THE EFFECT OF DIETARY COPPER AND ZINC ON FEAR EXTINCTION AND MOTOR COORDINATION

by

Caroline Leigh Copeland Neely 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

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DEDICATION

I dedicate this thesis to my parents, James and Deborah Neely, and my sister, Elizabeth Neely, for their support, sarcasm, and love throughout my undergraduate and graduate careers.

ACKNOWLEDGEMENTS

I would like to thank my family, friends, undergraduate research assistants, and cognitive/behavioral neuroscience program colleagues for their support throughout this project. I give special acknowledgement to Samantha Wilkins and Stephen Lippi for their support with training, consultation, and project execution. Cyrus Foroughi, Jacob Quartuccio, Molly Martini, and Samuel Monfort also deserve recognition for their assistance with R software and mixed effects models. I would like to thank my committee members – Dr. Jane Flinn, Dr. Jennifer Brielmaier, and Dr. Patrick McKnight – for their advice, guidance, and patience throughout this project. I would also like to thank David Cerri for laboratory management and David Myers and the husbandry staff for animal care. This study was partially funded by the George Mason University Students as Scholars OSCAR program.

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LIST OF ABBREVIATIONS AND SYMBOLS

Copper	Cu
Copper control	CC
Embryonic/prenatal day #	E#
Fear conditioning	FC
Fear extinction	FE
Fear extinction recall	FER
Fear extinction recall 2	FER2
Newly-formulated 7012	7012 _N
Newly-formulated 7012, copper-deficient	012 _{N-CD}
Newly-formulated 7012, zinc-supplemented	012 _{N+Zn}
Number of animals in experimental group	n
Postnatal day #	P#
Standard 7012	7012s
Total animals in cohort	N
Zinc	Zn

ABSTRACT

THE EFFECT OF DIETARY COPPER AND ZINC ON FEAR EXTINCTION AND MOTOR COORDINATION

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Dietary manipulation incorporates not only changes in nutritional content (e.g. biometal concentration) but also alterations in base formulas. This thesis is comprised of two separate experiments that aim to elucidate the precarious process of diet selection and how dietary manipulation can impact animal behavior. The first portion of this thesis examined two "control" diets previously administered in our laboratory: a Harlan 7012 feed (7012_s) and a Harlan Copper Control (CC). Animals underwent fear conditioning and extinction paradigms as well as rotarod, behavioral tests that assess associative learning and motor coordination, respectively. There were no significant differences between these control diets; however, the CC animals consistently performed worse compared to the 7012_s animals. The CC diet was created in order to mimic a 7012 base and ideally induce identical behavioral phenotypes, but our findings reveal that mere

manipulation of dietary base formula can produce weakened performance in animal behaviors.

The second portion of the study assessed the role of biometals – particularly copper (Cu) and zinc (Zn) – on behavior by utilizing a different 7012-base (7012_N) that controlled for the seasonal availability of ingredients. Copper deficiency has been under investigation due to possible causative roles in several pathological disease states that are characterized by learning and motor impairments. A lack of Cu intake in the diet can produce a direct Cu deficiency whereas excess Zn can lead to indirect Cu deficiency via competition with Cu for absorption in the gut. Previous work conducted in our laboratory revealed that Cu administered in conjunction to Zn in drinking water remediated some behavioral deficits associated with excess Zn; therefore it is suggested that Zn supplementation may be associated with Cu deficiency. Direct and indirect Cu deficiencies were studied by the respective administration of reduced Cu diets and Znsupplemented diets. Animals were raised on the 7012_N, the 7012_N with reduced Cu levels $(7012_{\text{N-Cu}})$, the 7012_{N} with 10 parts per million Zn added to drinking water $(7012_{\text{N+Zn}})$, or the CC diet. Fear extinction learning and open field activity were assessed at approximately four months of age, and rotarod performance was assessed at two months and four months of age. Statistical analyses indicated strong extinction-based learning throughout the three days of testing but failed to find a main effect of diet. Rotarod performance at four months of age was significantly better than performance at two months of age, and performance improved over the three days of testing. Likewise, dietary manipulation failed to contribute to our statistical models. There were significant

differences in open field activity: 7012_N controls and 7012_{N-Cu} animals moved significantly more compared to 7012_{N+Zn} and CC animals. This finding indicates that our dietary manipulation may have confounded general locomotion and weight of animals. We also believe that the recent changes to the 7012 formula base to form the 7012_N version may have impacted results. Both experiments demonstrate how dietary changes in base composition and biometal concentrations can impact behavioral performance in animal models. With these data, we propose stricter control over open-access diets and suggest stronger statistical analyses that incorporate diet assignments, weight, and behavioral outcomes.

CHAPTER ONE: INTRODUCTION

Metal homeostasis has gained attention within the past few decades due to its potential causative role in many neurodegenerative diseases. Substantial evidence suggests that both copper (Cu) and zinc (Zn), two abundant trace metal ions in the body, are involved in numerous biochemical interactions as well as several neurological diseases such as Alzheimer's disease and posttraumatic stress disorder. These diseases are not limited to one particular type of cognitive deficit; rather, altered biometal concentrations in the brain can induce impairment across a broad spectrum of behaviors that include, but not are limited to, learning, memory, and motor coordination. Disruptions in Cu and Zn availability during embryonic development and during adulthood can result in oxidative damage as well as disruption to signaling pathways involved with learning and memory, specifically those involved with long-term potentiation (Keen et al., 2003; Osredkar & Sustar, 2011). Recent results from our laboratory indicate that early exposure to Cu deficiencies (i.e. exposure during embryonic development) induces greater motor coordination and learning impairment (Lippi, 2014). This thesis explores the effects of biometal concentration via dietary manipulation on learned fear, extinction of learned fear, and motor coordination and also provides evidence for alternative mechanisms, such as poor weight gain, that could potentially mediate the effects of metal ion dyshomeostasis.

Fear conditioning and extinction

Fear conditioning, an adaptive form of associative learning, allows an individual to anticipate events and its surroundings and efficiently process future outcomes (Curzon, Rustay, & Borwman, 2009). These associations may be cue- or context-relevant such that an aversive stimulus or event becomes associated with a neutral stimulus (cue) or an environment (context). When the aversive stimulus is paired with a cue or context, the individual's response is said to be unconditioned. If the cue or context is presented but not reinforced with the aversive stimulus, then one would expect to see a conditioned response due to the presentation of the cue or context. These conditioned responses will decrease over time if the cue or context remains non-reinforced, but conditioned responses may remain elevated or become a "default mode" in pathological disease states.

Just as it is important to form these associations, it is also important for an individual to learn to extinguish fear of a stimulus to reduce mental workload. The process of fear extinction is not synonymous with forgetting; rather, it is a fragile yet flexible state in which newly-formed inhibitory memories compete with previously formed memories entailing regulation of responses to non-reinforced stimuli (Hartley et al., 2012; Herry et al., 2010; Lai, Franke, & Gan, 2012; Milad & Quirk, 2012). Once a conditioned stimulus is presented without its aversive component, the individual will experience less physiological arousal and fear, and the individual may proceed to allocate necessary mental resources toward other potential threats.

The most popular neural model of fear conditioning suggests that acquisition and consolidation of conditioned fear are heavily dependent on the basolateral amygdaloidal complex (BLA), the site in which the association between an unconditioned stimulus and a neutral stimulus is formed (Makkar, Zhang, & Cranney, 2010). The lateral division of the BLA receives sensory input from cortical regions such as the sensorimotor cortex, the parahippocampal gyrus, the orbitofrontal gyrus, and the anterior cingulate cortex (Mahan & Ressler, 2012). In turn, BLA GABAergic and glutamatergic projections are sent to the lateral (CeL) and medial (CeM) portions of the central nucleus (Ce) of the amygdala (Mahan & Ressler, 2012), as well as the intercalated neurons (ITCs). The CeL is involved with the acquisition of fear and produces tonic inhibitory control over the CeM, whereas the CeM is thought to be necessary for the expression of fear, such as freezing (Mahan & Ressler, 2012). The ITCs are thought to be involved with fear extinction because of their increased activation during extinction paradigms (Mahan & Ressler, 2012). Other regions that send inputs to the BLA include the hippocampus and the medial prefrontal cortex (mPFC) (Curzon et al., 2009). The hippocampus, specifically the dorsal portion and the cornu ammonis 3 (CA3) field, is thought to be required for learning of context but not necessarily important for learning of cued associations (Curzon et al., 2009). The mPFC, particularly the ventromedial prefrontal cortex (vmPFC), is suggested to be more involved with fear extinction; however, it plays a role in acquisition and attentional learning (Curzon et al., 2009).

Fear extinction is the decrement in conditioned responses with the repeated presentation of an unreinforced conditioned stimulus (Curzon et al., 2009) and is

analogous to the creation of inhibitory memories (Lai et al., 2012). These newly formed associations compete with memories formed during conditioning and regulate conditioned fear responses (Hartley et al., 2012). Although the same structures may be involved in both conditioning and extinction, plasticity induced by extinction may not mimic the plasticity induced by fear conditioning nor will plasticity exhibit the same excitatory or inhibitory effect across structures (Quirk & Mueller, 2007). Research suggests that the mPFC, particularly the vmPFC subregion, is the primary site of extinction consolidation (Makkar et al., 2010). The mPFC itself is influenced by inputs from the hippocampus which modulate consolidation and retrieval of contextual extinction memory (Quirk & Mueller, 2007). Additionally, thalamic input to the mPFC suppresses long-term potentiation and reduces input sensitivity to the amygdala via inhibitory interneuron modulation (Barad, Gean, & Lutz, 2006; Milad & Quirk, 2002). Neurons from the infralimbic cortex (the homologue of the mPFC in animal models) project to the ITCs adjacent to the amygdala as well as targets in the brainstem and hypothalamus, two regions that also modulate fear expression. These ITCs, which are activated during extinction learning, project inhibitory connections to the CeM resulting in a dampening fear response (Mahan & Ressler, 2012). Overall, the mPFC inhibits amygdalar responses to a conditioned stimulus, preventing conditioned responding (Makkar et al., 2010).

Copper

Copper, the third most abundant trace metal in the body, is obtained from diet and absorbed from the gut. It promptly binds to albumin and is transported to the liver where

it can be stored. Both ATP7A and ATP7B regulate Cu concentrations: ATP7A releases Cu into the portal vein of the liver in response to increased Cu levels, whereas ATP7B removes excess Cu via bile secretion (Osredkar & Sustar, 2011). Copper is mostly protein-bound and is tightly regulated through the nervous system; however, it is able to pass through the blood brain barrier (BBB) via CTR1 and ATP7A transporters expressed in endothelial and choroid plexus cells (Gaier, Eipper, & Mains, 2013). Levels of Cu in different brain regions are species-dependent and vary from individual to individual, but the dentate gyrus and CA1 of the hippocampus, the amygdala, the cerebellum, and parts of the diencephalon are generally cited as having the highest concentrations (Bolognin et al., 2012; Gaier et al., 2013). In addition, the lungs, kidneys, small intestines, heart, and liver also exhibit high Cu concentrations (Bolognin et al., 2012).

Copper serves as an important cofactor for the functioning of cuproenzymes and plays an important role in many biochemical interactions. Copper possesses redox-active characteristics that are essential for a plethora of biochemical activity. These include, but are not limited to, energy production, immune functioning, oxidation-reduction reactions, free radical damage prevention, myelination, thyroid functioning, and neuronal transmission (Gaier et al., 2013; Osredkar & Sustar, 2011; Uriu-Adams, Scherr, Lanoue, & Keen, 2010). For example, Cu plays a role in the functioning of cytochrome C oxidase and Cn/Zn superoxide dismutase (SOD1), two enzymes that are needed for energyproducing aerobic respiration and detoxification of damaging superoxides, respectively (Osredkar & Sustar, 2011). Copper-dependent lysyl oxidase aids in the production of structural proteins such as collagen and elastin (Smith-Mungo & Kagan, 1998) and serves as another example of the wide range of cuproenzymes with which Cu interacts. Of greatest focus are Cu's antioxidant properties and involvement in synaptic plasticity and neurotransmission. Dopamine B-hydroxylase requires Cu in the biotransformation of dopamine into norepinephrine (Osredkar & Sustar, 2011), an abundant neurotransmitter in the brain involved with arousal, attention, and stress response. Studies examining Cu deficiency in cattle, sheep, and in humans demonstrate Cu's role in myelination in the central nervous system (Follis, 1948; Kievay, 2013; Linder & Goode, 1991). On a cellular level, Cu is concentrated at excitatory NMDA receptors and influences glutamatergic transmission. Copper remains trapped within glutamatergic vesicles and is released in a calcium-dependent manner, often exhibiting inhibitory effects on excitatory synapses (Gaier et al., 2013). Ionotropic GABA_A receptors are an alternative mechanism by which Cu evokes cellular processes. Copper channel-gates chloride-conducting GABA_A receptors resulting in depressed synaptic transmission (Gaier et al., 2013).

