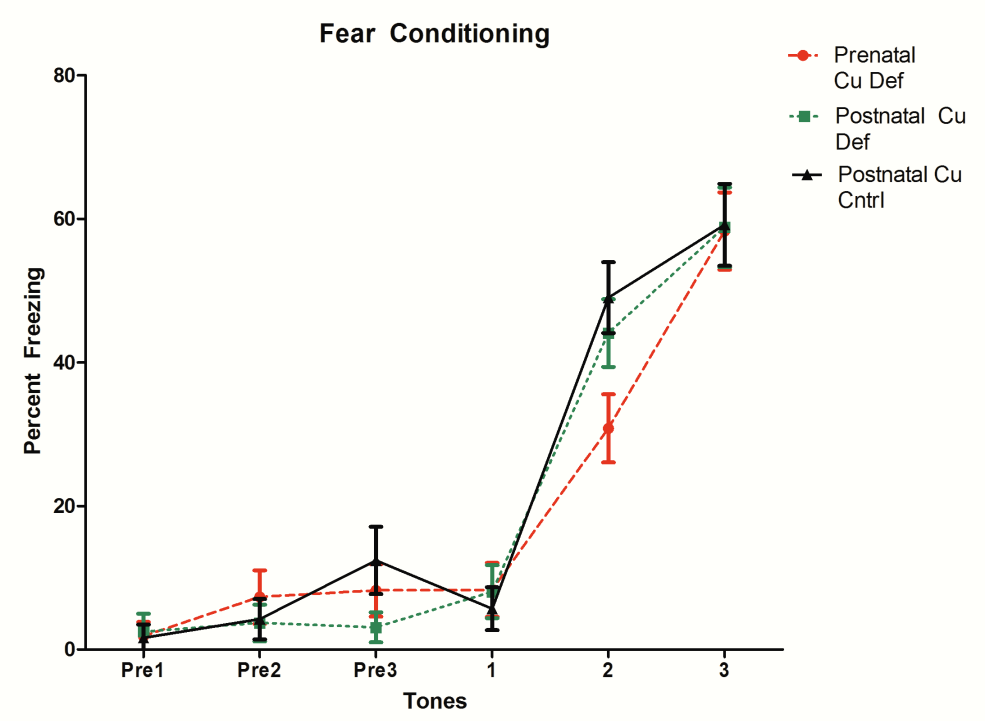

Figure 1: Fear Conditioning training day. Freezing significantly increased across tones similarly for the dietary groups. The animals were successfully conditioned. Error bars denote S.E.M.

3.2 RESULTS: FEAR EXTINCTION

Fear Extinction

A 3 (diet) x 18 (tones) repeated measures ANOVA was conducted on freezing rates to compare between diet conditions in fear extinction (24 hours after conditioning), with the Greenhouse-Geisser correction, revealed a significant main effect of time, $F(4.843, 203.395) = 9.491$, $p < .05$. There were no main effects found between diet groups $F(2,42) = .545$, $p > .05$. The following graph illustrates the 3 (diet) x 18 (tones) fear extinction results with an additional pre tone added

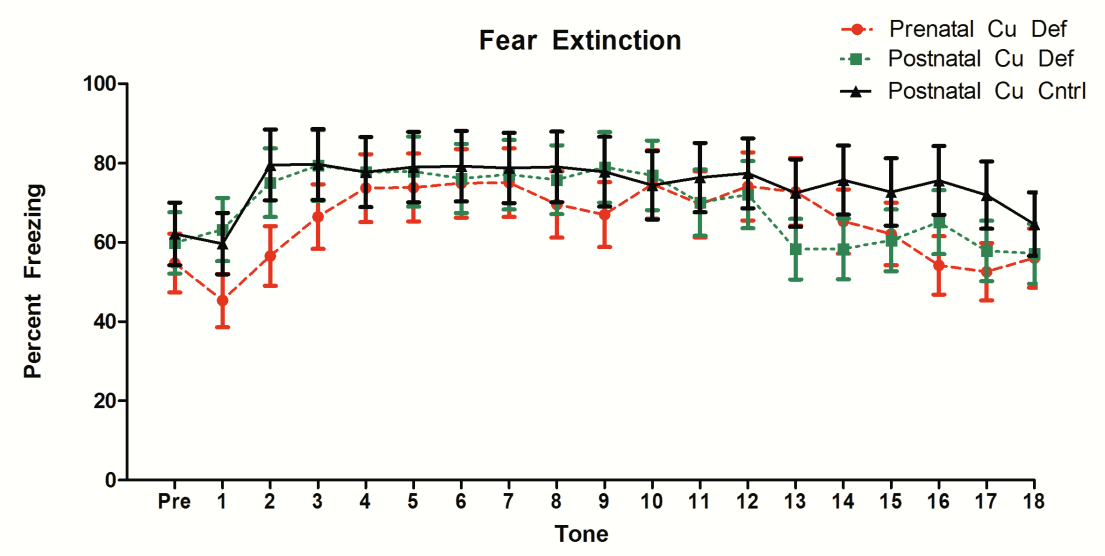

Figure 2: Fear Extinction. Freezing rates gradually declined with no difference detected between dietary groups; high freezing levels remained even after 18 tones. Error bars denote S.E.M.

3.3 RESULTS: FEAR EXTINCTION RECALL

Fear Extinction Recall

A 3 (diet) x 18 (tones) repeated measures ANOVA was conducted on freezing rates to compare between diet conditions in fear extinction recall (48 hours after conditioning). With the Greenhouse-Geisser correction, a significant main effect of time was seen $F(7.696, 323.253) = 19.392$, $p < .05$. No main effects were found for diet $F(2,42) = .582$, $p > .05$, and through post-hoc analysis, there were no significant differences between any of the diet conditions. The following graph illustrates the 3 (diet) x 18 (tones) fear extinction recall results with an additional pre tone added.

