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Metabolic and Behavioral Effects of Zinc Deficiency in Rats

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METABOLIC AND BEHAVIORAL EFFECTS OF
ZINC DEFICIENCY IN RATS

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DEDICATION

This thesis and all the hard work put into it is dedicated to my mother, Connie Evans, who has supported me through all my endeavors, and my son, John Evans, who has been my one and only inspiration. Without these two people, I would not be half the person I am today. Thanks, I love you both!
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TABLE OF CONTENTS

List of Tables ........................................................................................................................................ vii

List of Figures ....................................................................................................................................... viii

Abstract ................................................................................................................................................ ix

1. INTRODUCTION .......................................................................................................................... 1
   Regulation of Food Intake .............................................................................................................. 1
   Zinc .............................................................................................................................................. 3
   Other Causes of Anorexia ............................................................................................................. 7
   Conclusion .................................................................................................................................... 7

2. METHODS ..................................................................................................................................... 8
   Metabolic and Consummatory Experiments .............................................................................. 8
   Behavioral Experiments .............................................................................................................. 9
   Statistical Analysis ...................................................................................................................... 9

3. RESULTS ....................................................................................................................................... 10
   Food Intake and Body Weight ..................................................................................................... 10
   Metabolic Variables and Locomotor Activity ......................................................................... 14
LIST OF TABLES

1. Summary of Gaetke et al., 2002 ......................................................................................... 4
LIST OF FIGURES

1. Effect of zinc deficiency on body weight and caloric intake .........................11
2. Effect of zinc deficiency on food events ...............................................................12
3. Effect of zinc deficiency on feeding time ...............................................................13
4. Effect of zinc deficiency on MR ........................................................................15
5. Effect of zinc deficiency on RQ .........................................................................16
6. Effect of zinc deficiency on locomotor activity ...................................................17
7. Effect of zinc deficiency on BMR .......................................................................18
8. Effect of zinc deficiency on novelty-seeking behavior .......................................20
9. Effect of choice on total fluid intake of zinc deficient rats ................................21
10. Saccharin and water intake in zinc deficient rats ...............................................22
11. Effect of choice on food intake and body weight of zinc deficient rats .............23
Disruptions in the regulation of food intake and metabolism can result in obesity or anorexia. It is clear that zinc deficiency results in anorexia and previous research suggests the existence of alterations in energy efficiency and metabolism. Zinc deficiency results in changes in neuropeptides that regulate energy intake and expenditure. Numerous diagnostic conditions also result in anorexia and wasting, similar to that of zinc deficiency. However, the mechanism underlying these abnormalities remains unknown, and the behavioral and metabolic effects of zinc deficiency have not been fully established. Therefore, the purpose of this work is to fully characterize the behavioral and metabolic consequences of zinc deficiency and its association with anxiety, and to suggest mechanisms underlying the anorexia associated with zinc deficiency and other clinical conditions. Despite differences in locomotor activity between zinc deficient (ZD, <1 ppm zinc, ad lib) and pair-fed (PF, 28 ppm zinc, amount consumed by ZD), there were no differences in MR, RQ or BMR. This suggests a greater metabolic cost of activity may exist in ZD. Contrary to previous studies, this work shows a decrease in consummatory food intake with zinc deficiency without evidence of alterations in appetitive motivational behaviors. This suggests that zinc deficiency alters the hedonic impact of food reward, but not the motivation to seek food. The data presented here also suggests an anxiogenic effect associated with zinc deficiency, which may be involved in the hedonic changes in food intake. Furthermore, this work suggests that alterations of the opioid reward system may be involved in the anorexia and anxiety-like behaviors produced by zinc deficiency.
Appetite and food intake are influenced by internal and external factors. Externally, learned behaviors and environment can influence meal patterns and food choices. Internally, numerous endogenous compounds regulate hunger and satiety signals, which control the amount of food consumed over long intervals. Disruption in the synchrony of these factors can result in conditions such as obesity and anorexia-induced wasting. While environmental factors play key roles in the development of these conditions, it is important that we understand the internal mechanisms involved in the regulation of food intake. Dietary zinc deficiency results in anorexia in laboratory animals and has been implicated in anorexia produced by other conditions. Furthermore, many psychological conditions, such as anxiety, and physiological conditions, such as cancer and inflammation, lead to anorexia and increased energy expenditure. Gaining an understanding of the parallels between zinc deficiency, these conditions and internal mechanisms controlling food intake and metabolism may provide opportunities for pharmacological and therapeutic strategies to prevent the anorexia and the subsequent wasting which results.