Zinc

Similar to Cu, Zn is obtained through diet and is absorbed through the gut and small intestine. The BBB and cerebrospinal fluid systems tightly regulate Zn distribution throughout the central nervous system, and Zn L-histidine in plasma and cerebrospinal fluid transfers Zn to its appropriate target sites (Mocchegiani, Bertoni-Freddari, Marcellini, & Malavolta, 2005; Takeda, 2001; Takeda et al., 2002). It is primarily found in the hippocampus, the stratum lucidum of CA3, the amygdala, and the striatum (Frederickson, Kasarkis, Ringo, & Frederickson, 1987) as well as muscle, bones, kidneys,

the liver, eyes, and the prostate gland (Bolognin et al., 2009; Osredkar & Sustar, 2011). Zinc influx into neurons and glial cells is not fully understood.

Zinc has been identified as an essential component of numerous biochemical interactions. Approximately 200 enzymatic processes involve Zn in some fashion, and roughly 10% of human proteins bind to Zn (Osredkar & Sustar, 2011). It is suggested that Zn is heavily involved with the glutamatergic neuromodulation such that simultaneous Zn-glutamate release inhibits NMDA-receptor activity while potentiating non-NMDA synaptic activity (Mocchegiani et al., 2005; Peters, Koh, & Choi, 1987). Zinc release also enhances nearby AMPA conduction on glutamatergic neurons and can induce cell death (Koh et al., 1996). In addition, evidence has shown that Zn modulates GABAergic signaling by reducing amplitude, decreasing onset rate, and accelerating the decay rate of postsynaptic currents at GABA_A receptors, thereby inhibiting GABAergic activity (Barberis, Cherubini, & Mozrzymas, 2000; Westbrook & Mayer, 1987).

Direct and indirect copper deficiency

Although not empirically studied and somewhat rare, Cu deficiency has been considered an under-recognized cause of neurological problems. Metal dyshomeostasis is not uncommon as age increases, but pathological disease states can derail tight regulation of metal ions in the brain and can favor abnormal protein-metal ion interactions (Bolognin et al., 2012), often leading to protein misfolding and activation of detrimental cellular pathways. Thus, it is paramount to explore the potential effects of this deficiency on aforementioned cuproenzymes, neurological processes, and overall behavior.

Copper deficiency can occur primarily by reduced dietary Cu intake and secondarily through competitive nutrient absorption (such as Zn), genetic mutations and polymorphisms, and physiological stressors (Chhetri, Mills, Shaunak, & Emsley, 2014). For example, during the absorption phase, Zn actively competes with Cu in the gut and small intestines; thus, excess Zn levels can lead to indirect Cu deficiency (Maret & Sanstead, 2006; Nations et al., 2008). Short-term administration of Zn-supplemented diet fails to elicit substantial changes in Zn levels in the brain, potentially indicating Znsupplemented diets induce an indirect effect on Cu levels (Knight, 2000). Recent research has elucidated the precarious relationship between Zn and Cu, emphasizing the importance of a stable, non-elevated ratio of Zn to Cu rather than the level of each biometal by itself (Guo, Chen, Yeh, Hsiung, & Wang, 2011; Osredkar & Sustar, 2011), but it has yet to clarify how excess Zn or reduced Cu can induce morphological changes in certain brain regions and changes in behavior.

Regardless of the mechanism by which Cu is reduced, Cu deficiency has been linked to serious developmental and health complications. Reduced Cu levels prevent SOD1 from detoxifying superoxide and hydroxyl radicals produced during metabolism, resulting in oxidative stress and cellular damage (Bolognin et al., 2009; Keen et al., 2003; Osredkar & Sustar, 2011). Reduced lysyl oxidase activity negatively impacts heart and brain embryonic development due to oxidative damage, altered angiogenesis, and compromised energy production (Keen et al., 2003). Mutations in Cu-relevant proteins also illustrate detrimental processes that can occur. Copper transporter ATP7A mutations are linked to Menkes Disease, a neurological disorder characterized by a lack of Cu,

psychomotor deficits, mental retardation, convulsive seizures, and neuronal loss in the hippocampus and cerebellum (Lyons & Prohaska, 2010). Mutations in ATP7B, the protein necessary for metallation of ceruloplasmin and biliary excretion, are linked to Cu toxicity found in Wilson disease which accounts for 10% of human hypocupremia (Chhetri et al., 2014; Hedera et al., 2009; Prohaska, 2011).

In regard to learning and memory, Cu influences glutamatergic and GABAergic signaling in brain regions associated with fear conditioning and extinction such as the hippocampus, the amygdala, the ITCs, and the prefrontal cortex (Bolognin et al., 2012; Flinn et al., 2005). As previously mentioned, Cu targets GABA receptors in conjunction with NMDA receptors located in the mPFC (Barberis et al., 2000; Davis, 2002; Davis & Myers, 2002). The BLA contains NMDA receptors sensitive to the presence of Cu (Kardos, Kovacs, Hajos, Kalman, & Simonyi, 1989). It has been shown that the primary mechanism by which extinction takes place is the mPFC-driven NMDA-mediated plasticity that occurs in the BLA and the ITCs (Parsons, Gafford, & Helmstetter, 2010; Quirk, Likhtik, Pelletier, & Paré, 2003). Copper's interactions with GABA and NMDA receptors suggest that it may play a role in regulating long-term potentiation which is the putative biological explanation for memory formation and retention.

Zinc is also found in learning- and memory-relevant regions such as the hippocampus, the amygdala, the striatum, and neocortex. Zinc is present in a substantial amount of connections between the mPFC and the BLA (Cunningham, Ames, Christensen, & Sorensen, 2007), and Zn influences the glutamatergic system by enhancing AMPA activity while inhibiting NMDA activity which has been shown to

affect spatial memory in rats and mice (Flinn et al., 2005). Evidence from a Morris water maze task conducted with 3 and 9 month old Sprague-Dawley rats suggests that Zn supplementation (10 parts per million [ppm] ZnCO₃) interrupts cellular processes involved with reference and working memory, and Zn supplementation further exacerbates these processes when rats are older (i.e. the 9 month condition) (Flinn et al., 2005). In another experiment, Railey and colleagues (2010) tested 4 month old Sprague-Dawley rats in both fear conditioning and extinction paradigms and a Morris Water Maze task. Results indicated that rodents that consumed Zn-supplemented water (10 ppm Zn) exhibited higher freezing rates during contextual retention and extinction and cued extinction compared to animals on standard lab water, indicating that Zn may affect longterm memory and negatively impact fear extinction of both context and cue associations while sparing working memory (Railey et al., 2010). The same study noted similar results compared to Flinn and colleagues' work conducted in 2005: nine month old rats raised on a Zn-supplemented diet also exhibited longer latencies to reach the platform compared to controls in the Morris Water Maze indicating impaired spatial memory. The addition of Cu to Zn-supplemented water decreased freezing and latency levels closer to those of the control animals demonstrating the balance of these two biometals (Railey et al., 2010).

The effect of Cu deficiency in particular has been linked to motor impairment and abnormal cerebellar and total brain growth since the 19th century (Chhetri et al., 2014; Lyons & Prohaska, 2010). Previous research has indicated that brain regions responsible for motor coordination – specifically the cerebellum – are negatively affected by prenatal Cu deficiencies (Everson, Tsai, & Wang, 1967; Georgeiff, 2007) and that limited Cu

availability is often associated with abnormal gating and ataxia in both animal models and humans (Chhetri et al., 2014; Penland & Prohaska, 2004). The consequences of lacking proper Cu levels can be seen with patients with Menkes disease and Wilson's disease, two disorders characterized by psychomotor deficits and limb spasticity, respectively (Lyons and Prohaska, 2010). Purkinje cells in the cerebellum are particularly interesting targets due to their deep projections into cerebellar nuclei and their ability to modify activity patterns of upper motor neurons. It is believed that these efferent projections mediate cerebellar coordination and somatomotor activity, and dysfunction of these cells could lead to abnormal phenotypes (Lyons & Prohaska, 2010). Copper deficiency may disrupt Purkinje conduction yielding abnormalities in motor coordination; how this is done on a molecular and cellular basis, however, is not fully understood.

Purpose

This thesis is divided into two separate experiments. The first experiment (herein referred to as "pilot portion" of the study) addresses the precarious process of selecting proper diets in animal experimentation. Behavior can differ not only due to the primary manipulation (e.g. Cu restrion or Zn supplementation) but also due to the ingredient-base itself. Omission and inclusion of ingredients such as wheat, grain, and soy in formula "base-makeups" are contingent upon the research question. For example, isoflavones, a class of phytoestrogens found in soy-based products, have been studied for potential effects in a variety of age-related diseases and cancers (Lephart et al., 2002) due to their tumorigenesis-suppressant, antioxidant, and anti-inflammatory properties (Patisaul & Jefferson, 2010). Research suggests that phytoestrogens and soy have anxiolytic effects

as shown by decreased anxiety levels in open-field testing (McCarthy, Schwartz-Giblin, & Wang, 1997), social interaction testing (Johnston & File, 1991), and elevated plusmaze testing (Friedman & Frye, 2011; Lephart et al., 2002; Patisaul, Blum, Luskin, & Wilson, 2005). In addition to these effects on learning, memory, and anxiety, soy can also impact weight regulation. Cederroth and colleagues (2007) found that rats fed on a soyrich diet had reduced body weight and exhibited improved body metabolism possibly due to phytoestrogens affecting hypothalamic neuropeptide expression. These findings prompt consideration of dietary interactions due to the presence of natural (e.g. wheat and soy) and purified (e.g. casein, non-isoflavone-containing products) ingredients.

Previous experiments from our laboratory (Howell, 2014; Lippi, 2014) have utilized two different control diets produced by Harlan Laboratories: a natural, commonly used 7012 rodent diet ("7012s") and a purified, Cu-control diet ("CC"). Ideally, animals raised on these diets should exhibit similar performance on behavioral measures due to their "controlled" designation; however, we note that "control designation" is a somewhat arbitrary process in regard to behavioral neuroscientific research. No experiment has been conducted to determine if there are any beneficial effects of using a more natural diet (7012_s) over a purified diet (CC); thus, the pilot portion of the study compares the two aforementioned control diets and considers the best alternative for studies conducted after the completion of this thesis.

The second experiment (herein referred to as "experimental portion" of the study) utilizes a more controlled diet ("7012_N") in order to explore the effects of direct and indirect Cu deficiency. The proportion of ingredients tends to fluctuate throughout the

year which can result in several caveats in research that rely on homogenous mixtures from batch-to-batch or lot-to-lot (Watson, 1996). Although not extensively documented, research does recommend that feeds originate from the same batch and/or lot for dietary manipulation (Barnard, Lewis, Teter, & Thigpen, 2009). As advised by Harlan nutritionists, we implemented a controlled, open-formula 7012 diet (7012_N) that controlled for seasonal availability of ingredients in the base formula. We utilized this formula to investigate the role of direct and indirect Cu deficiency by reducing Cu in the food pellets in one condition and adding Zn to the water supply in another condition.

General findings indicate that Cu deficiency is particularly damaging on both the heart and brain, and that disruptions in Cu availability during embryonic development can result in altered skeletal, pulmonary, and cardiovascular abnormalities as well as an increase in oxidative damage on a cellular level (Keen et al., 2003; Osredkar & Sustar, 2011). Copper deficiency in prenatal development can also lead to lower levels of superoxide dismutase (SOD1), promoting the production of highly toxic free radicals (Bolognin et al., 2012; Keen et al., 2003). Excess Zn consumption, which leads to indirect Cu deficiency, also negatively impacts embryonic development. Symptoms of excess Zn are similar to those of direct Cu deficiency but also include poor weight gain, anemia, ataxia, and other neurological impairments (Everson et al., 1967). Unfortunately it appears that Cu repletion does not reverse the damage caused by deficiency during development (Urui-Adams et al., 2010) which incites greater need to explore the mechanisms by which direct and indirect Cu deficiencies induce detrimental cellular processes.

Previous research in our lab has indicated that Zn supplementation impaired rodents' fear extinction and spatial memory as measured by the Morris Water Maze task (Railey et al., 2010). In the same experiment, Cu added to Zn-supplemented diets induced a remediating effect: rodents on Zn-supplemented diet with additive Cu exhibited similar learning compared to rodents on a control diet. A separate experiment conducted by Lippi (2014) examined fear conditioning, extinction learning, and motor coordination (as measured by an accelerating rotarod task) in rodents that either had pre- or post-natal exposure to a Cu-deficient diet or a control diet. Although the experiment yielded unexpected results in the postnatal conditions, consistent data were obtained suggesting that prenatal exposure to a Cu-deficient diet induced greatest impairment in both fear extinction learning and motor coordination (Lippi, 2014; see Figure 1 in the appendix). In light of this recent finding, this thesis includes diets that were administered prenatally in order to induce the greatest behavioral deficits.