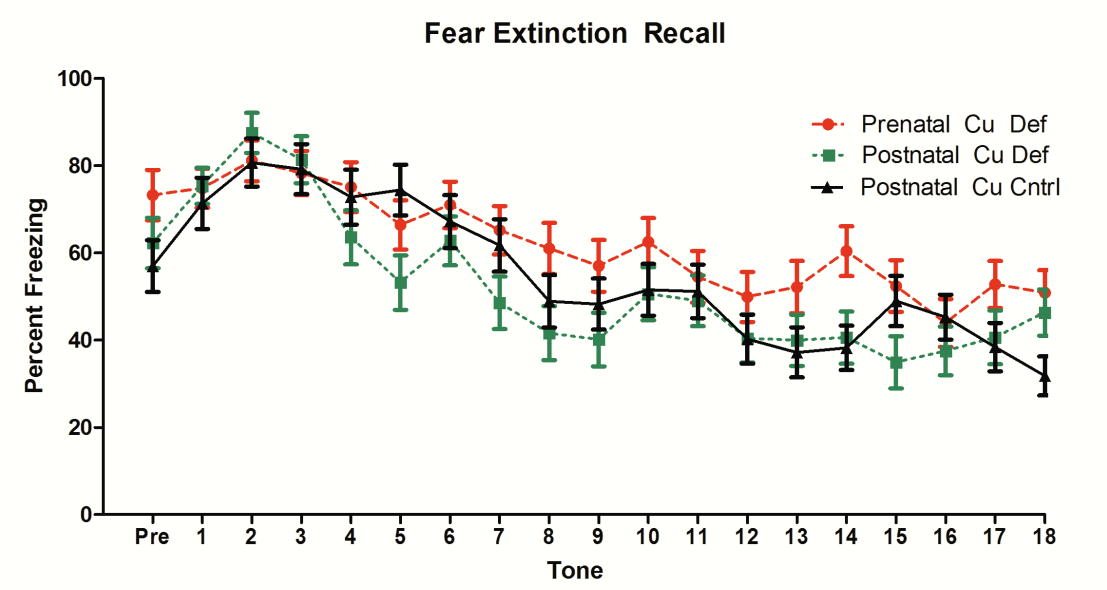

Figure 3: Fear Extinction Recall. Although a decrease in freezing is present, no significant differences was found between dietary groups. Error bars denote S.E.M.

3.4 RESULTS: FEAR EXTINCTION RECALL DAY 2

Fear Extinction Recall Day 2

A 3 (diet) x 18 (tones) repeated measures ANOVA was conducted on freezing rates to compare between diet conditions in the second day of fear extinction recall (done 72 hours after conditioning). With the Greenhouse-Geisser correction, a significant main effect was found for time, $F(6.920, 290.638) = 9.766$, $p < .05$. No main effect was found for diet $F(2,42) = 2.511$, $p > .05$, ($p = .093$). However, a 3 (diet) x 6 (tones 5-10) repeated measures ANOVA was conducted on freezing rates to see if there were any significant differences between the diet groups since the graph showed a limited time period with large differences through tones 5 and 10 between diet groups; a significant effect was found for diet, $F(2, 42) = 4.928$, $p < .05$. Tukey's post-hoc analysis revealed that there was a significant difference between the prenatal Cu deficient and both the postnatal Cu deficient ($p < .05$) and the postnatal Cu control ($p < .05$) with the prenatal Cu deficient group exhibiting a larger percent freezing. The following graph illustrates the 3 (diet) x 18 (tones) fear extinction recall results with an additional pre tone added

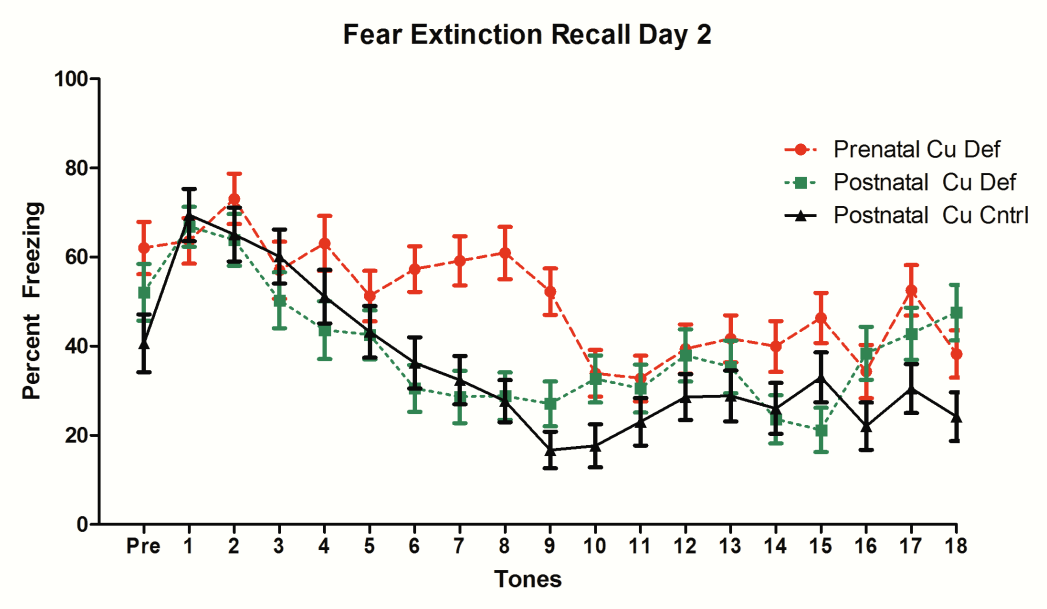

Figure 4: Fear Extinction Recall Day 2. Percent freezing levels declined across the 18 tones but no differences were observed overall between dietary groups. Between tones 5 and 10, there was a significant difference between the prenatal Cu deficient group and the postnatal Cu deficient and control groups. Error bars denote S.E.M.

3.5 RESULTS: ACCELERATING ROTAROD "TRAINING" DAY

Experiment 2—Accelerating Rotarod

A 3 (diet) x 3 (trials) repeated measures ANOVA was conducted on the 3 trials of the accelerating rotarod procedure for each day. After the trials were analyzed for each day, repeated measures ANOVAs were run across average times for each day and for all 9 trials across the three testing days.

Training Day (Day 1)

A 3 (diet) x 3 (trials) repeated measures ANOVA was conducted on latency to fall for the 3 trials of the accelerating rotarod procedure on day 1. There was a main effect of trials, with the Greenhouse-Geisser correction, $F(1.645, 67.427) = 7.547$, $p < .05$. There was no significant difference in performance between dietary groups found $F(2,41) = 1.994$, $p > .05$).

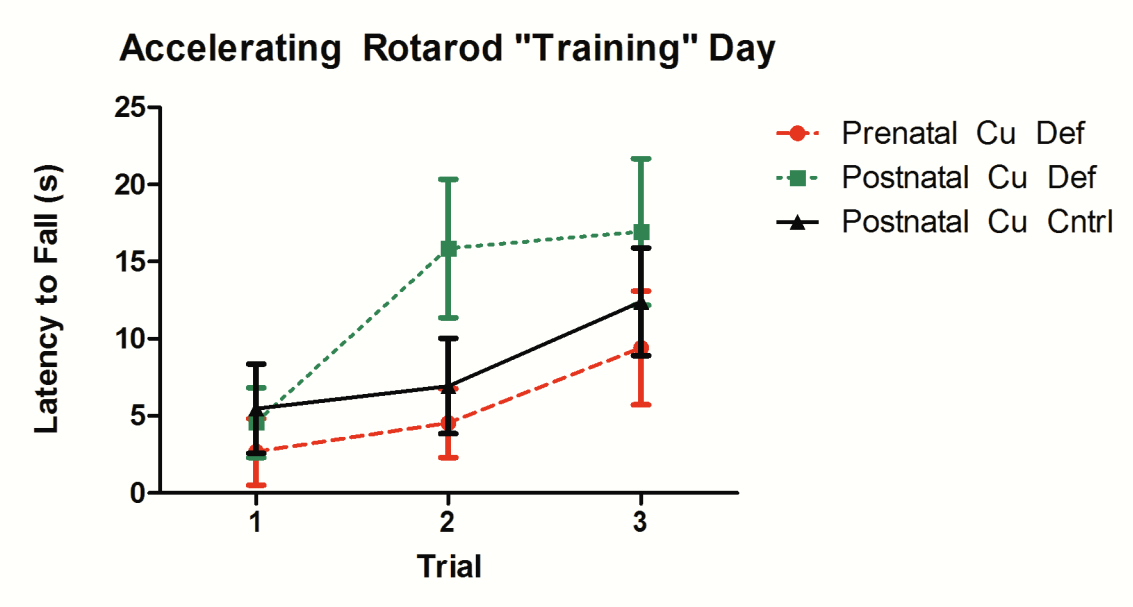

Figure 5: Accelerating Rotarod "Training" Day. There was a main effect of trials but no significant difference between dietary groups. Error bars denote S.E.M.