**Regulation of Food Intake**

Feeding behavior can be divided into two primary phases, 1) the appetitive phase, which involves searching for food, and 2) the consummatory phase, which involves actual eating of the food (Hillebrand et al., 2002). Hunger and satiety are internal cues involved in the initiation, duration, and termination of a meal. These cues are regulated by multiple peripheral and central signals, including hormones, peptides and neurotransmitters, that sense and respond to changes in the external and internal environment. Additionally, rewarding properties of food influence the composition and amount of food that is consumed.

*Peripheral Systems.* Peripheral signals which influence food intake include sensory inputs, such as taste and smell, gastrointestinal inputs, blood-borne factors, such as circulating glucose and leptin, metabolic signals, and neural input from the vagus nerve. The type of food consumed influences the level of hormones and peptides in circulation. For example, consumption of carbohydrate results in production and secretion of insulin, while cholecystokinin (CCK) is released in response to fat ingestion. Leptin is an example of how
nutrient stores can affect food intake and metabolic rate. Leptin is hormone produced by adipose tissue in relation to adipose mass, which, along with many other functions, decreases food intake. All of the aforementioned hormones and peptides have central targets involved in the regulation of food intake and energy expenditure.

Central Systems. While numerous brain regions are involved in food intake regulation, the hypothalamus is regarded as the main feeding center of the brain. Several hypothalamic nuclei are involved in food intake. Neurons of the arcuate nucleus (ARC) are considered ‘first order neurons’ because of their location near the median eminence and contact with peripheral blood-borne factors (Hillebrand et al., 2002). ARC neurons project to ‘second order neurons’ in the paraventricular nucleus (PVN), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), and lateral hypothalamic area (LHA). These second order neurons project to the nucleus of the solitary tract (NTS) and dorsomotor nucleus of the vagus (DMV). In addition to the hypothalamus, the central nucleus of the amygdala (CeA) is involved in the emotional or rewarding aspect of food (Norgren, 1976). Among others, interconnections between the PVN, NTS, and CeA exist, which communicate to regulate food intake.

Neuropeptides. The modulation of food intake by the central as well as the peripheral system is modulated by neuropeptides. Orexigenic peptides are those that increase food intake and decrease energy expenditure. Anorexigenic peptides decrease food intake and increase energy expenditure. The ARC contains two distinct groups of neurons, those that contain orexigenic peptides and those that contain anorexigenic peptides. Orexigenic hypothalamic peptides include neuropeptide tyrosine (NPY) and agouti-related protein (AgRP). Anorexigenic hypothalamic peptides include pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). POMC is a precursor molecule that is typically considered anorexigenic, because it can be processed into the anorexigenic melanocortins (MCs), including α-melanocyte-stimulating hormone (α-MSH), but it can also be processed into the orexigenic endogenous opioid, β-endorphin. Processing of POMC depends on numerous factors and is cell-specific. MC receptors 3 (MC3-R) and 4 (MC4-R) are distributed throughout the brain, including in the PVN and LHA, and interfere with food intake and increase energy expenditure upon MC binding, especially that of α-MSH (Forbes et al., 2000). Other peptides regulating food intake and energy expenditure are the orexigenic peptides melanin-concentrating hormone
(MCH) and galanin, and the anorexigenic peptides corticotropin-releasing hormone (CRH) and glucagon-like peptide-1 (GLP-1).

**Opioids and Reward.** Endogenous opioids (β-endorphin, enkephalins, and dynorphins) are a family of peptides that increase the intake of palatable foods (Glass et al., 1999). Opioids and their receptors are widely distributed in the central nervous system (CNS) and abundantly localized within feeding centers in the brain, including the PVN, NTS, and CeA (Mansour et al., 1995). These peptides are associated with the rewarding properties of food as well as the maintenance, but not the initiation, of a meal (Kirkham et al., 1984). Recently, opioids have been shown to be involved with the actions of other orexigenic peptides such as NPY and AgRP (Britton and Southerland, 2001; Hagan et al., 2001).