In conclusion, the pilot portion of the study intends to examine differences in behavioral performance between rodents raised for 4 months on a natural diet (standard 7012) and rodents raised for 4 months on a purified Cu-control diet used in Howell, 2014 and Lippi, 2014. The experimental portion of this thesis provides information regarding learning and motor impairments linked to biometal dyshomeostasis. These findings may be insightful for researchers and future students that intend to study diseases characterized by learning and motor impairments such as posttraumatic stress disorder and Alzheimer's disease.

CHAPTER TWO: MATERIALS AND METHODS

Food and water preparation

Food and water were offered *ad libitum* and diet and water intake were measured in grams by utilizing a subtraction method (weight filled from previous administration – weight consumed). In consultation with nutritionists at Harlan Laboratories (Indianapolis, IN), five diets were manufactured and used for this thesis: a Cu-control diet Cu previously used in Lippi, 2014 ("CC"), a commonly used Teklad 7012 standard animal feed (" 7012_{s} "), a newly formulated 7012 controlling for seasonal availability of ingredients (" $7012_{\rm N}$ "), the newly formulated 7012 diet with reduced Cu levels (" $7012_{\rm N}$ - $_{CD}$ "), and the newly formulated 7012 with added zinc carbonate (ZnCO₃) to the water supply ("7012_{N+Zn}"). The first four diet conditions consumed standard lab water. Methods for Zn water preparation have been utilized in previous studies (Chrosniak et al., 2006; Linkous et al., 2009; Railey et al., 2010). To prepare the Zn water, tap water was flushed for approximately 20 minutes prior to collection. Ten parts per million (ppm) ZnCO₃enhanced water (referred to as "Zn water") was prepared using a solution of 10,000 mg/L of Zn solution dissolved in in 5% HNO₃ (SPEX CertiPrep, Metuchen, NJ). The final solution was buffered with approximately 3 mg/L sodium carbonate (Na₂CO₃) (Sigma-Aldrich, St. Louis, MO) in order to obtain an approximate pH level of 7.0. Water was stored in polycarbonate carboys and administered weekly in glass bottles. Water samples

were analyzed by the United States Geological Survey (USGS, Reston, VA) to ensure accurate dosage. Diet manipulation lasted approximately 4 months prior to the start of behavioral testing for both pilot and experimental portions of the study. Diet manipulation ceased when animals were sacrificed at 4 months and two weeks for the pilot portion and 4 months and three weeks for the experimental portion. Table 1 summarizes diet administration for both pilot and experimental portions of the study. Figures 2 - 5 in the appendix provide detailed information about nutrition and composition of each diet.

Table 1Diets administered and metal concentration

Diet	Used in thesis	When administered	Cu deficiency	Control for ingredients	Water type	Cu (ppm)	Zn (ppm)
7012s	Pilot	Postnatal (P0+)	No	no	Standard	23	63
CC	Pilot Experimental	Postnatal (P0+) Prenatal (E7)	No	yes	Standard	16	40
7012 _N	Experimental	Prenatal (E7)	No	yes	Standard	23	63
7012 _{N-CD}	Experimental	Prenatal (E7)	Yes-direct	yes	Standard	7 - 12	63
$7012_{N\!+\!Zn}$	Experimental	Prenatal (E7)	Yes - indirect	yes	+ Zn	23	73

Note. Diets were administered for approximately 4 months. Cu = copper; Zn = zinc; $7012_S = standard 7012$ diet; CC = Cu-control diet from Lippi, 2014; $7012_N = newly$ formulated diet controlling for extraneous ingredients; $7012_{N-CD} = new 7012$ diet with reduced Cu levels; $7012_{N+Zn} = new 7012$ diet with normalized Cu levels but with added Zn to water supply; ppm = parts per million. Supplemental information regarding other ingredients are located in the appendix.

Breeding and housing

Pilot breeding. This portion of the study examined potential differences between a purified Cu-control diet (CC) and a natural, standard 7012 (7012_s) diet. Six female and three male Sprague-Dawley rats were ordered from Harlan Laboratories (Indianapolis, IN) for breeding purposes. Breeders were administered the 7012_s diet upon arrival to the David King Hall animal facility at George Mason University and were given one week for acclimatization. Two female rats were housed per cage and three male rats were housed per cage during acclimatization. After the one week acclimatization period, one male rat was paired with two female rats in the female home-cage for a one week breeding period. Males were removed after one week and females were monitored for pregnancy. After exhibiting signs of pregnancy, mothers were individually housed, and male breeders were grouped housed. All breeder mothers gave birth within three days of each other. Before postnatal day 5 (P5), pups were sexed and five males were retained to serve as testing subjects. Excess males and females were sacrificed using CO₂ asphyxiation and decapitation. To minimize potential stress on newborn pups from handling cages, three litters were randomly assigned to switch to the CC diet approximately one week after birth. This random assignment yielded two diet conditions: the 7012s and the CC conditions. Pups remained with their mothers until P21 when they were weaned, ear-punched for identification purposes, weighed, and group-housed in groups of two or three. The mothers were then sacrificed. Due to premature death (illness and cannibalization by dams), we obtained a total N = 25, with n = 13 animals in the $7012_{\rm S}$ condition and n = 12 animals in the CC condition.

Experimental breeding. This portion of the study utilized direct and indirect Cudeficient diets administered prenatally. Sixteen time pregnant females were ordered from Harlan and were scheduled to arrive to the animal facility on embryonic day 7 (E7). Upon arrival, the females were randomly assigned to receive one of four diet conditions: CC, 7012_{N} , 7012_{N-CD} , and 7012_{N+Zn} . All dams gave birth to offspring two weeks later, indicating that all dams arrived on E7. Before P5, pups were sexed and five males per

dam were retained. Excess males and females were sacrificed. The male pups remained with their mothers until P21 when they were weaned, ear-punched, weighed, and group-housed in groups of two, three, or four. The mothers were then sacrificed. Due to cannibalization and a low birth rate for two of our dams, we obtained the following group sizes for diets: the 7012_N , 18; 7012_{N-CD} : 13; 7012_{N+Zn} : 17; CC: 18. Due to a delivery delay and inclement weather, five 7012_N and five 7012_{N+Zn} cages were temporarily put on 7012_S at six weeks of age for eight days out of the four month manipulation. Table 2 summarizes the number of animals per condition for both pilot and experimental portions of the study.

Table 2 Animals per condition

Portion of study	ortion of study CC		7012 _s 7012 _N		7012_{N+Zn}
Pilot	12	13	Not assessed	Not assessed	Not assessed
Experimental	18	Not assessed	18	13	17

Note. Cu = copper; Zn = zinc; $7012_s = standard 7012$ diet; CC = Cu-control diet from Lippi, 2014; $7012_N =$ newly formulated diet controlling for extraneous ingredients; $7012_{N-CD} =$ new 7012 diet with reduced Cu levels; $7012_{N+Zn} =$ new 7012 diet with normalized Cu levels but with added Zn to water supply.

Housing. The colony was maintained on a 12-hour light/dark cycle. Each cage was lined with Tek-Fresh bedding (Harlan Laboratories, Indianapolis, IN) and was cleaned by husbandry staff twice per week using a standard cage wash machine. The same cage lids and hoppers were used throughout the experiments to ensure that the weight of the hopper that contained remaining food was consistent. After P21, trained research assistants gently handled animals twice weekly to minimize stress from handling during behavioral testing and to assess for injury or illness. Handling did not occur during

behavioral testing procedures. Room temperature and relative humidity of the colony were recorded two times a week during handling sessions. Abnormal occurrences were reported to the principal investigator and lab management.

Materials and procedures

General testing. All procedures were approved by the George Mason Institutional Animal Care and Use Committee (IACUC) and were in concordance with National Institutes of Health (NIH) guidelines for animal testing. Pilot testing (N = 25) occurred in one wave, and experimental testing (N = 66) occurred in two waves with 33 animals in each wave. In each wave, there were approximately equal numbers of animals per condition. Table 3 and Figure 6 in the appendix illustrate the number of animals per wave and the testing schedule, respectively.

Experimenter's gender. In light of recent research (Sorge et al., 2014), the research assistants' genders (herein referred to as "RA gender") were recorded to control for potential effects on behavior during testing.

Diet and water consumed, and water dosage. For the pilot portion of the study, diet consumption (in grams) for each cage was measured by utilizing a subtraction method (weight filled from previous administration – weight consumed). Water was administered through a cage rack system during the pilot portion; thus water consumption could not be measured. In addition, water was not the primarily manipulation during the experiment, and both diet conditions received water from the same cage rack system. For the experimental portion of the study, diet and water intake per cage were measured in grams by utilizing the subtraction method.

To ensure accurate dosage, water samples (experimental portion only) were sent to USGS (Reston, VA), and calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), Cu, and Zn concentrations were measured and expressed in ppm. pH levels were also recorded to ensure appropriate pH for both lab and Zn water conditions.

Animal weights. For pilot testing, animals' weights (in grams) were measured on P21 and one hour before death which occurred approximately at four months and two weeks of age. For experimental testing, animals' weights were measured on P21, at two months, at three months and one day (due to inclement weather), four months, and one hour before death which occurred at approximately four months and three weeks of age.

Fear conditioning and extinction. Fear conditioning and extinction procedures began at four months and two days for both pilot and experimental animals. Order was determined such that no animal was left alone in its homecage nor a non-conditioned animal was paired with a conditioned animal in the homecage. Fear conditioning and extinction procedures were completed in one wave (N = 25) for the pilot portion and in two waves for the experimental portion (N = 66; 33 per wave). Fear conditioning and extinction occurred in two identical clear plexiglass chambers (26 cm long by 26 cm wide by 18 cm high) inside sound attenuating boxes (Coulbourn Instruments, Whitehall, PA). FreezeScan software (Clever Sys., Inc., Reston, VA) monitored and recorded freezing behavior in terms of percentage.

Conditioning. Animals underwent delayed conditioning in which a tone (the NS, CS) was paired with a footshock (the US). Animals were placed in a wire-cage transport unit and were wheeled down to a dark (3 - 4 lux), quiet room in the animal colony where

they habituated for 10 minutes. Two animals were brought into a moderately bright testing room (74 - 75 lux) and were quickly placed into their assigned chambers. The animal was allowed to explore the chamber for 180 seconds prior to tone onset. After 180 seconds, a 20-second, 85 decibel (dB), 2 kilohertz (kHz) tone played; a two (2) second, 0.5 milliamp (mA) scrambled foot shock coterminated with the tone. Shocks/USs coterminated with the tones at 200 seconds, 260 seconds, and 320 seconds for a total of three NS/US (tone/shock) pairings. An additional 10 seconds passed before the animal was carefully removed from the chamber and returned to its transport cage. Researchers returned conditioned animals in their transport cages to the habituation room, thoroughly cleaned the chambers with 2:1 diluted store-bought vinegar (distilled water: 5% vinegar dilution), and brought the next animals into the testing room. Researchers changed gloves between trials to eliminate potential odor/pheromone cues. After all animals underwent fear conditioning, researchers thoroughly cleaned chambers twice with 70% ethanol, and fans aired out the room to remove potential odors. Figure 7A in the appendix illustrates the conditioning paradigm.

Extinction, extinction recall, and extinction recall 2. Twenty-four hours after conditioning, animals underwent fear extinction. Animals were placed in opaque white containers with clean bedding and were transported on a cart to a dark (3 - 4 lux), quiet habituation room different from the room used in fear conditioning where they habituated for 10 minutes. The testing chamber apparatus was altered such that the walls were covered with black and yellow strips of laminated paper, and the door and back wall were covered in black star-shaped cut outs to increase contrast. Black plexiglass and bedding

were also used to hide the bars and to produce tactile differences. Diluted vanilla extract was used in the pilot portion of the study to induce olfactory differences; however, the extract was excluded from the experimental portion because Zn supplements have been shown to affect sense of smell in both rodents and humans (Alexander & Davidson, 2006; McBride, Slotnick, & Margolis, 2003). Overhead lighting was altered $(1 - 2 \ln x)$ and a fan was utilized to minimize contextual interference in the testing room. Similar to fear conditioning procedures, animals habituated in the chambers for 180 seconds with no tone presented. Eighteen 20-second tones (CS) were played with a 40 second interval. An additional 10 seconds passed before animals were carefully removed and returned to their transport cages. Researchers then brought the transport cages back to the habituation room. Chambers were thoroughly cleaned with 70% ethanol, vanilla extract was reapplied (for pilot portion only), and bedding was changed between trials. Researchers changed gloves between animals to reduce potential odor/pheromone cues. After all testing, the chambers were thoroughly cleaned twice with 70% ethanol and a fan was used to air out the room. This procedure was repeated 48 hours and 72 hours after conditioning to test for fear extinction recall and fear extinction recall 2, respectively. Extinction recall is an extension of extinction learning and is used to further test the effects of learning beyond the first day of extinction acquisition. Extinction recall 2 is a procedure that was run in order to maintain consistency with procedures conducted in Lippi, 2014. Figure 7B in the appendix demonstrates the fear extinction/recall/recall 2 paradigm.