3.6 RESULTS: ACCELERATING ROTAROD DAY 2

Day 2

A 3 (diet) x 3 (trials) repeated measures ANOVA was conducted on latency to fall for the 3 trials of the accelerating rotarod procedure on day 2. There was a main effect of trials, $F(2, 82) = 11.383$, $p < .05$. A significant main effect was found between dietary groups $F(2,41) = 3.579$, $p < .05$. Tukey's post-hoc analyses revealed that there was a significant difference between the postnatal Cu deficient group and the prenatal Cu deficient group ($p < .05$) with the prenatal Cu deficient rats exhibiting lower latencies to fall. Means and standard errors of the three groups are listed in table 2.

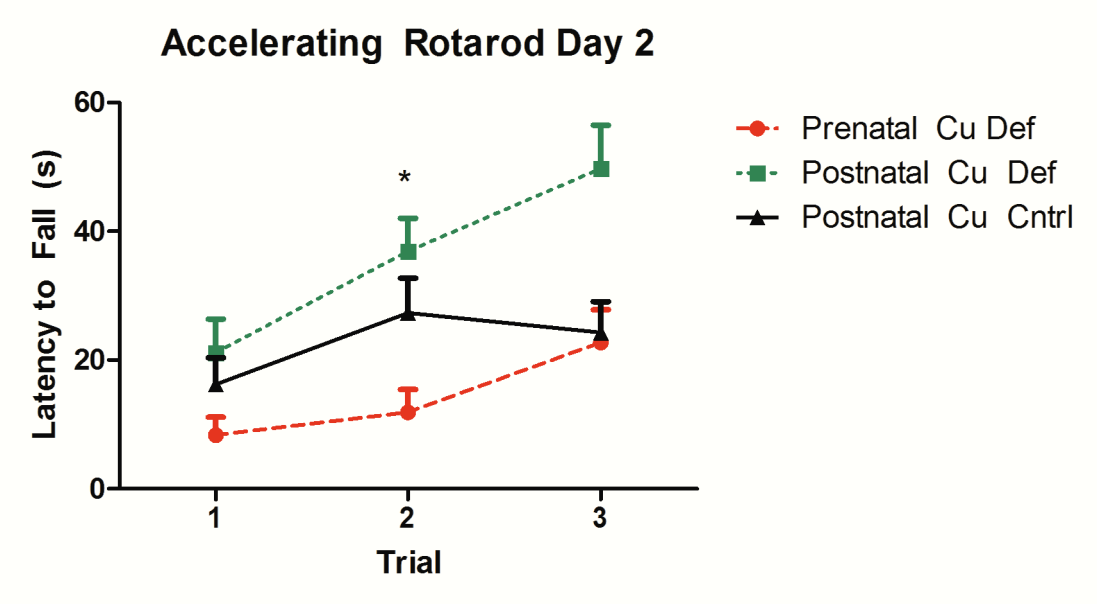

Figure 6: Accelerating Rotarod Day 2. The postnatal Cu deficient rats exhibited higher latencies to fall and differed significantly from the prenatal Cu deficient group. * $p < .05$ vs, prenatal Cu deficient and postnatal Cu deficient. Error bars denote S.E.M.

Table 2 Accelerating Rotarod Day 2 Average times and standard errors

Accelerating Rotarod Day 2	Trial 1	Trial 2	Trial 3
Prenatal Cu Deficient Mean (seconds) ± SEM	8.33 ± 2.78	11.87 ± 3.54	22.73 ± 5.10
Postnatal Cu Deficient Mean (seconds) ± SEM	21.14 ± 5.22	36.86 ± 5.16	49.71 ± 6.76
Postnatal Cu Control Mean (seconds) ± SEM	16.2 ± 4.17	27.33 ± 5.40	24.27 ± 4.79

3.7 RESULTS: ACCELERATING ROTAROD DAY 3

Day 3

A 3 (diet) x 3 (trials) repeated measures ANOVA was conducted on latency to fall for the 3 trials of the accelerating rotarod procedure on day 3. Performance increased across trials for all 3 groups except for the Postnatal Cu deficient group who decreased slightly on trial 3. There was a main effect of trials, $F(2, 82) = 10.815$, $p < .05$. There was no main significant difference found between dietary groups $F(2,41) = 2.763$, $p > .05$ but the postnatal Cu deficient group again had the largest latencies to fall. Repeated measures ANOVA revealed a trending difference between dietary groups ($p = .075$) but yielded no significant difference at the α level of .05.

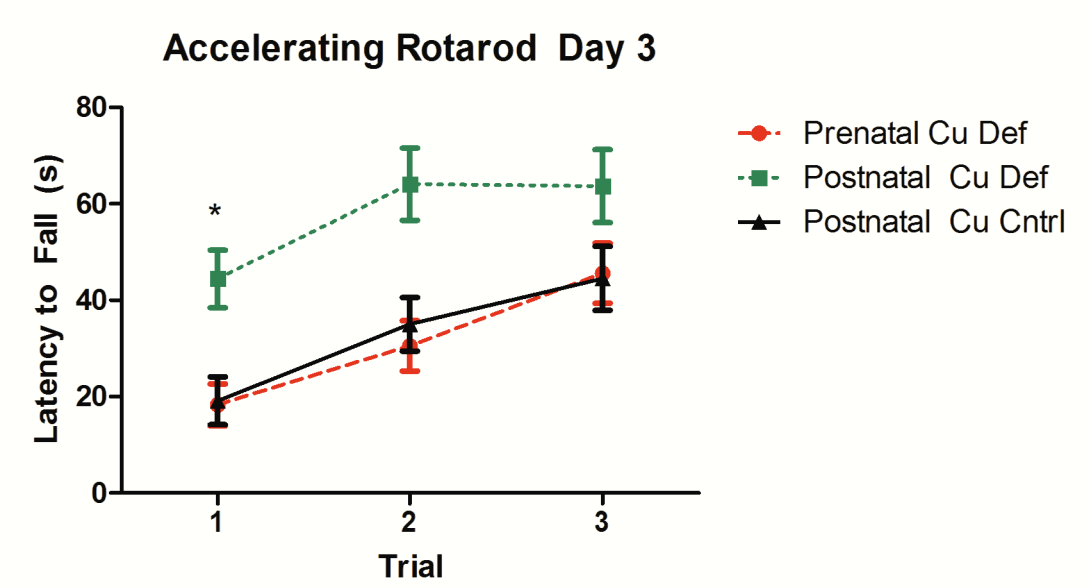

Figure 7: Accelerating Rotarod Day 3. No significant differences were found between the dietary groups; however, a trending difference was found between the groups ($p = .075$). * $p < .05$ vs, prenatal Cu deficient and postnatal Cu deficient; vs, postnatal Cu deficient and postnatal Cu control. Error bars denote S.E.M.