**Mesocorticolimbic Structures and Reward.** The CeA, as well as other limbic structures in the brain (ventral tegmental area (VTA), nucleus accumbens (NAc), and prefrontal cortex (PFC)), are directly involved in the hedonic impact of food. Neurons in these brain areas comprise the mesocorticolimbic system. These areas are involved in the ‘wanting’ and ‘liking’ of food, and the relative palatability of the food taste (Levine and Billington, 1997). The mesocorticolimbic system has connections with the food centers of the brain, including the PVN and NTS, impacting the motivation to eat and the hedonic value of food. While opioids are prevalent within the mesocorticolimbic system, dopamine (DA) is the most prevalent neurotransmitter, and seems to be involved in ‘wanting’ of food and other stimuli, but does not influence the ‘liking’ of the stimuli (Pecina and Berridge, 2000).

**Zinc**

**Zinc in Energy Metabolism.** Zinc is an essential trace metal involved catalytically or structurally in 100+ enzymes. As a requirement for DNA and RNA polymerases, it is involved in regulation of gene transcription and protein synthesis. Many enzymes involved in nutrient metabolism and digestion are zinc-dependent. Zinc is incorporated into insulin and plays a role in carbohydrate metabolism (Gaizer, 1989). Zinc deficiency has been shown to decrease insulin response resulting in impaired glucose tolerance (Faure et al., 1992). Carboxypeptidase A is a zinc-dependent metallo-enzyme necessary for protein metabolism (Li and Solomon, 2001). Carbonic anhydrase is an enzyme essential for disposal of CO₂ in respiration and requires zinc (Komai et al., 2000).
Given the role of zinc in both food intake regulation and metabolic enzymes, it is reasonable to hypothesize that zinc deficiency will have an effect on energy metabolism and metabolic rate (MR). Zinc deficiency results in a reduced anabolic response to food due to a reduced capacity for, and activation of, protein synthesis in muscle and thymus (Giugliano and Millward, 1987). While both zinc deficient and pair-fed rats have reductions in weight compared to controls, zinc deficient rats have an increase in carcass fat compared to pair-fed rats (White, 1988). Furthermore, zinc supplementation has been shown to significantly increase the rate of weight gain in malnourished children without significantly affecting food intake (Golden and Golden, 1981). These studies suggest a disruption of energy metabolism in zinc deficiency.

The methods and results from a recent study are summarized in Table 1 (Gaetke et al., 2002). While this work attempted to address the important issues of MR, activity and food efficiency in zinc deficiency, this study had numerous shortcomings. For example, MR and locomotor activity were measured on days 0, 10, 20, and 30 of zinc deficiency. For measurement of MR, rats were placed in metabolic cages for only 40 minutes in the light phase. Thus the influence of circadian rhythms and light cycle were not measured. Furthermore, locomotor activity was measured only in the dark phase. Dark phase activity in rats is associated with feeding behavior, thus data are currently unavailable for the effect of zinc deficiency on overall activity changes not associated with feeding. Our preliminary data suggest that pair-fed animals exhibit increases in both dark and light phase activity. Without knowing the light cycle activity levels of the animals, it is not known if changes in MR in the Gaetke study are the result of changes in activity. Additionally, the time of feeding was not reported. This would be

<table>
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<tr>
<th>Measurement</th>
<th>Method</th>
<th>Results</th>
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<tr>
<td>Metabolic Rate</td>
<td>O_2 consumption adjusted for body weight</td>
<td>+Zn &gt; -Zn, PF</td>
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<tr>
<td></td>
<td>Light cycle only</td>
<td>PF &gt; -Zn only on day 10</td>
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<td>40 minute period on days 0, 10, 20, 30</td>
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<tr>
<td>Activity</td>
<td>Number of movements (horizontal and vertical)</td>
<td>activity associated with feeding (first 2 hr period)</td>
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<td></td>
<td>Dark phase only</td>
<td>day 10 +Zn, PF &gt; -Zn</td>
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<td></td>
<td></td>
<td>day 20 Control &gt; PF, -Zn</td>
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<tr>
<td></td>
<td></td>
<td>day 30 +Zn, PF &gt; -Zn</td>
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<tr>
<td>Food Efficiency</td>
<td>Weekly basis weight gain/food intake</td>
<td>+Zn &gt; PF &gt; -Zn</td>
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Table 1. Summary of Gaetke et al., 2002
expected to have a profound affect on food intake and anticipatory activity, especially in pair-fed animals. Food efficiency was measured on a weekly basis. It is not clear whether measurements were made for the entire week or just on the day reported. No attempt was made by the researchers to explain food efficiency differences between groups. Furthermore, activity differences were not accounted for in their measurements.