Fecal boli. After each trial for fear conditioning, extinction, recall, and recall 2, research assistants counted the number of fecal boli left in the testing chambers. A higher number of fecal boli indicated higher levels of anxiety (Archer, 1973).

Accelerating rotarod. One week after fear extinction recall 2, motor function was assessed using the accelerating rotarod task. The accelerating rotarod task was completed in one wave for the pilot portion (N = 25) at approximately four months of age, and in two waves for the experimental portion (N = 66; 33 per wave) at approximately nine weeks and again at four months and one week of age. Order was determined by randomly selecting cages. Order of animals was randomized each day, but the order was kept the same within day to ensure that each rat had a long enough intertrial interval (ITI). This task was completed in a dimly lit (4 - 5 lux) and quiet testing room completely separate from fear conditioning and extinction procedures.

The first day served as an exposure day to familiarize the animal with the apparatus (Coulbourn Instruments, PA). The animal was placed on the non-rotating rod and was allowed one minute to remain on the rod. If the animal jumped off the rod or remained on the rod for one minute, then the animal was returned to its homecage, ending exposure training. Once all animals in the selected cage were exposed, the homecage was brought back into the animal colony. The apparatus was thoroughly cleaned with 70% ethanol between trials and between cages.

The following three days served as the experimental testing days. The trial began when the animal was placed facing away from the researcher on a rotating rod spinning at four rotations per minute (RPM). Once the researcher removed his/her hand and the
rodent stood on the rotating rod without support, another researcher started the apparatus's time recorder. The rod increased speed by one RPM every eight seconds. The trial ended when the animal fell from the rod and hit the underlying panel and stopped the apparatus's recorder, or if the animal remained on the rod for five minutes (which ever came first). The animal was returned to its homecage for an ITI of 10 minutes. Based off the results from pilot data, the ITI of the experimental cohort was reduced from 10 to five minutes. This procedure was repeated two more times for a total of three trials for each experimental day. The apparatus was thoroughly cleaned with 70% ethanol between trials and between cages. The researchers recorded the duration of the trial in seconds.

Open field testing. The open field test is a measure of exploratory behavior and general activity, and it is commonly used as a "control" assay for other behavioral tests that involve locomotion (e.g. freezing in fear conditioning, motor coordination in rotarod) (Gould, Dao, & Kovascics, 2009). These diets could have induced motor impairment which could have been incorrectly interpreted as a learning – rather than motor – impairment. Open field testing permits more "natural" motor activity and eliminates the potential effects of stress from being placed in a confined space or on a rotating rod; thus it was decided to add open field testing prior to the start of behavioral testing for the experimental cohort.

Open field testing was completed for only the experimental portion of the study, and all animals (N = 66) completed testing in one day at approximately four months and three weeks of age. Order was determined by randomizing cages. Animals were transported individually in opaque transport cages to a dimly lit testing room

(approximately $4 - 6 \ln x$) where they habituated for 10 minutes prior to testing. Each rat had a single five minute trial where it was placed in the center of a steel pool (100 cm in diameter, 61 cm in height) facing the same direction. Movement in the pool was recorded via Videotrack tracking system (Viewpoint Life Sciences, Montreal, Canada), and the number of fecal boli after each animal's trial was recorded. The pool was cleaned with 70% ethanol and any debris from the transport cages (e.g. bedding) was removed. One research assistant familiar with the project and two research assistants not involved in the project and blind to the diet conditions scored each video by recording the number of seconds spent moving in the apparatus.

Sacrifice. Order for sacrifice was randomized, and animals were sacrificed approximately 24 to 72 hours after all behavioral testing. Animals were transported to a lab specialized in surgical procedures and were placed under a fume hood where CO_2 was pumped into the cage. CO_2 exposure lasted less than five minutes and did not exceed 60 pounds per square inch (psi). Research assistants pinched paws and the base of the tail, brushed whiskers, and checked for heart beat to confirm loss of life. Death was confirmed with rapid decapitation using a guillotine specialized for rodents.

Pilot. Brains were quickly extracted and placed into dry ice. All four paws from each animal were removed by a guillotine or pair of surgical scissors. Paws were retained in order to potentially measure the amount of Zn in nail content. Brains were then collected from the dry ice and were temporarily stored in brain bags which were kept on dry ice. Samples were transferred and stored in a -80°C freezer until needed for future

studies involving histology and/or western blot analysis for proteins involved with learning and stress response.

Experimental. Brains were quickly extracted and immediately sectioned down the medial longitudinal fissure using a razor blade thoroughly cleaned with 70% ethanol. The left hemisphere was immediately placed into a 15% sucrose solution and stored at 4°C, whereas the right hemisphere was immediately placed on dry ice. The left hemispheres were later transferred into a 30% sucrose solution, followed by a 4% paraformaldehyde solution, and stored at 4°C until needed for future histology studies. The flash-frozen right hemispheres were removed from the dry ice, placed in individual brain bags, and stored in a -80°C freezer for future protein expression/western blot studies. Left and right adrenal glands were extracted, immediately weighed together in grams, placed in tubes, and placed on dry ice. The tubes were later stored in a -80°C freezer until needed for future western blot studies for corticotropin-releasing factor and other stress-related neurochemicals. Left and right eyeballs were extracted and placed in a 4% paraformaldehyde solution in tubes and were stored at 4°C until further analyses involving Zn and macular degeneration.

Statistical analyses

Primary outcome measures for the pilot portion of the study were food consumption for each dam prior to weaning (P21), food consumption for test rats, changes in animal weight (from weaning to approximately four months of age), fear conditioning, fear extinction, extinction recall, extinction recall 2, and rotarod performance. Primary outcome measures for the experimental portion of the study were

food consumption for each dam prior to P21, food consumption for test rats, metal content and pH of prepared drinking water, changes in animal weight (from weaning, at two months, at approximately three months, at four months, and to approximately four months and three weeks of age), fear conditioning, fear extinction, extinction recall, extinction recall 2, rotarod performance, open field testing, and the ratio of adrenal gland weight to body weight. All animals were included in analyses unless otherwise noted.

All outcomes were examined using mixed effects models in *lme4* and *lmerTest* packages in R (Bates, Maechler, Bolker, & Walker, 2014; Kuznetsova, Brockhoff, & Christensen, 2014; R Core Team, 2014) unless otherwise noted due to small amounts of data or due to a lack of a repeated-measures design. In this case, data were analyzed using linear regression. Mixed effects modeling eliminates problems posed by repeated measures and missing data (e.g. non-independence violations found in mixed analyses of variance, reduced power) and permits incorporation of continuous and categorical predictors (Baayen, Davidson, & Bates, 2008). In addition, mixed effects modeling has an advantage over other statistical procedures (e.g. mixed analyses of variance) because it incorporates individual differences in nested terms. Time variables were added to account for repeated-measures designs which allowed analysis of time by day by diet interactions. Time variables and other predictors of interest were transformed into z scores when necessary to avoid inappropriate scaling and to facilitate coefficient interpretation. Analyses were conducted in a stepwise fashion starting with a null model followed by the addition of nested terms. Analyses incorporated time/trials nested within day and repeated observations nested within individual rats. Nested models were compared to one

another by sequentially adding predictors as main effects followed by interactions if model fit improved. Bayesian Inclusion Criterion, rather than Akaike's An Information Criterion or p value, was utilized to assess model improvement. Sattherwaite (1946) approximations were utilized for degrees of freedom as well as t values and p values. Relevant main effects and interactions were reported and differences were considered significant if p < 0.05 and marginally significant if p < 0.10. The additional packages *data.table* (Dowle, Short, Lianoglou, & Srinivasan, 2014), *lsmeans* (Lenth & Hervé, 2015), and *psych* (Revelle, 2015) were used to format data, to obtain contrasts from linear regression and mixed effects analyses, and to obtain means. For open field testing, interrater reliability among the three coders was assessed using the *irr* package (Gamer, Lemon, Fellows, & Singh, 2012). Pertinent results were illustrated using *ggplot2* (Wickham, 2009). Mean differences and standard errors were reported as $M \pm S.E.M$.

CHAPTER THREE: RESULTS

Pilot

Diet consumed

Pups primarily rely on dams' milk for a food source rather than solid food located in cage hoppers; thus potential differences in consumption for approximately two weeks between dams assigned to $7012_{\rm s}$ or CC diets were assessed. Due to a small sample size (N = 6) and a lack of comparability analysis, mixed effects modeling could not be used. A linear regression model with diet as the predictor variable found no difference in amount of diet eaten between the $7012_{\rm s}$ dams (715.3 ± 44.49 g) and the CC dams (801.20 ± 46.61 g), B = -85.90, t(4) = -1.33, p = 0.25.

After P21, rats were able to reach the food hopper and were separated from their dams. In compliance with NIH and IACUC guidelines, test rats were grouped housed; thus, exact diet consumption for each individual rat could not be directly assessed. Differences in total consumption among cages over a four month period were examined using linear regression analyses with diet entered as a predictor variable. The model suggested that total diet consumption over a four month period was not significantly different between rats on the 7012s diet (5526.92 \pm 276.74 g) and rats on the CC diet (5112.04 \pm 504.73 g), B = -414.9, SE = 548.8, p = 0.47. Water was not manipulated

during this portion of the experiment nor was it easily measured due to the cage's water administration system; therefore, water consumption was not analyzed.

Animal weight

The selected mixed effects model incorporated ID as a nested term. Figure 8 illustrates the main effect of time such that animals were heavier at 4 months of age $(7012_{\rm S} = 484.92 \pm 7.59 \text{ g}; \text{CC} = 498.10 \pm 14.56 \text{ g})$ compared to weaning age $(7012_{\rm S} = 57.70 \pm 1.97 \text{ g}; \text{CC} = 64.07 \pm 4.08 \text{ g})$, B = 430.49, SE = 6.49, p < 0.001. Subsequent models failed to find a significant main effect of diet and a significant time by diet interaction, indicating that diet did not influence weight differences at three weeks or four months of age.

Fear conditioning

Due to technical difficulties, one 7012s animal was removed from the data yielding n = 12 for the 7012s group and n = 12 for the CC diet group across conditioning, extinction, extinction recall, and extinction recall 2. Freezing behavior during habituation (the 180 seconds prior to tone onset) was first examined. The selected model incorporated ID as a nested term and minute (first, second, and third minute) as a predictor. The model yielded a main effect of minute, B = 4.01, SE = 1.18, p < 0.01, such that freezing increased from the first minute (1.06 ± 0.39%) to the third minute (9.06 ± 3.02%) during habituation. This behavior is considered not uncommon and has occurred in experiments previously conducted in our lab (Lippi, 2014; Neely, unpublished data). Nested model comparisons failed to find any effect of RA gender, box placement, weight, or diet and any significant interactions among predictors on activity during habituation. Freezing behavior during conditioning (at which tones were paired with shocks) was analyzed with ID entered as a nested term. The selected model yielded a main of effect of tone/shock presentation, B = 0.39, SE = 0.06, p < 0.001, indicating that rodents significantly froze more over three tone/shock pairings. Pairwise comparisons indicated a significant increase in freezing percentage from the first tone/shock ($10.75 \pm 3.33\%$) to the third tone/shock ($57.17 \pm 5.88\%$), t(46) = -5.75, p < 0.001. Subsequent nested model comparisons failed to indicate a main effect of diet, weight, RA gender, and box placement as well as significant interactions among predictors, indicating that animals, regardless of their diet condition, box placement, weight, and the RA gender, froze similarly over the three tone/shock pairings. Specific results are illustrated in Figure 9.

Fear conditioning, extinction, recall, and recall 2

Freezing during habituation periods was analyzed using a model that incorporated ID and day as nested terms with the expectation that each rat would exhibit correlated freezing behavior (as expected with repeated-measures designs) and that freezing behavior would be similar within day. The selected model yielded a main effect of minute, B = 8.14, SE = 1.01, p < 0.001, indicating that freezing increased during habituation periods. As mentioned, this behavior was not considered uncommon. There was no main effect of day, or a minute by day interaction, which indicated that freezing during habituation prior to conditioning and extinction did not significantly differ, although further investigation showed that freezing during habituation prior to conditioning was lower (4.66 ± 1.12%) compared to freezing during habituation prior to extinction (35.97 ± 2.82%) indicating possible contextual interference. There was a main

effect of diet, B = 15.397, SE = 5.30, p < 0.01, such that freezing during habituation was on average higher in the CC animals (31.01 ± 2.48%) compared to the 7012_s animals (17.36 ± 1.79%). Lastly, there was a main effect of RA gender, B = 27.20, SE = 10.23, p < 0.01. It appeared that rats froze more when female RAs handled them prior to be placed in the box; however, this finding warranted caution due to the overwhelming number female RA interactions (273) compared to the number male interactions (15) with the rats. Nested model comparisons failed to find main effects of box placement and weight on freezing behavior during habituation as well as any significant interactions among predictors of interest.