3.8 RESULTS: ACCELERATING ROTAROD AVERAGE TIMES BY DAY

Average Accelerating Rotarod Performance across Days

A 3 (diet) x (3 day) repeated measures ANOVA was conducted on the three days of the accelerating rotarod procedure where for each day, the three trials were averaged together. There was a main effect of day, with the Greenhouse-Geisser correction, $F(1.392, 57.06) = 38.18$, $p < .05$. Across the three days of testing, performance increased in each group. A significant main effect was found between diet groups, $F(2,41) = 3.594$, $p < .05$). Tukey's post-hoc analyses revealed that there was a significant difference between the prenatal Cu deficient group and the postnatal Cu deficient group ($p < .05$) with the prenatal Cu deficient animals exhibiting a lower latency to fall than the postnatal Cu deficient animals. The means and standard error for the 3 groups are reported in table 3.

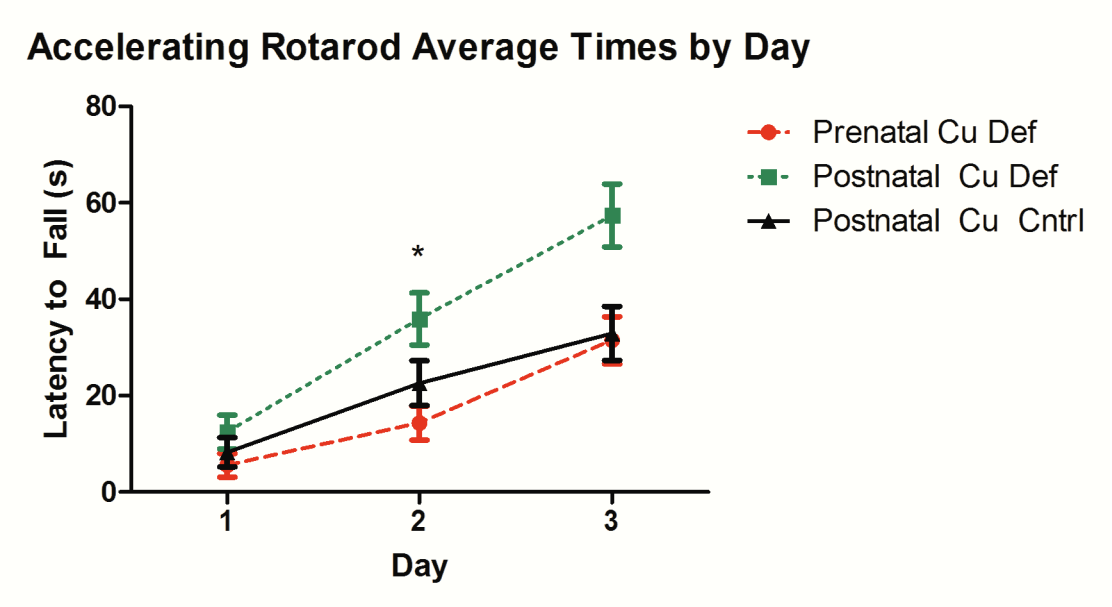

Figure 8: Accelerating Rotarod Average Times by Day. The prenatal Cu deficient animals exhibited a lower latency to fall than postnatal Cu deficient animals. * $p = <.05$ vs, prenatal Cu deficient and postnatal Cu deficient. Error bars denote S.E.M.

Table 3 Accelerating Rotarod Averages Times and standard errors by Day

Accelerating Rotarod Average Time by Day	Day 1	Day 2	Day 3
Prenatal Cu Deficient Mean (seconds) ± SEM	5.53 ± 2.47	14.31 ± 3.54	31.47 ± 4.88
Postnatal Cu Deficient Mean (seconds) ± SEM	12.45 ± 3.48	35.90 ± 5.38	57.38 ± 6.52
Postnatal Cu Control Mean (seconds) ± SEM	8.27 ± 3.02	22.60 ± 4.64	32.89 ± 5.60

3.9 RESULTS: ACCLERATING ROTAROD PERFORMANCE ACROSS TOTAL TRIALS

Performance across all 9 Trials

A 3 (diet) x 9 (trials) repeated measures ANOVA was conducted on all of the trials across the three days of the accelerating rotarod procedure (9 trials: 3 per day). There was a main effect of trial, with the Greenhouse-Geisser correction, $F(3.427, 140.511) = 23.489$, $p < .05$. A significant main effect was found between diet groups, $F(2,41) = 3.594$, $p < .05$ and Tukey's post-hoc analyses revealed that there was a significant difference between the prenatal Cu deficient group and the postnatal Cu deficient group ($p < .05$) with the prenatal Cu deficient animals exhibiting lower latencies to fall than the postnatal Cu deficient ones. The postnatal Cu deficient group performed consistently better than the other groups. Means and standard error for the 3 groups are reported in table 4.