Subsequent data analyzed were the cued-based freezing behavior. The full fear learning model incorporated fear conditioning, extinction, recall, and recall 2 in order to measure freezing behavior over the four days of testing. Time in seconds and weight in grams were transformed into z scores in order to improve scaling and interpretation of coefficients. The selected model incorporated Rat ID and day as nested terms. The final model yielded a significant main effect of day, B = 366.93, SE = 33. 43, p < 0.001, as well as a main effect of tone presentation, B = -584.54, SE = 54.82, p < 0.001. These main effects were qualified by a significant day by tone interaction, B = 79.39, SE = 8.28, p < 0.001, such that freezing for all animals during conditioning increased with each tone/shock pairing as previously discussed. The following day during extinction training, average freezing across tones was higher ($87.67 \pm 0.90\%$) than that of mean freezing across tones/shocks in conditioning ($39.54 \pm 3.69\%$), indicating successful cuedbased learning. Freezing decreased with each tone presentation during extinction from the

first tone (81.50 \pm 3.45%) to the final tone (79.56 \pm 4.98%), but notable decreases in freezing were not noted until extinction recall and extinction recall 2. Freezing during extinction recall decreased from the first tone (76.96 \pm 3.55%) to the last tone (38.72 \pm 5.84%), demonstrating strong extinction-based learning. In extinction recall 2, freezing decreased from the first tone (60.04 \pm 5.73%) to the last tone (48.07 \pm 7.20%), with the lowest freezing percentage (18.73 \pm 5.09%) recorded at tone 9. Nested model comparisons failed to find main effects on freezing behavior due to weight, diet, box placement, and RA gender as well as significant interactions among these predictors on testing day and the tones played throughout each day. Figure 10 illustrates results.

Analyses were also conducted on the number of fecal boli counted after each training session throughout the four days of testing. As expected, there was a main effect of day such that the number of fecal boli decreased throughout the four days, B = -0.90, SE = 0.19, p < 0.001, indicating a decrease in anxiety throughout the extinction paradigm. There was a significant main effect of RA gender in the full fear model, B = -1.20, SE = 0.55, p = 0.04, such that there was a higher number of counted fecal boli from trials with female RAs (3.7 ± 0.27) compared to trials with male RAs (2.5 ± 0.46). Despite efforts to balance male/female interaction with the rats, there were 76 trials with female contact compared to 20 trials with male contact. Nested model comparisons failed to find any effects or significant interactions on fecal boli count due to diet and box placement.

Accelerating rotarod performance

Linear regression was utilized to examine differences in duration spent on a nonrotating rod during exposure training. Statistical analyses indicated significant differences during the 60-second exposure training, B = 5.47, t(23) = 2.84, p < 0.01, such that CC animals remained on the non-rotating rod longer (7.70 ± 1.96 s) compared to 7012s animals (2.22 ± 0.41 s).

The full rotarod model excluded exposure training and assessed performance over the three days of experimental testing. Weight in grams was transformed into z scores in order to improve scaling. The selected model consisted of Rat ID and day as nested terms. The final model yielded a significant main effect of day, B = 18.60, SE = 4.64, p < 0.001, and a significant day by trial interaction, B = 4.19, SE = 1.62, p = 0.01, indicating that performance improved from the first day of testing $(8.79 \pm 1.69 \text{ s})$ to the third day of testing (58.44 \pm 6.36 s). The model failed to find a main effect of trial, B = -5.05, SE = 3.49, p = 0.15, indicating that performance was relatively the same across the three trials on a given testing day. The significant interaction, however, indicated significant improvement on the third testing day between trial 1 (47.68 \pm 8.98 s) and trial 2 (64.44 \pm 11.41 s). The main effect of effect of RA gender indicated that animals remained on the rod longer when female RAs were in the room $(31.13 \pm 3.97 \text{ s})$ compared to when male RAs were in the room (25.99 \pm 3.67 s), B = -15.09, SE = 5.71, p = 0.01. Similar to findings from the fear learning paradigm, the number of female interactions (129) exceeded the number of male interactions (96) with the rats which may explain this difference. Nested model comparisons failed to find significant main effects

of weight, diet, and any significant interactions among the entered predictors. Results are illustrated in Figure 11.

Experimental

Diet and water consumed

Four dams were assigned to each diet condition when they arrived to the animal testing facility. Diet and water consumption were analyzed using linear regression analyses for reasons previously explained. Analyses demonstrated a significant difference between the 7012_N (1009.52 ± 118.55 g) and the 7012_{N-Cu} (753.18 ± 78.68 g) dams, B = 256.35, t(12) = -2.25, p = 0.04. Follow-up analyses also indicated that the 7012_N dams consumed more than the CC dams (702.85 ± 17.39 g), B = 306.68, t(12) = -2.69, p = 0.02. There were no other significant differences in consumption among groups. In addition, there were no significant differences in water consumption among the four dietary conditions (all p > 0.10).

Experimental animals' total consumption was analyzed using linear regression with diet entered as a predictor. The overall model revealed that CC animals (5831.35 ± 80.18 g) consumed less diet than the other three conditions, B = -2194.00, SE = 411.9, p< 0.001. There were no significant differences in diet consumption among the 7012_N (8025.37 ± 267.94 g), 7012_{N-Cu} (8132.83 ± 192.06 g), and 7012_{N+Zn} (8269.10 ± 460.96 g) animals (all p > 0.90). This difference may be explained by pellet density differences between the CC base and the 7012_N base. Statistical analyses also indicated a significant difference in water consumption, B = -1482.50, SE = 468.6, p < 0.01, such that CC animals (9614.95 ± 290.24 g) consumed less water than 7012_N animals (11097.45 ± 174.81 g) and 7012_{N-Cu} animals (11293.27 \pm 299.21 g) (both *p* < 0.05). No significant differences in water consumption were reported between the CC animals and the 7012_{N+Zn} animals (10307.75 \pm 494.59 g) or among 7012_N, 7012_{N-Cu}, and 7012_{N+Zn} animals.

Water dosage

Five lab water samples and two zinc water samples from the beginning of the experiment were lost and could not be analyzed. Because three out of four conditions were on lab water, more lab water samples were made. Sixteen lab water samples and six zinc water samples were taken and analyzed. Linear regression analyses indicated a difference in pH between Zn and water samples, B = -0.25, SE = 0.09, t(20) = -2.90, p =0.01. Most likely, the Na₂CO₃ treatment did not sufficiently reduce pH of Zn water (6.93 \pm 0.03) to the pH of lab water (7.19 \pm 0.05); however, with the exception of two lab water samples (pH = 7.6 and 7.5), all samples were in acceptable pH range for consumption (6.8 - 7.2). Linear regression analyses confirmed that Zn content was significantly higher in Zn water samples compared to lab water samples, B = 8.71, SE = 1.05, t(20) = 8.32, p < 0.001. We note, however, that one Zn water sample appeared to not contain Zn, but this may have been due to mislabeling of a test tube. Other metal ions in the water samples remained consistent between water sample types. Abnormally high Cu levels were reported in one lab water sample. This was attributed to the testing facility losing cold water pressure for unknown reasons on the day of water bottle refilling.

Animal weight

The selected mixed effects model incorporated ID as a nested term. Figure 12 illustrates the main effect of time, B = 93.07, SE = 2.35, t(328) = 39.57, p < 0.001, indicating that rats gained significant amounts of weight between each weighing over the experiment. Model comparisons failed to find a significant effect due to diet or a time by diet interaction.

Fear conditioning

Freezing behavior during the 180 seconds prior to tone onset (habituation) was first examined. The selected model incorporated ID as a nested term and minute (first, second, third) as a predictor. The model yielded a main effect of minute, B = 3.03, SE = 0.68, p < 0.001, such that freezing increased from the first minute ($5.06 \pm 0.84\%$) to the third minute ($11.11 \pm 1.35\%$) during habituation. As mentioned, these results are not uncommon and were similar to results obtained in the pilot portion of this thesis. Nested model comparisons failed to find any effect of diet, weight, RA gender, wave, and box placement and any significant interactions among predictors on freezing during habituation.

Freezing behavior during conditioning was analyzed with ID listed as a nested term. The selected model yielded a main of effect of tone presentation, B = 0.33, SE = 0.04, p < 0.001, indicating that rodents significantly froze more over three tone/shock pairings. There was an increase in freezing percentage from the first tone ($6.50 \pm 0.99\%$) to the third tone ($46.21 \pm 3.64\%$). Subsequent nested model comparisons failed to indicate a main effect of diet, weight, RA gender, wave, and box placement as well as significant interactions among predictors, indicating that animals froze similarly over the three tone/shock pairings. Results are illustrated in Figure 13.

Fear conditioning, extinction, recall, and recall 2

Freezing during the habituation periods was first analyzed. The model incorporated ID and day nested terms. The selected model yielded a main effect of minute, B = 8.99, SE = 0.68, p < 0.001, indicating that freezing increased during habituation periods. There was also a main effect of day, B = 3.94, SE = 1.25, p < 0.01, but no significant minute by day interaction. Freezing during habituation was relatively low on conditioning ($8.19 \pm 0.65\%$) but was significantly higher on the first day of extinction ($49.90 \pm 1.79\%$). Freezing continued to decrease from extinction recall habituation period ($42.90 \pm 1.99\%$) to the extinction recall 2 habituation period ($23.65 \pm 1.54\%$). This may indicate that contextual interference may have led to the relatively high freezing rate on the first day of extinction but that animals exhibited decreased anxiety throughout the extinction learning paradigm. Nested model comparisons failed to find any effect of diet, weight, RA gender, wave, and box placement and any significant interactions among predictors on freezing during habituation.

Cued-based freezing data were analyzed next. The full fear learning model incorporated fear conditioning, extinction, recall, and recall 2 in order to measure freezing behavior over the four days of testing. Time in seconds and weight in grams were transformed into z scores in order to improve scaling and interpretation of coefficients. The selected model incorporated Rat ID and day as nested terms. The final model yielded a significant main effect of day, B = 339.79, SE = 20.24, p < 0.001, a

significant main effect of tone presentation, B = -540.52, SE = 33.25, p < 0.001, and qualifying significant interaction, B = 73.58, SE = 5.03, p < 0.001. Freezing for all animals during conditioning increased with each tone/shock pairing as discussed. The following day during extinction training, average freezing across tones was higher (77.92 $\pm 0.82\%$) compared to freezing on conditioning (34.41 $\pm 2.17\%$) indicating successful cue-based learning. Freezing began to decrease starting at tone 5 (92.68 $\pm 2.12\%$) and continued to decrease through the final tone (67.67 $\pm 3.73\%$) during extinction. Freezing during extinction recall decreased from the first tone (72.44 $\pm 2.51\%$) to the last tone (32.08 $\pm 3.15\%$), demonstrating strong extinction-based learning. In extinction recall 2, freezing decreased from the first tone (55.34 $\pm 3.03\%$) to the last tone (37.24 $\pm 3.02\%$), with the lowest freezing percentage recorded at tone 6 (24.21 $\pm 2.96\%$). Nested model comparisons failed to indicate a main effect of diet, weight, RA gender, wave, and box placement as well as significant interactions among predictors. Results are illustrated in Figure 14.

Analyses were also conducted on the number of fecal boli counted after each training session throughout the four days of testing. As expected, there was a main effect of day such that the number of fecal boli decreased throughout the four days, B = -0.41, SE = 0.14, p < 0.01, indicating a decrease in anxiety throughout the extinction paradigm with the highest number of fecal boli recorded on fear extinction (5.89 ± 0.24) and the lowest number of fecal boli recorded on extinction recall 2 (2.74 ± 0.32). Unlike the pilot portion of the study, we failed to find any effects due to RA gender. Nested model

comparisons also failed to find any main effects of diet, weight, wave, and box placement as well as significant interactions among entered predictors.

Accelerating rotarod performance

Mixed effects modeling was used to examine differences in duration spent on a non-rotating rod during exposure training at both nine weeks and four months of age. ID was entered as a nested term. Statistical analyses indicated a main effect of age, B = 20.40, SE = 1.29, p < 0.001, indicating that rats spent more time on the non-rotating rod at four months of age (58.38 ± 0.75 s) compared to nine weeks of age (17.62 ± 2.48 s). This finding does not exclude the possibility that mere exposure to the rod, rather than age, may have improved performance during exposure training. Model comparisons failed to find any effects on training at nine weeks and four months of age due to diet, wave, RA gender, and weight.