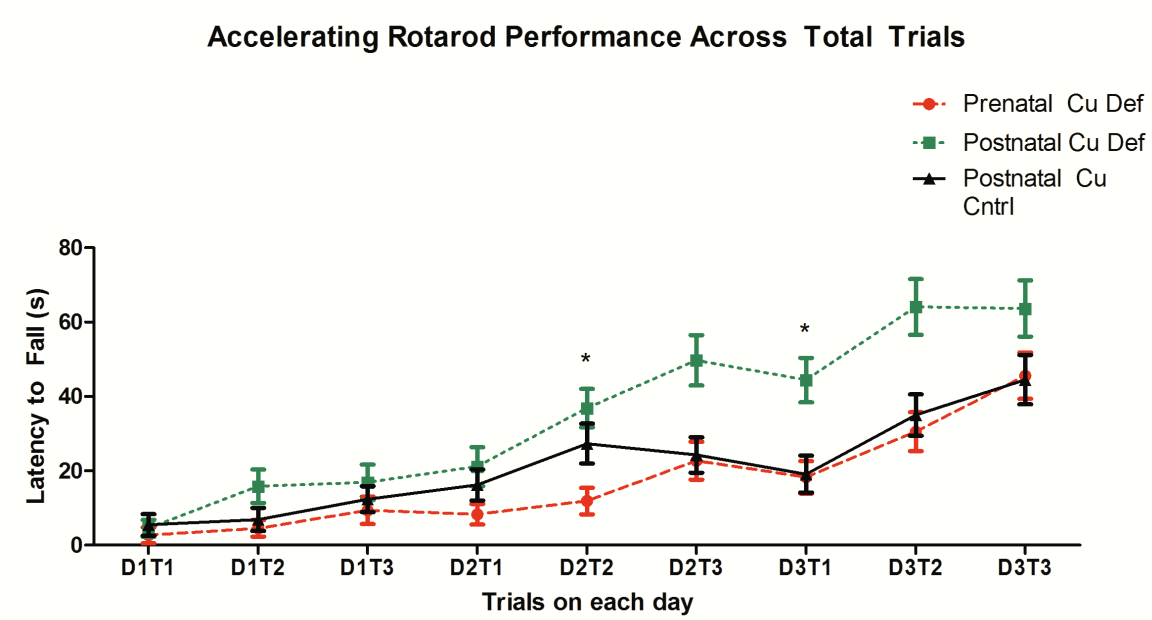

Figure 9: Accelerating Rotarod Performance Across Total Trials. The prenatal Cu deficient animals exhibited lower latencies to fall than the postnatal Cu deficient animals. The postnatal Cu deficient group consistently performed better than the other dietary conditions. * $p < .05$ vs, prenatal Cu deficient and postnatal Cu deficient; $p < .05$ vs, postnatal Cu deficient and postnatal Cu control (Day 3 Trial 1). Error bars denote S.E.M.

Table 4: Accelerating Rotarod Average times and standard errors across all trials

Accelerating Rotarod Performance Across All Trials	Day 1 Trial 1	Day 1 Trial 2	Day 1 Trial 3	Day 2 Trial 1 (4)	Day 2 Trial 2 (5)	Day 2 Trial 3 (6)	Day 3 Trial 1 (7)	Day 3 Trial 2 (8)	Day 3 Trial 3 (9)
Prenatal Cu Deficient Mean (seconds) ±SEM	2.67 ± 2.16	4.53 ± 2.23	9.40 ± 3.69	8.33 ± 2.78	11.87 ± 3.54	22.73 ± 5.10	18.27 ± 4.35	30.53 ± 5.21	45.60 ± 6.20
Postnatal Cu Deficient Mean (seconds) ± SEM	4.57 ± 2.26	15.86 ± 4.50	16.93 ± 4.75	21.14 ± 5.22	36.86 ± 5.16	49.71 ± 6.76	44.43 ± 5.97	64.07 ± 7.48	63.64 ± 7.57
Postnatal Cu Control Mean (seconds) ± SEM	5.47 ± 2.90	6.93 ± 3.09	12.4 ± 3.48	16.2 ± 4.17	27.33 ± 5.40	24.27 ± 4.79	8.27 ± 3.02	22.60 ± 4.64	32.89 ± 5.60

3.10 RESULTS: WEIGHTS

Weights

Weights were obtained at 2 times in the study: at weaning (PND 21) and at 4 months. A 3 (diet) x 2 (weight time) repeated measures ANOVA was conducted between the three dietary groups and there was a significant main effect of diet, $F(2,41) = 5.909$, $p < .01$. Tukey's post-hoc analyses revealed that there was a significant difference between the postnatal Cu deficient group and the postnatal Cu control group ($p < .01$). A main effect of time was found, with the Greenhouse-Geisser correction, $F(1.000, 41.000) = 15563.34$, $p < .001$. Means and standard error for the 3 groups across both weight times are reported in table 5.

Weaning

Weights were obtained at PND 21 when the pups were weaned from their mother. A one-way ANOVA was conducted and there was a significant main effect of diet, $F(2,41) = 21.490$, $p < .001$ and Tukey's post-hoc analyses revealed that there were significant differences between the dietary groups. The prenatal Cu deficient animals weighed more than the postnatal Cu deficient animals ($p < .001$), and more than the postnatal Cu controls ($p < .001$). Also, the postnatal Cu deficients weighed less than the postnatal Cu controls ($p < .05$, $p = .010$).

4 months

Weights were taken at 4 months of age. A one-way ANOVA was conducted and there was a significant main effect of diet, $F(2,41) = 21.490$, $p < .01$ and Tukey's post-hoc analyses revealed that there was a significant differences between the dietary groups. The postnatal Cu controls weighed more than the prenatal Cu deficients ($p < .01$) and more than the postnatal deficient ($p < .05$).

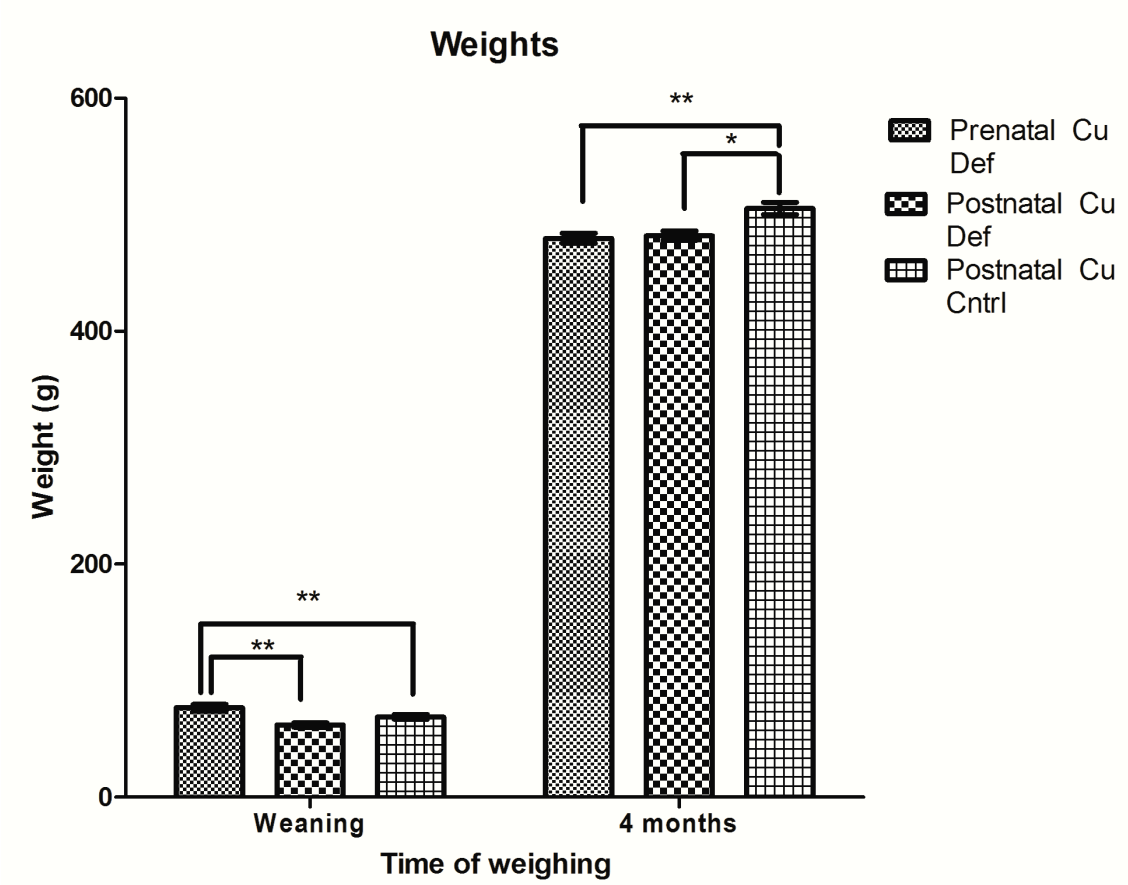

Figure 10: Weights. At weaning, the prenatal Cu deficient group weighed more than the postnatal Cu deficient animals and the postnatal Cu controls. ** $p < .01$ vs, prenatal Cu deficient and postnatal Cu deficient; vs, prenatal Cu deficient and postnatal Cu control; vs, postnatal Cu deficient and postnatal Cu control. Also, the postnatal Cu deficient animals weighed less than the postnatal Cu controls. At 4 months, the postnatal Cu controls weighed more than the pre- and postnatal Cu deficient animals. * $p < .05$ vs, postnatal Cu deficient and postnatal Cu control; ** $p < .01$ vs, prenatal Cu deficient and postnatal Cu control. Error bars denote S.E.M.

Table 5: Weights at Weaning (PND 21) and at 4 Months

Weights	Weaning	4 months
Prenatal Cu Deficient Mean (grams) ± SEM	76.79 ± 2.95	479.73 ± 4.45
Postnatal Cu Deficient Mean (grams) ± SEM	62.01 ± 1.81	481.98 ± 4.14
Postnatal Cu Control Mean (grams) ± SEM	68.94 ± 2.18	505.31 ± 5.30

4. DISCUSSION

The aim of the study was to examine the effect of moderate Cu deficiency on fear learning, extinction, and motor coordination in the male Sprague-Dawley rat. Results from this study indicate that a mild Cu deficient diet over a period of four months has an impact on certain behaviors. Another important point illustrated by the current study is that the formulation of the control diet is crucial and if the control diet is not properly formulated, the results of the research in question may be compromised. It was initially hypothesized that animals raised prenatally on the Cu deficient diet (4ppm) would perform worse than those raised postnatally on a Cu deficient diet and Cu control diet (16ppm). The results were surprising due to the fact that in fear extinction, no between-group differences were found; however, statistically significant between-group differences in the rotarod procedure were found. Weight was also taken to analyze whether there were significant differences between the dietary groups at weaning (PND 21) and at four months. However, a limitation remains considering the control diet's formulation.

Results from the prenatal Cu deficient group given the diet continually for four months did not support the hypothesis that they would perform significantly worse than the other groups for extinction tasks. This group did not exhibit a significant difference in extinguished fear compared to the postnatal Cu deficient or postnatal Cu control groups.

Although no significant difference was observed in the behavioral tasks of fear extinction or extinction recall, the issue of the control diet should be addressed. Based on previous research using a standard lab diet "7012" (Railey et al., 2010), the current research predicted that the control group should have performed better throughout the extinction paradigm due to the relatively higher amount of Cu present in the diet. However, throughout the course of the extinction and extinction recall procedures, the postnatal Cu control group performed poorly and seemed to be a poor choice for a control diet. Through analysis of previous lab data utilizing the "7012" diet in a similar fear extinction and extinction recall paradigm, it was seen that there were no significant differences between the diets used in this study and the "7012" diet used previously; however, the "7012" diet did result in lower levels of fear extinction and fear extinction recall compared to the current dietary groups as can be seen in figure 11. Results from the fear extinction and recall days showed rats exhibiting high levels of freezing even before the first tone sounded (indicated by high percent freezing value at the pretone mark). This high level of freezing may indicate contextual freezing. Although a large amount of cues were changed transitioning from conditioning to extinction days (carrying the rats in different containers, cleaning the chambers with a different solution, adding stripes, bedding, altering the lighting, and adding an additional odorant to the chambers), future experiments may need to make additional changes to further mask the conditioning context in order to avoid such high pretone freezing.

Besides contextual freezing, another possibility for such high pretone freezing levels may be due to dietary makeup. The TD.130230 and TD.130231 diets used in this

study differed from the “7012” diet through nutritional factors such as the addition of soybean oil, lower fiber levels, and a different level of Zn. These dietary features might explain the differences in fear extinction noted between the “7012” and the current diets. Based on the fact that pretone levels were so much higher in the Cu deficient and Cu control diets compared to the “7012”, the diets could have a more significant role in fear extinction than previously thought.