The full rotarod model excluded exposure training and assessed performance over the three days of experimental testing at both nine weeks and four months of age. Weight in grams was transformed into z scores in order to improve scaling. The selected model consisted of Rat ID, age, and day as nested terms. Statistical analyses indicated a main effect of age, B = 41.59, SE = 3.61, p < 0.001, as well as a significant age by day interaction, B = -12.96, SE = 1.50, p < 0.001. Rodents performed better at this task when they were four months of age compared to nine weeks of age with improvement noted on the first day of testing. Nine-week old rodents had relatively low scores on the first day of testing (26.94 ± 1.77 s) compared to their scores at four months of age (83.91 ± 2.35 s). This finding indicated that exposure to the test may have improved scores. There was

also a main effect of trial, B = 6.06, SE = 1.01, p < 0.001, indicating that scores improved over trials on a given testing day. Nested model comparisons failed to find main effects or interactions on training at nine weeks and four months of age due to diet, wave, RA gender, and weight. Results are illustrated in Figure 15.

Open field testing

Due to technical difficulties, two animals' (one CC animal and one 7012_{N+Zn}) files were not saved and could not be included in analyses. One non-blind and two blind coders independently scored all animals; thus inter-rater reliability (IRR) was examined using a two-way, agreement, average-measures intraclass correlation (ICC). Based on existing criteria (Cicchetti, 1994; Hallgren, 2012; McGraw & Wong, 1996), the ICC yielded good agreement among coders, *ICC* = 0.62. Scores were then averaged across the three coders to produce a final score for each animal.

Linear regression analyses with follow-up post-hoc tests indicated a main effect of diet such that 7012_N animals moved significantly more than 7012_{N+Zn} animals (B =29.09, SE = 9.68, p = 0.02) and CC animals (B = 41.53, SE = 10.45, p = 0.001). 7012_{N-Cu} animals moved significantly more than 7012_{N+Zn} animals (B = 41.43, SE = 10.76, p =0.002) and CC animals (B = 53.86, SE = 11.99, p < 0.001). There were no significant differences between 7012_N animals and 7012_{N-Cu} (p = 0.63). In addition, there were no significant differences between the 7012_{N+Zn} and CC animals (p = 0.63). There was a main effect of weight such that heavier animals moved more compared to lighter animals, B = 0.42, SE = 0.12, p = 0.001; however, there was no significant interaction between diet condition and weight. Results are reported in Figure 16.

3.2.8: Adrenal to body weight ratio

Linear regression indicated no significant differences in adrenal gland weight to body weight ratios among animals. Adrenal gland weights are reported in Figure 17.

CHAPTER FOUR: DISCUSSION

This thesis was composed of two experiments that utilized dietary manipulation to induce changes in fear extinction learning and motor coordination. The first part of this thesis examined two different control diets and the effects on the aforementioned behaviors. This experiment demonstrated the need to scrutinize ingredient-base for all diets selected for dietary manipulation studies. The second part of this thesis explored the effects of Cu deficiency and Zn supplementation on behavior. Although the second experiment yielded unexpected outcomes, we were able to propose alternative mechanisms by which learning and motor impairment occurred.

The pilot portion of the study saw few behavioral differences between animals on the 7012_S diet and animals on the CC diet. Although much research is dedicated to the study of ingredients that compose diets from manufacturers, assignment or labeling a feed as a "control" diet is a rather arbitrary and hastily process. For this thesis, we compared two control diets in order to benefit future studies that will potentially utilize one of these diets as a control condition. The 7012_S animals weighed less than the CC animals which could have impacted behavioral data involving motor coordination. In this case, we did not find differences in freezing or rotarod performance but 7012_S animals consistently performed better than CC animals. 7012_S animals exhibited faster fear

extinction and greater latency to remain on a rotating rod, indicating improved extinction learning and motor coordination, respectively.

The experimental portion of this thesis examined the effects of Cu deficiency and Zn supplementation on behavior. This portion of the study utilized an open-formula 7012_N base that strictly controlled for seasonal availability of ingredients. Although differences among diet conditions were nonsignificant, the 7012_{N-Cu} and 7012_{N+Zn} animals exhibited faster fear extinction learning compared to 7012_N and CC animals on the first day of extinction training. On extinction recall and extinction recall 2, differences among 7012_{N} , $7012_{\text{N-Cu}}$, and $7012_{\text{N+Zn}}$ animals were reduced, but the CC animals exhibited higher freezing (Figure 14). The CC animals' freezing levels remained consistently higher than those of the 7012_N , 7012_{N-Cu} , and 7012_{N+Zn} animals which suggested that weight could have impacted our behavior; however, statistical analyses failed to find a main effect of weight on freezing. Likewise, statistical analyses failed to find differences among diet conditions for rotarod performance. Weight also failed to explain differences in performance as indicated by failure to improve model fit in mixed effects modeling. Performance did improve from approximately two months of age to four months of age (Figure 15) which suggests that mere exposure to the rod prior to the second round of testing improved scores. Day by trial interactions indicated that performance did improve over three days of testing with improvements occurring between specific trials. We recommend maintaining this protocol and/or increasing the number of testing days in order to assess motor coordination over time.

In contrast to null findings from fear extinction and rotarod performance, there were dietary differences in open field testing (Figure 16A). 7012_N and 7012_{N-Cu} animals moved more in the chamber compared to 7012_{N+Zn} and CC animals, and there were no differences between the 7012_N and 7012_{N-Cu} animals nor the 7012_{N+Zn} and CC animals. There was also a main effect of weight in open field activity such that heavier animals moved more than lighter animals, but this effect was seen only in the 7012_{N-Cu} , 7012_{N+Zn} , and CC animals (Figure 16B). It is possible that these diets interacted with weight gain which confounded general locomotion in both open field activity and fear learning. Further investigation utilizing weight-matched animals and control behavioral measures are needed to explore the effects of dietary manipulation.

Addressing limitations in this thesis is paramount for preserving the validity and quality of research concerning dietary manipulation. The modalities for inducing Cu deficiency and Zn supplementation were different; thus dietary manipulation is confounded by delivery method prompting the need for alternative methods for accurately measuring Cu and Zn in food and water supplies. It is important to note, however, that simply adding given amounts of biometals (i.e. Cu or Zn) to diet can alter the physical properties of the diet entirely; thus Zn could not have been easily added to the food supply without potentially reducing Cu levels even more. Copper could not be removed from the water supply because water chelation, or the removal of metals from water, is not cost-effective and would reduce the ecological validity of the project design. Future studies should utilize the same modality for manipulating biometal content.

For the experimental portion of this thesis, we were advised to implement stricter control over the amount of batch-to-batch ingredients of our diet orders; thus experimental design was strengthened by including a controlled, open-formula 7012 diet (7012_N) and excluding the commonly-used yet uncontrolled 7012 diet (7012_S) that was first assessed in the pilot portion of the study. The 7012_N and 7012_S diets have not been compared, but 7012_N and 7012_S comparisons could reveal potential behavioral differences due to seasonal availability of ingredients. Although the composition of the 7012_N diet was controlled throughout the four month manipulation, Cu levels in the 7012_{N-CD} diet varied from 7 to 12 ppm whereas the 7012_N and 7012_{N+Zn} diets remained stable at 23 ppm. Manipulation of Cu, the primary manipulation in this study, should not be approximated, warranting greater need for stricter open-formulas from manufacturers.

Results also prompt consideration of dietary interactions due to the presence of natural and purified ingredients. The concentration of isoflavones, a specific class of phytoestrogens, correlates with soybean content (Thigpen, Locklear, Caviness, Stokes, & Setchell, 1992), an ingredient found in both the 7012_s and 7012_N diets, but not the CC diet. In this thesis, animals on the isoflavone/phytoestrogen-containing 7012 feed exhibited faster fear extinction learning on the first and second days of extinction compared to animals on the phytoestrogen-free CC diet. Animals on the 7012 feed in both the pilot and experimental portions of the study also exhibited lower levels of freezing, indicating lower levels of anxiety prior to tone onset. In addition, animals raised on a 7012 feed weighed significantly lighter than animals raised on the CC diet, and animals on the CC diet also were consistently the worst in the rotarod task in the pilot

portion of the study. In the experimental portion, two-month-old CC animals also performed poorly in the rotarod task although they exhibited improvement at four months of age. It is possible that the lack of soy content in the CC diet, which is composed of casein rather than soy and wheat, produced both weight and behavioral differences, explaining the poorer performance of the CC animals in pilot and experimental testing. Phytoestrogen content (or lack thereof) may have impacted the $7012_{\text{N-CD}}$ as well due to a potential association between Cu deficiencies and accelerated genital growth in both rats and mice (Thigpen et al., 2003; Thigpen et al., 2004). Phytoestrogens could have exacerbated the effect of Cu deficiency, explaining the low count of 7012_{N-CD} animals in the experimental study. Several institutions suggest using phytoestrogen-free diets in order to eliminate unanticipated dietary hormone/biometal interactions (Brown & Setchell, 2001). It should be emphasized that the macronutrient content and other ingredients are not held constant across the 7012-base diets and CC diet, and that soy and phytoestrogens cannot be confidently designated as the driving mechanisms behind the CC animals' poor behavioral scores and weight gain.

The CC animals in both the pilot and experimental portions weighed heavier than the other groups. Recent research suggests a relationship among high fat/sugar diets, obesity, and cognitive deficits in a variety of learning and memory paradigms. Reichelt and colleagues (2015) administered a nutrient-poor, high fat diet to six-week-old Sprague-Dawley male rats for eight weeks and conducted trace fear conditioning. As expected, the high-fat diet rats weighed significantly more than their control-diet counterparts at the time of testing. The high-fat diet rats froze less during contextual

testing indicating impaired contextual retention, but froze more when exposed to the CS in a different context. Researchers suggest that contextual information failed to be encoded due to compromised hippocampal function and that cued-learning was impaired (Reichelt et al., 2015). Other experiments and reviews propose that high food-intake and excessive weight interfere with cognitive-related, hippocampal-dependent behavioral tasks (Davidson et al., 2013; Yamada-Goto et al., 2012. Experimental designs incorporating proper control or elimination of phytoestrogens, weight-matched animals, and possibly controlled food administration would be ideal for future studies in our lab. In addition, weight-matched rodents would allow for us to examine direct causal relationships between dietary manipulation and behavioral performance.

It is possible that the Cu and Zn levels utilized in the experimental portion failed to impact mechanisms underlying learning and memory but still impacted weight gain. The Cu concentration was mildly lowered compared to lowered concentrations to less than 1 ppm Cu in other studies. In one particular mouse study, C57BL/6J mice on a 0.6 ppm Cu diet for nine weeks weighed approximately the same as mice on a diet that contained 15 ppm Cu, but Cu-deficient mice displayed more anxious behavior compared to control animals when tested in the elevated zero maze (Bousquet-Moore et al., 2010). In contrast, poor weight gain was evident in Prohaska and Smith's study (1982) that found significant body and brain weight differences in young C57BL mice and Sprague-Dawley rats on a 0.05 ppm Cu-deficient diet. A more recent study found that 25 day-old Holtzman Sprague-Dawley offspring, whose dams were given a 0.04 ppm Cu diet during gestation and lactation, were born ataxic and were much smaller in size compared to offspring whose dams were given 20 mg Cu/L through the water supply (Lyons & Prohaska, 2010). We chose not to administer a diet with such low Cu levels to avoid the risk of premature death due to severe Cu deficiency; thus it is possible that Cu levels were not lowered enough to impact the neural mechanisms behind learning and memory in the offspring that survived.

Likewise, the 10 ppm Zn supplementation in the water supply, in conjunction with the 63 ppm Zn in the food, may have been too low to truly induce an indirect Cu deficiency and associated behavioral deficits. Studies that assess Zn toxicity have used Zn levels as high as 100 mg/Kg to 180 mg/Kg, but also note that peripheral Zn may not influence total brain Zn levels due to strict regulatory mechanisms and binding proteins (Yang et al., 2013). Yang and colleagues (2013) utilized a standard rodent diet containing 30 ppm Zn in conjunction with deionized water (control), with 15 ppm Zn supplementation ("low" dose), or 60 ppm Zn supplementation ("high" dose). After three months of diet administration, data revealed that ICR mice on the 60 ppm "high" dose Zn water exhibited impairment in reward-alternation working memory and contextual discrimination whereas mice on the 15 ppm "low" dose group exhibited improved performance on spatial memory tasks and contextual discrimination (Yang et al., 2013). Researchers concluded that mice in the 60 ppm Zn water condition exhibited impairment of hippocampal-dependent learning and memory. In this thesis, rats on 10 ppm Znsupplemented water exhibited faster fear extinction and improved rotarod performance compared to control rats which mimics results from the aforementioned study. Unlike Yang and colleagues' study, the experimental design for this thesis utilized cued-based

fear learning, but contextual-based learning should also be assessed for future research studies.