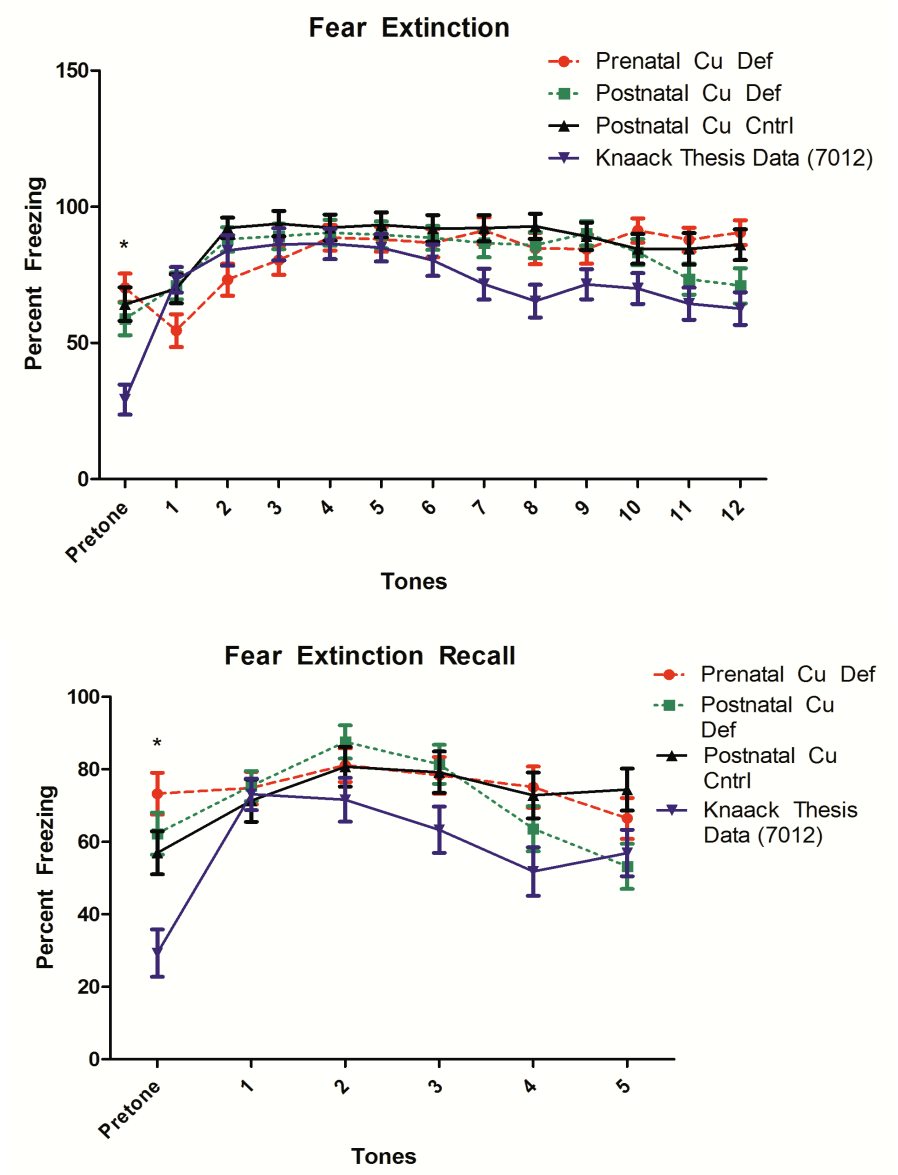

Figure 11: Differences in current research diets (TD.130230 & TD.130231 and "7012"). Compared to current results, control animals from a previous research experiment on "7012" exhibited lower latencies to freeze. * $p < .05$, vs prenatal Cu deficient and "7012". Error bars denote S.E.M.

One possible explanation for the lack of significant results could be due to the Zn/Cu ratios between the two control diets.

The ratio of Zn to Cu may be clinically more important than the concentration of either of the metals alone (Osredkar & Sustar, 2011). A diet that solely differed in Cu levels was suggested as a necessary control diet. The choice of control diet was to use one that either has served as a control diet in the past for previous experiments (Railey et al, 2010) ("7012") or one which has a similar Zn/Cu ratio. In order to make the results as comparable as possible, the control diet with a similar Zn/Cu ratio as "7012" was chosen. Future studies should take into account the effects that the control diet can have in experimental procedures.

Another dietary concern that should be addressed is the inclusion of soybean oil in the makeup of the diets used in the current study which was not present in the "7012" diet. Soy could have led to the differences observed between past results using the "7012" diet and the new control since soy is a source of phytoestrogens in rodent diets and was not included in the "7012" makeup. These phytoestrogens can affect research dealing with behavior, learning, and reproduction to name a few and it has been seen in a study using Long-Evans rats that anxiety decreased with exposure to phytoestrogens (Lephart, E., Setchell, K., Handa, R. & Lund, T., 2004; Mickelson, B., 2009).

The accelerating rotarod task was chosen for its sensitivity in detecting cerebellar dysfunction and abnormal motor coordination due to perinatal Cu deficiency and its effects on the cerebellum (Penland & Prohaska, 2004; Shiotsuki, et al., 2010). In this task, the postnatal Cu deficient animals consistently demonstrated higher latencies to fall

than the prenatal Cu deficient and postnatal Cu control animals. Again, the accelerating rotarod task hypothesis was that the postnatal Cu control (16ppm) diet group would perform better than the deficient groups; however, the control group performed intermediately compared to the postnatal Cu deficient and prenatal Cu deficient groups in each day of training and across all trials completed (nine). Based on the results obtained from the accelerating rotarod task, the Cu control diet may not actually be serving as an appropriate control. The Cu control diet group did not perform the best, as was hypothesized, and a potential reason for this could be that 16ppm Cu could still be relatively deficient. Future research should run another standard control diet, such as the "7012" diet, on the rotarod in order to compare how the 16ppm Cu control diet compares with one that has a higher Cu content (23ppm).

All three groups performed poorly based on their short latencies to fall even after several training sessions. Control rodents in the study by Penland and Prohaska (2004) exhibited high mean times on the rotarod even on day 1 whereas rats in the current study started to reach similar levels by the last trial on day 3. Suggested improvements to the current set-up include a day of exposure with no rod rotation prior to test day. Doing so may allow for the rats to become familiar with the apparatus before being placed on it for testing.

Measuring weight proved to be statistically significant, both at weaning (PND 21) and at four months of age. Over the course of the study, weights increased in each group. The prenatal Cu deficient rats weighed the most at weaning and the least at 4 months; at weaning, the postnatal Cu deficient animals weighed the least and the postnatal Cu

control ended up weighing the most at 4 months. At least for the 4 month weight data, although there were significant differences between the diets, the percent change between diets is probably not meaningful. At weaning the heavier weights of the prenatal deficient animals was surprising. One idea to consider is possible differences between pups bred in house (at George Mason) for the experiment (the postnatal Cu deficient and control rats) and those from timed- pregnant mothers (the prenatal Cu deficient rats). Those from timed-pregnant mothers could differ in some ways from the rats born from paired-in-lab mothers; further research needs to be done to see if any significant changes exist between these conditions. The significance achieved when analyzing weights must be considered carefully due to the make-up of the diet itself. The diets used, TD.130230 and TD.130231, were developed to support gestation and lactation and unbeknownst to the researchers, continuing such a diet for four months led to heavy rats, which may have actually led to confounds in other behavioral results (particularly the fear conditioning paradigm whereby more weight could have led to a heavier placement on the bars giving more of a shock to the heavier rats than to lighter rats). Weight could have also affected performance on the accelerated rotarod task. However, this is unlikely based on data from Penland and Prohaska (2004) who evaluated 3 month and 6 month old control and repleted rats that weighed an average of over 600 grams in each condition, similar to the rats in this study.

Lastly, an important note that warrants caution is whether or not the ratio of Zn to Cu may have been playing a role. As previously mentioned, the ratio of Zn to Cu may be more important than the concentration of either metal alone (Osredkar & Sustar, 2011)

and this ratio may actually be playing more of a role in behavior than previously thought. Based on previous research demonstrating learned fear impairment caused by excess Zn levels, Cu supplements remediated the impairment and brought freezing levels closer to control animals in fear extinction (Railey et al, 2010). It was hypothesized that a diet deficient in Cu would lead to poor behavior in comparison with a control group; however, in this study, the control diet was not as effective as intended and posed a serious issue. This problem demands attention for future researchers looking to create appropriate lab control diets. Nutrition is a vital aspect of an experiment since nutritional insult can outweigh the brain's plasticity, in turn affecting myelination and the development of critical brain structures (Georgieff, 2007). The standard lab diet used in previous experiments was considered a poor control by the Harlan nutritionist due to a variety of different ingredients and other dietary aspects such as fiber and additional Zn. Based on the results of the current study, a future study ought to compare the effects of the control diet used in most labs (typical standard lab diets, e.g. "7012") to those diets specifically formulated to serve as control diets.

The current results do show that nutrition can play a role in behavior but show that caution must be exercised when choosing an appropriate diet to feed animals used in research, especially those experiments that intend to manipulate dietary conditions. Researchers should scrutinize formulated control diets in order to provide an appropriate control that does not compromise manipulations or contradict expectations. In addition, the current experiment studied manipulated Cu levels that represent mild Cu deficiencies. Since the rats in this experiment were to be raised on their respective diets for four

months, a diet sufficient for long-term health was needed as opposed to diets that are administered for less than two months and incorporate severely deficient Cu levels in order to study enzymatic levels and developmental consequences of Cu deficiencies (Keen et al, 2003; Prohaska & Brokate, 2001). Based on the current results of a mildly Cu deficient diet, a greater deficiency in Cu may produce more severe behavioral effects; however, how long rats will survive given such a severe deficiency is not known.