"Zinc supplementation" should not be confused with "Zn toxicity" due to the growing body of evidence that suggests that Zn supplements may remediate functional impairment found in several neuropsychiatric disorders. Zinc deficiency and lowered Zn intake have been linked to depression as demonstrated by numerous human clinical trials (Amani, Saeidi, Nazari, & Nematpour, 2010; Maes et al., 1994; Nowak et al., 1999; Siwek et al., 2010). Animal studies also provide evidence that suggests Zn deficiency is linked with depression as demonstrated by increased immobility in the forced-swim test and tail suspension test (Mlyniec et al., 2012; Mlyniec & Nowak, 2012; Tamano, Kan, Kawamura, Oku, & Takeda, 2009; Whittle Lubec, & Singewald, 2009). Animal and human studies have linked Zn supplements with several improved mental health outcomes and anti-depressant therapeutic response (Franco et al., 2008; Nowak, Siwek, Dudek, Zieba, & Pilc, 2003; Nowak et al., 2003; Ranjbar et al., 2014; Siwek et al., 2009); however, results have yet to remain consistent across the several facets of depression, and the consensus for other behaviors have yet to be fully established. For example, research has shown an improvement in rat pups' spatial memory (in a Morris Water Maze task) whose dams were administered 16 mg/kg and 32 mg/kg Zn during pregnancy (Piechal, Blecharz-Klin, Pyrzanowska, & Widy-Tyszkiewicz, 2012). Other studies conducted in our lab, however, emphasize that Zn supplementation of 10 ppm can impair spatial memory as well as long-term memory in rats and mice (Flinn et al., 2005; Howell, 2014; Railey et al., 2010). Studies assessing dose-response curves for Zn supplementation and

well as Cu deficiency should be conducted in order to determine the appropriate amount of Zn and Cu needed to evoke both behavioral improvements and deficits.

Results from this study can be used to support the neuroprotective role of Zn following TBI (Cope, Morris, Scrimgeour, VanLangdingham, & Levenson, 2011; Morris & Levenson, 2013). Cope and colleagues (2012) examined Zn as a potential treatment for traumatic brain injury using two different diets: a Zn-adequate diet that contained 30 ppm Zn, and a Zn-supplemented diet that contained 180 ppm. After receiving injury to the medial frontal cortex, rats were administered one of the two diets. Additional rats in each diet condition received a 30 mg/Kg intraperitoneal injection of Zn one hour after injury. Injury itself resulted in an increase in anxiety-like and depressive behaviors and impairment in learning and memory-related tasks. Both dietary supplements in conjunction to the Zn injection improved scores on a 2-bottle saccharin test, indicating that these two treatments reduced anhedonia following injury (Cope et al., 2012). In addition, the 180 ppm Zn supplementation, but not the Zn injection, improved performance in the Morris Water Maze task, demonstrating improvement in spatial memory of rats (Cope et al., 2012). Although this study utilized different modalities for Zn supplementation (e.g. food and injections versus water supply), the results provide evidence that links Zn supplementation with resiliency following TBI and long-term potentiation, the underlying mechanism for learning and memory (Bliss & Collingridge, 1993). It should be noted, however, that the relationship between Zn and its therapeutic effects are not perfectly linear, and that the efficacy of Zn may be dependent on the

severity of TBI, duration of injury, and time of treatment administration (Portbury & Adlard, 2015).

In summary, the pilot portion of this thesis explored the effects of two different control diets, and the experimental portion assessed the impact of Cu deficiency and Zn supplementation on learning, memory, and motor behaviors. In the pilot portion, we were able to provide evidence that suggests that the CC diet, which is higher in fat content and phytoestrogen-free, may have produced behavioral deficits as shown by slower fear extinction and poorer rotarod performance compared to animals on a 7012-base diet. In the experimental portion, the Cu-deficient animals and Zn-supplemented animals exhibited faster fear extinction learning and better rotarod performance compared to both the 7012-control animals and CC animals. We proposed that the new formulation of the $7012_{\rm N}$ diet may have impacted our behavioral data. In addition, the Cu-deficient animals' improved scores may have been due to low body weight. Zn-supplemented animals, on the other hand, may have had improved scores due to receiving a "healthy" dose of Zn which coincides with several studies previously mentioned, although there is work that contradicts our assumption. Despite these unexpected data, this thesis provides valuable insight for diet selection and strongly supports the notion that dietary manipulation can impact behavior in animal models. This type of manipulation may be of interest to those who wish to study biometal dyshomeostasis in several neurological diseases.

APPENDIX

Table 3	
Animals per wave for conditioning/extinction of	and rotarod testing

Portion of study/Diet	CC	7012s	7012 _N	7012 _{N-CD}	7012 _{N+Zn}
Pilot – 1 wave	12	13	Not assessed	Not assessed	Not assessed
Experimental – wave 1	9	Not assessed	9	7	8
Experimental – wave 2	9	Not assessed	9	6	9

Note. Cu = copper; Zn = zinc; 7012_s = standard 7012 diet; CC = Cu-control diet from Lippi, 2014; 7012_N = newly formulated diet controlling for extraneous ingredients; 7012_{N-CD} = new 7012 diet with reduced Cu levels; 7012_{N+Zn} = new 7012 diet with normalized Cu levels but with added Zn to water supply. Open field testing occurred in a single wave (N = 66) for the experimental portion of the study.











Day (D) / Trial (T)

Figure 1. Data from Lippi, 2014. **[A]** Fear extinction recall 2 from Lippi (2014). Percent freezing decreased across 18 tones on the third day of extinction training, with significant differences reported between the prenatal Cu deficient group and the postnatal control and postnatal Cu deficient groups for tones 5 through 10. In light of this finding, this thesis utilized prenatal exposure to Cu deficiency and Zn supplementation in order to potentially induce greatest impairment in behavior. Error bars denote S.E.M. **[B]** Accelerating rotarod task from Lippi (2014). Time spent on the road increased across the three days of testing, with significant differences reported between the prenatal Cu deficient group and the postnatal Cu deficient group on day 2. The prenatal Cu deficient group consistently performed the worst across all three days of testing. In concordance with fear extinction findings, prenatal exposure to Cu deficiency and Zn supplementation was selected for this thesis. Error bars denote S.E.M.

Teklad LM-485 Mouse/Rat Sterilizable Diet

Product Description- 7012 is a fixed formula, autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from 300 to 600 mg/kg. Absence of animal protein and fish meal minimizes the presence of nitrosamines. 7012 is supplemented with additional vitamins to ensure nutritional adequacy after autoclaving. Also available certified (7012C) and irradiated (7912).

Ingredients (in descending order of inclusion)- Ground corn, dehulled soybean meal, ground oats, wheat middlings, dehydrated alfalfa meal, soybean oil, corn gluten meal, calcium carbonate, dicalcium phosphate, rewers dride veast. iodized salt. choline cholride, kaolini. maanseium oxide.
L-lysine, DL-methionine, locust bean gum, gelatin, ferrous sulfate, menadione sodium bisulfite complex (source of vitamin K activity), vitamin E acetate, thiamin mononitrate, calcium pantothenate, manganous oxide, niacin, copper sulfate, zinc oxide, vitamin A acetate, pyridoxine
hydrochloride, riboflavin, vitamin D ₃ supplement, vitamin B ₁₂ supplement, folic acid, biotin, calcium iodate, cobalt carbonate.

Standard Product Form: Pellet

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% 51 % 1.1 % 0.: % 0.7 % 0.1
% 1. % 0. % 0. % 0. % 0.
% 1.0 % 0.1 % 0.1 % 0.1 % 0.1
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Helping you do research better

vitamins		
Vitamin A * f	IU/g	30.0
Vitamin D ₃ ^{*,9}	IU/g	2.4
Vitamin E	IU/kg	150
Vitamin K ₃ (menadione)	mg/kg	80
Vitamin B, (thiamin)	mg/kg	95
Vitamin B ₂ (riboflavin)	mg/kg	14
Niacin (nicotinic acid)	mg/kg	100
Vitamin B ₆ (pyridoxine)	mg/kg	17
Pantothenic Acid	mg/kg	87
Vitamin B12 (cyanocobalamin)	mg/kg	0.09
Biotin	mg/kg	0.77
Folate	mg/kg	7
Choline	mg/kg	2200
Fatty Acids		
C16:0 Palmitic	%	0.6
C18:0 Stearlc	%	0.2
C18:1w9 Oleic	%	1.3
C18:2w6 Linoleic	%	2.6
C18:3w3 Linolenic	%	0.3
Total Saturated	%	0.8
Total Monounsaturated	%	1.3
Total Polyunsaturated	%	2.9
Other		
Cholesterol	mg/kg	1
	2.201.20	

^a Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

^b Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

^c Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber

⁴ Energy density is a calculated estimate of metabolizable energy based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

* Indicates added amount but does not account for contribution from other ingredients

⁹ 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

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Figure 2. Harlan Teklad LM-485 "7012s" rodent diet. This diet, which contains isoflavones, is often used as a control diet in diet-manipulation studies. This diet will be used in the pilot portion of the study. Ratio of copper to zinc: 23 ppm Cu/63 ppm Zn.

¹ 1 IU vitamin A = 0.3 µg retinol

Teklad Custom Research Diet Data Sheet

TD.130231 Cu Control Rodent Diet (16 ppn	n)	Key F	eatures	
Formula	a/Ka	Purified D	Diet	
Casein, low Cu & Fe	200.0	Control D	liet	
L-Cystine	3.0	• Copper		
Corn Starch	394.772	• Zinc		
Maltodextrin	132.0			
Sucrose	100.0	Selected N	utrient Information	n'
Soybean Oil	70.0		% by weight	% kcal from
Cellulose	50.0	Protein	17.7	19.1
Vitamin Mix, AIN-93-VX (94047)	10.0	сно	59.0	63.5
Choline Bitartrate	2.5	Fat	7.2	17.4
		Kcal/g	3.7	
Calcium Carbonate	17.136	1 Values are	calculated from ingredi	ent analysis or
Potassium Phosphate, monobasic	9,41	manufactur	ter data	
Potassium Citrate, monohydrate	3.4	Takind Diels	are designed & man	declured for researc
Sodium Chloride	3.55	ourposes onl	v. V.	industrica for reactance
Potassium Sulfate	2.23	14 - Di		
Magnesium Oxide	1.17	Key Pla	nning Inforn	nation
Ferric Citrate	0.6	Products	are made fresh to ord	ler
Zinc Carbonate	0.065	 Store prod 	fuct at 4°C or lower	
Manganous Carbonate	0.03	 Use within 	6 months (applicable	to most diets)
Cupric Carbonate	0.029	 Box labele 	d with product name	
Potassium Iodate	0.0005	manufactu	uring date, and lot nur	mber
Sodium Selenate	0.0005	Replace d	liet at minimum once	per week
Ammonium Paramolybdate, tetrahydrate	0.0004	More fre	ouent replacement ma	v be advised
Sodium Meta-Silicate, nonahydrate	0.07	Lead time		,
Chromium Potassium Sulfate, dodecahydrate	0.013	· 2 week	s non-irradiated	
Lithium Chloride	0.0008	· 4 week	s irradiated	<u> </u>
Boric Acid	0.004	1 1000	0 110010100	
Sodium Fluoride	0.003	Sector Sector Sector		Contraction of the local division of the loc
Nickel Carbonate Hydroxide, tetrahydrate	0.0015	Product	Specific Inform	mation
Ammonium Meta-Vanadate	0.0003	 1/2" Pellet 	or Powder (free flow	ing)
		Minimum	order 3 Kg	
FBHO, antioxidant	0.014	 Irradiation 	not advised	
		· Contac	t a nutritionist for reco	ommendations
		Options (Fe	es Will Apply)	
		 Rush orde 	er (pending availability	()
		 Irradiation 	(see Product Specific I	Information)
		 Vacuum p 	ackaging (1 and 2 Kg))
		Speak With	A Nutritionist	
		• (800) 48	3-5523	
		 askanutri 	tionist@harlan.com	1
		Internation	al Inquiry	-
		· Outside U.	S.A or Canada ·	
		 askanutri 	tionist@harlan.com	SGS
Footnote		Place Your	Order (U.S.A. & Canada))
A rodent diet formulated to support gestation and lactation with 1	6 ppm Cu added via	· Place Ord	er · Obtain Pricing ·	
cupric carbonate. Zinc carbonate adds approx. 40 ppm Zn. The e	estimated	· Check (Order Status	
background level of Cu is 0.3 - 0.7 ppm and for Zn 4 - 8 ppm. Fo	rmulated as a	• (800) 48	3-5523	
control diet to TD.130230.		• (608) 27	7-2066 facsimile	
		tekladinfo	@harlan.com	
		Help	oing you do rese	arch better
Iarlan Laboratories - PO Box 44220 - Madison, WI 53744-4220	- 1	00		090
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Figure 3. Harlan Teklad "copper control – CC" diet. This diet was formulated to be a control diet for Lippi et al. (2013) copper deficiency study. This diet will be used for both pilot and experimental portions of the study. Ratio of copper to zinc: 16 ppm Cu/40 ppm Zn.