5. CONCLUSION

Dietary manipulation had no effect on the ability to extinguish fear; however, significant differences were seen between dietary groups in the accelerating rotarod task with the prenatal Cu deficient group exhibiting lower latencies to fall compared to the other groups. This may indicate possible cerebellar abnormalities. Diet is a very important variable to consider when implementing a study and a proper control diet must be used in order to be able to obtain meaningful results.

REFERENCES

Askwith, C. & Kaplan, J. (1998). Iron and copper transport in yeast and its relevance to human disease. *TIBS, 23,* 135-138.

Barone, A., Ebesh, O., Harper, R. G., & Wapnir, R. A. (1998). Placental copper transport in rats: Effects of elevated dietary zinc on fetal copper, iron and metallothionein. *The Journal of Nutrition, 128,* 1037-1041.

Bertinato, J. & L'Abbé, M. R. (2004). Maintaining copper homeostasis: regulation of copper-trafficking proteins in response to copper deficiency or overload. *Journal of Nutritional Biochemistry, 15,* 316-322.

Bousquet-Moore, D., Eipper, B., Prohaska, J., & Mains, R. (2009). Variations in dietary copper alter physiological and behavioral function [Abstract]. *FASEB J. 23,* 231.5

Burgos-Robles, A., Vidal-Gonzalex, I., Santini, E. & Quirk, G.J. (2007). Consolidation of fear extinction requires NMDA receptor-dependent bursting in the ventromedial prefrontal cortex [Abstract]. *Neuron, 53*(6), 871-880.

Collinson, N., Kuenzi, F.M., Jarolimek, W., Maubach, K.A., Cothliff, R., Sur, C., Smith, A., Out, F.M., Howell, O., Atack, J.R., McKernan, R.M., Seabrook, G.R., Dawson, G.R., Whiting, P.J. & Rosahl, T.W. (2002). Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. *The Journal of Neuroscience, 22*(13), 5572-5580.

Desai, V. & Kaler, S.G. (2008). Role of copper in human neurological disorders. *The American Journal of Clinical Nutrition, 88,* 855S-858S.

Gaier, E.D., Eipper, B.A. & Mains, R.E. (2012). Copper signaling in the mammalian nervous system: Synaptic effects. *Journal of Neuroscience Research.* Wiley Periodicals, Inc, 1-18.

Georgieff, M. K. (2007). Nutrition and the developing brain: nutrient priorities and measurement. *The American Journal of Clinical Nutrition, 85,* 614S-620S.

Groff, J. L., Gropper, S. S. & Hunt, S. M. (1995). Advanced Nutrition and Human Metabolism. West Publishing Company, New York.

Gybina, A.A. & Prohaska, J.R. (2003). Increased rat brain cytochrome c correlates with degree of perinatal copper deficiency rather than apoptosis. *The Journal of Nutrition, 133,* 3361-3368.

Harris, E. D. (2001). Copper homeostasis: The role of cellular transporters. *Nutr Rev, 59:* 281-285.

Johnson, W.T. (2005). Copper and brain function. In R.B. Kanarek (Ed.), *Nutritional Neuroscience* (pp. 289-305). Boca Raton, FL: CRC Press.

Keen, C. L., Hanna, L. A., Lanoue, L., Uriu-Adams, J. Y., Rucker, R. B. & Clegg, M. S. (2003). Developmental consequences of trace mineral deficiencies in rodents: Acute and long-term effects. *The Journal of Nutrition, 133,* 1477S-1480S.

Lephart, E.D., Setchell, K.D., Handa, R.J. & Lund, T.D. (2004). Behavioral effects of endrocrine-disrupting substances: Phytoestrogens. *ILAR J, 45,* 443-454.

Lönnerdal, B. (2000). Dietary factors influencing zinc absorption. *The Journal of Nutrition, 130,* 1378S-1383S.

Maret, W. & Sandstead, H. H. (2006). Zinc requirements and the risks and benefits of zinc supplementation. *J Trace Elem Med Biol 20*(1), 3-18.

Mickelson, B. (2009, December 01). Formula for Success. *ALN magazine.*

Mickelson, B. (2013, March 05). How do I choose a feed for my research animals? *ALN magazine.*

Osredkar, J. & Sustar, N. (2011). Copper and zinc, biological role and significance of copper/zinc imbalance. *J Clinic Toxicol S3:*001.

Penland, J.G. & Prohaska, J.R. (2004). Abnormal motor function persists following recovery from perinatal copper deficiency in rats. *The Journal of Nutrition, 134,* 1984-1988.

Prohaska, J.R. & Brokate, B. (2001). Dietary copper deficiency alters protein levels of rat dopamine β-monooxygenase and tyrosine monooxygenase. *Experimental Biology and Medicine, 226,* 199-207.

Prohaska, J.R. & Gybina, A.A. (2005). Rat brain iron concentration is lower following perinatal copper deficiency. *Journal of Neurochemistry, 93,* 698-705.

Prohaska, J.R. & Smith, T.L. (1982). Effect of dietary or genetic copper deficiency on brain catecholamines, trace metals and enzymes in mice and rats. *The Journal of Nutrition, 112,* 1706-1717.

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

Railey, A.M., Groeber, C.M. & Flinn, J.M. (2010). *J Alzheimers Disease, 24*(2), 375-381.

Sandstead, H. H., Penland, J. G. & Alcock, N. W. (1997). Zinc: Report of an international meeting. National Environmental Health Forum Monographs, 1-9.

Schauz, C. & Koch, M. (2000). Blockade of NMDA receptors in the amygdala prevents latent inhibition of fear conditioning. *Learn Mem, 7*(6), 393-399.

Shiotsuki, H., Yoshimi, K., Shimo, Y., Funayama, M., Takamatsu, Y., Ikeda, K., Takahashi, R., Kitazawa, S. & Hattori, N. (2010). A rotarod test for evaluation of motor skill learning. *J Neurosci Methods, 189*(2), 180-185. doi: 10.1016/jneurmeth.2010.03.026.

Vlachová, V., Zemková, H. & Vyklicky L Jr. (1996). Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons [Abstract]. *Eur. J. Neurosci. 8*(11), 2257-2264.

Weiser, T. & Wienrich, M. (1996). The effects of copper ions on glutamate receptors in cultured rat cortical neurons [Abstract]. *Brain Res, 742*, 211-218.

Zatta, P. & Frank, A. (2007). Copper deficiency and neurological disorders in man and animals. *Brain Research Reviews, 54,* 19-33.

BIOGRAPHY

Stephen Lippi graduated from Colonial Forge High School, Stafford, Virginia, in 2010. He received his Bachelor of Science from George Mason University in 2013. As part of the Accelerated Master's Program in CBN, he continued on to earn a Masters of Arts in Psychology with a concentration in Cognitive and Behavioral Neuroscience in 2014. He will be continuing at George Mason University to earn a Doctorate degree.