Teklad Custom Research Diet Data Sheet

TD.140386	Custom Control Diet (701	2) Key Features
Ingredients <i>(in descen</i> Ground corn, dehulled sc dehydrated alfalfa meal, dicalcium phosphate, bre kaolin, magnesium oxide	ding order of inclusion) sybean meal, ground oats, whe soybean oil, corn gluten meal, wers dried yeast, iodized salt, , L-lysine, DL-methionine, locu	e Natural Ingredient Diet e Teklad LM-485 7012 Diet e Closed/Proprietary Formula choline chloride, st bean gum, gelatin,
ferrous sulfate, menadior	ne sodium bisulfite complex (so	wrce of vitamin K Key Planning Information
activity), vitamin E acetat manganous oxide, niacin pyridoxine hydrochloride, supplement, folic acid, bi	(e, triamin mononitrate, carciun), copper sulfate, zinc oxide, vit , riboflavin, vitamin D ₃ supplem otin, calcium iodate, cobalt car	 Products are made fresh to order Products are made fresh to order Store product at 4°C or lower Use within 6 months (applicable to most diets) Box labeled with product name, manufacturing date, and lot number Replace diet at minimum once per week More frequent replacement may be advised Lead time: 2 weeks non-irradiated 4 weeks irradiated
Footnote		Product Specific Information
The Teklad 7012 formula b	wilt from scatch	 1/2" Pellat or Pewder (free flowing)
		Minimum order 3 Kg
Calastad Nutriant Inform	mation ¹	Ontione (See Will Apple)
Selected Nutrient inform	% by weight	% kcal from Rush order (pending availability)
Protein	19.4	25.3 Irradiation (see Product Specific Information)
Carbohydrate?	44.4	58.1 • Vacuum packaping (1 and 2 Kg)
Fat	5.6	16.5
Kcal/g	3.1	Contact Us
¹ Values are calculated from ingre ² Estimated digestible carbohydra	adiant analysis or manufacturer data ne	Obtain Pricing - Check Order Status tekladinfo@harlan.com (800) 483-5523 option 3
Teklad Diets are designed & /	manufactured for research purposes	askanutritionist@harlan.com International Inquiry (Ousside USA or Canada) askanutritionist@harlan.com
 Speak With A Nutritionist (800) 483-5523 opti 	t ion 4	Place Your Order (USA & Canada) · Please Choose One ·
askanutritionist@harlau Harlan Laboratories, Inc. PO Box 44220	n.com	https://webapps.harlan.com/teklad-orders/ tekladinfo@harlan.com (800) 483-5523 option 3 (608) 277'-2066 facsimile
Madison, WI 53744-4220		www.harlan.com Helping you do research better
Hartan, Hartan Labonrioles, Hitping you do toteo and the Hartan togo are trademarks and trade rate in 2016 (doi:10.1000/clime.html	anth bellar, mes of Harlan Labortorics, br.	and party for to restarting better

Figure 4. Harlan Teklad custom 7012 ("7012_N") diet. This diet will be utilized in two conditions in the experimental portion of the study: the control (7012_N) condition and the zinc-supplemented condition (7012_{N+Zn}) which will induce indirect copper deficiency by including Zn in the water supply. This diet strictly controls for seasonal availability of ingredients and is a more natural chow. Ratio of copper to zinc for 7012_{N+Zn}: 23 ppm Cu/63 ppm Zn. Ratio of copper to zinc for 7012_{N+Zn}: 23 ppm Cu/73 ppm Zn.

Teklad Custom Research Diet Data Sheet

ngredients (In desce Ground corn, dehulled s Jehydrated alfalfa meal dicalcium phosphate, br caolin, magnesium oxid	nding order of inclusion) soybean meal, ground oats, wheat mid I, soybean oil, corn gluten meal, calciuu rewers dried yeast, iodized salt, cholin- ie, L-lysine, DL-methionine, locust bea	dlings, n carbonate, e chloride, n gum, gelatin,	 Natural Ingredient Diet No Supplemented Copper Teklad LM-485 7012 Diet Closed/Proprietary Formula
errous sulfate, menadio	one sodium bisulfite complex (source o	of vitamin K	Key Planning Information
activity), vitamin E aceta nanganous oxide, niac hydrochloride, riboflavir acid, biotin, calcium iod	ate, mamin mononitrate, carcium pant in, zinc oxide, vitamin A acetate, pyride n, vitamin D ₃ supplement, vitamin B ₁₂ s ate, cobalt carbonate.	onerate, oxine upplement, folic	 Products are made fresh to order Store product at 4°C or lower Use within 6 months (applicable to most diets) Box labeled with product name, manufacturing date, and lot number Replace diet at minimum once per week More frequent replacement may be advised Lead time: 2 weeks non-irradiated 4 weeks irradiated
Footnote			Product Specific Information
Footnote Modification of the Teklad copper. Background Cu is	i 7012 that does not included a suppleme s estimated to be 7 - 12 ppm.	nted source of	Product Specific Information 1/2" Pellet or Powder (free flowing) Minimum order 3 Kg Irradiation available upon request
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ootnote Modification of the Teklad opper. Background Cu is Selected Nutrient Info Protein Carbohydrate ²	t 7012 that does not included a suppleme s estimated to be 7 - 12 ppm. mmation ⁵ % by weight 19.4 44.4 5.6	Med source of % kcal from 25.3 58.1 16.5	Product Specific Information • 1/2" Pellet or Powder (free flowing) • Minimum order 3 Kg • Irradiation available upon request Options (Faes Will Apply) • Rush order (pending availability) • Irradiation (see Product Specific Information) • Vacuum packaging (1 and 2 Kg)
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Figure 5. Harlan Teklad minimal copper ("7012_{N-CD}") diet. This diet will be used in the experimental portion of the study. This diet also controls for seasonal availability of ingredients but is modified to induce a direct copper deficiency. Cu levels are estimated to be 7 to 12 ppm. Ratio of copper to zinc: 7 - 12 ppm Cu/63 ppm Zn.



Figure 6. Schedule for behavioral testing for the experimental portion of this thesis. Testing occurred in two waves for conditioning/extinction and rotarod whereas testing was completed in one wave for open field testing. Animals were sacrificed over a period of three days at the conclusion of behavioral testing.



Figure 7. Fear conditioning and extinction, recall, and recall 2 paradigms. **[A]** The first line depicts the fear conditioning protocol. Rats were given 180 seconds to explore the chamber. A 20 second, 85 dB, 2 kHz tone played and coterminated with a 2 second, 0.5 mA scrambled footshock. Shocks coterminated at 260 seconds and 320 seconds. **[B]** The second line depicts fear extinction, recall, and recall 2 protocols. Rats explored the altered chamber for 180 seconds prior to tone onset. Eighteen tones separated by 40 second intervals played in a similar fashion to fear conditioning; however, no shock will was delivered.


Figure 8. Weight (in grams) of pilot animals at weaning age and four months. Data indicated a main

Figure 8. Weight (in grams) of pilot animals at weaking age and four months. Data indicated a main effect of time such that animals were heavier at 4 months ($7012_8 = 484.92 \pm 7.59$ g; CC = 498.10 ± 14.56 g) compared to weaking age ($7012_8 = 57.70 \pm 1.97$ g; CC = 64.07 ± 4.08 g). There were no significant differences between diet conditions at weaking or four months of age.



Pilot: Freezing percentage during fear conditioning

Figure 9. Freezing percentage of pilot animals during fear conditioning. Animals received three tone shock pairings on the first day of testing. Data indicated a main effect of time such that animals exhibited increased freezing from the first tone/shock to the third tone/shock, with 7012_s animals exhibiting lower freezing percentages on all three tones/shocks. The 7012_s animals exhibited lower freezing ($5.58 \pm 2.56\%$) on the first/tone shock compared to CC animals ($15.92 \pm 5.92\%$). This trend was repeated for the second tone/shock (7012_s : $48.42 \pm 6.76\%$ vs. CC: $52.72 \pm 7.93\%$) and for the third tone/shock (7012_s : $50.42 \pm 8.18\%$ vs CC: $63.91 \pm 8.33\%$). These differences between diet conditions were not significant which was indicated by diet failing to contribute to model fit in statistical analyses.





Figure 10. Graphical representation of averaged freezing percentage across tones for fear conditioning, extinction, extinction recall, and extinction recall 2 during pilot testing. Statistical modeling revealed a significant day by tone interaction which indicated that freezing for all animals increased during conditioning and began to decrease during extinction, with notable freezing decreases occurring on recall and recall 2 days. Freezing during extinction recall 2 decreased from the first tone ($60.04 \pm 5.73\%$) to the last tone ($48.07 \pm 7.20\%$), with lowest freezing percentage reported at tone 9 ($18.73 \pm 5.09\%$). Model comparisons failed to find an effect due to diet, weight, box placement, and RA gender on freezing.



Pilot: Rotarod performance across three days at approximately 4 months of age (P122)

Figure 11. Pilot animals' latency to fall in rotarod testing. A significant day by trial interaction indicated that testing improved from the first day of testing $(8.79 \pm 1.69 \text{ s})$ to the third day of testing $(58.44 \pm 6.36 \text{ s})$ and revealed that latency improved on the third day of testing between trial 1 (47.68 ± 8.98 s) and trial 2 (64.44 ± 11.41 s). Although CC animals consistently performed poorer than 7012_S, statistical models failed to find significant effects of weight or diet.



Average weight of experimental animals throughout experiment

Figure 12. Experimental animal weight throughout four month diet manipulation. Analyses indicated that rats gained a significant amount of weight throughout the course of the four month manipulation; however, there was no main effect of diet and no diet by time interaction. It is important to note that rats were not weight-matched at time of testing. At the start of testing which was at approximately four months (P114) of age, CC animals weighed more (447.49 \pm 5.34 g) compared to 7012_N (409.33 \pm 6.88 g), to 7012_{N-Cu} (394.72 \pm 10.22 g), and to 7012_{N+Zn} (415.85 \pm 4.90 g) animals.



Experimental: Freezing percentage during fear conditioning

Figure 13. Experimental fear conditioning at four months of age. There was a main effect of tone presentation such that freezing increased from the first tone $(6.50 \pm 0.99\%)$ to the third tone $(46.21 \pm 3.64\%)$. There were no significant differences in freezing among dietary conditions as indicated by a non-significant main effect of diet. These findings indicate successful conditioning although freezing levels were somewhat low (below 60%) for this paradigm.



Experimental: Averaged freezing percentage across tones for fear conditioning, extinction, extinction recall, and extinction recall 2

Day

Figure 14. Graphical representation of averaged freezing percentage across tones for fear conditioning, extinction, extinction recall, and extinction recall 2 during experimental testing. Statistical modeling revealed a significant day by tone interaction which indicated that freezing for all animals increased during conditioning (46.21 \pm 3.64%) and began to decrease during extinction (77.92 \pm 0.82%), with notable freezing decreases occurring on recall and recall 2 days. Freezing during extinction recall 2 decreased from the first tone (55.34 \pm 3.03%) to the last tone (37.24 \pm 3.02%), with lowest freezing percentage reported at tone 9 (24.21 \pm 2.96%). Model comparisons failed to find an effect due to diet, weight, box placement, wave, and RA gender on freezing.



Day (D) / Trial (T)

[B] Experimental: Rotarod performance across three days at approximately 4 months of age (P124)



Figure 15. Accelerating rotarod performance. **[A]** Rotarod performance at approximately two months of age. There was a main effect of day such that performance improved over three days of testing; however, there was no main effect of diet indicating that diet did not induce motor impairment at this age. **[B]** Rotarod performance at approximately four months of age. Statistical analyses indicated a main effect of age such that animals were more skilled at this task at four months. A day by trial interaction indicated that animals improved over testing days with notable improvement occurring on day 3. Both diet and weight failed to improve model fit; thus they were determined to not have effect of performance.





Figure 16. Open field activity in four month old rats. **[A]** Significant differences were reported between the 7012_N ($118.87 \pm 6.85s$) compared to 7012_{N+Zn} ($93.46 \pm 6.98s$) and CC ($93.18 \pm 7.21s$) animals (*p < 0.05). 7012_{N-Cu} animals ($126.21 \pm 9.88s$) moved significantly more than the 7012_{N+Zn} and CC animals (**p < 0.01). There were no significant differences between the 7012_N and 7012_{N-Cu} animals, nor differences between 7012_{N+Zn} and CC animals. **[B]** Analyses indicated no weight*diet interaction. It was noted, however heavier animals in the 7012_{N-Cu} , 7012_{N+Zn} , and CC groups moved more than lighter animals, but this effect was not seen in 7012_N animals.



Adrenal gland weight in grams at time of death (P136)

Figure 17. Adrenal gland weight at time of death (approximately four months and three weeks of age). Adrenal gland weight to body weight ratios did not differ among animals. This was also confirmed through linear regression analyses that showed that adrenal gland weights did not differ among the 7012_N (0.08 ± 0.00 g), the 7012_{N-Cu} (0.08 ± 0.01 g), the 7012_{N+Zn} (0.10 ± 0.01 g), or CC (0.10 ± 0.01 g) animals, as shown in the figure above.

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