**Zinc in Anorexia.** Zinc deficiency results in anorexia, growth retardation, and hypogonadism. In experimental models, a decrease in food intake occurs 3-5 days after the initiation of dietary zinc restriction, with a peak in food intake every 3-4 days (Tamaki et al., 1995). This cyclic anorexia persists as long as zinc is restricted. Previous analyses of food intake have shown that zinc deficient rats reduce the number of meals but not the relative size of each meal (Rains et al., 1998) and carbohydrate consumption is largely reduced, while fat and protein are spared (Kennedy et al., 1998). Zinc deficient animals also have altered NPY and galanin metabolism (Selvais et al., 1997).

Associations of zinc in human anorexia nervosa (AN) exist, though they are less clear. Nearly half of all AN patients tested are zinc deficient (Humphries et al., 1989). This may be due in part to the fact that absorption of zinc is diminished in AN, but could also be attributed to the dietary and activity patterns of AN patients. Zinc supplementation in the treatment of AN nearly doubles the rate of weight gain in AN patients (Su and Birmingham, 2002; Birmingham et al., 1994). This could be explained by a restoration of appetite, but could also be attributed to abnormalities in protein synthesis and energy metabolism seen with inadequate zinc status.

**Zinc Deficiency and Hypothalamic Neuropeptides** While it is clear that zinc deficiency results in a profound anorexia, the mechanisms responsible for the reductions in food intake are not understood. Previous work has suggested that the regulation of NPY may be involved. Zinc deficiency leads to a significant increase in NPY mRNA in the ARC (Rains et al., 1998; Selvias et al., 1997) and a modest peptide increase in the PVN (Rains et al., 1998). Other work (Selvias et al., 1997) reported no increase in hypothalamic NPY peptide, but looked at whole hypothalamus rather than specifically in the PVN. If there is an increase, it does not result in increased feeding behavior. Thus investigators have examined the effect of dietary zinc restriction in NPY receptor function by administering exogenous NPY into the ventricles of zinc deficient rats (Lee et al., 1998). Initial reports showed that NPY injections stimulated feeding in these animals. However, it has now been shown that, while feeding was stimulated, it was not
restored to the amount seen in controls (Williamson et al., 2002). Recent work using microdialysis in control, zinc deficient, and pair-fed rats suggests that NPY may not be functional because it is not released from the PVN of zinc deficient rats (Huntington et al., 2002). However, NPY knockout animals do not exhibit anorexia (Erickson et al., 1996), suggesting compensatory mechanisms exist. Thus the role of NPY, if any, in zinc deficiency-induced anorexia is not known. Studies of other hypothalamic peptides, such as galanin (Selvias et al., 1997) and neuroactive peptides, such as leptin (Ott and Shay, 1998) have also failed to identify the mechanisms responsible for reductions in food intake seen in zinc deficiency.

Zinc Deficiency in Psychosocial Disorders Major depression and depression-related disorders such as anxiety are often associated with reductions in food intake (Zauszniewski and Ahmad, 2000). Zinc deficiency is also commonly seen in illnesses that have a depression component, such as schizophrenia, eating disorders, and senile dementia (Fan et al., 2002; Humphries et al., 1989; Ronzoni et al., 2001). Furthermore, clinically there is a link between zinc deficiency and co-morbid depression and anxiety. Lower serum zinc is seen in unipolar depressed patients and in the chronic mild stress animal model of depression (Nowak et al., 1999). There is a negative correlation between serum zinc levels and the severity of depression (Maes et al., 1994). Moreover, altered serum zinc concentrations are normalized following successful antidepressant treatment (Maes et al., 1997) and zinc supplementation produces antidepressant-like effects in the forced swim test, a rodent test for depression-like behaviors (Kroczka et al., 2001).

Preliminary data from our laboratory showed that zinc deficient rats displayed anhedonia-like behaviors when given a choice between a solution of 0.05% saccharin and deionized water. Zinc deficiency reduced the ratio of saccharin to water intake ratios (8.2±0.7 vs. 3.0±0.7 mL/d). This suggests that zinc deficiency may induce depression-like behaviors in this model. Interestingly, during this test for anhedonia, it was noted that when rats were given ad libitum access to the saccharin solution and deionized water they did not develop anorexia. A repetition of this experiment confirmed these results.
Other Causes of Anorexia

While decreased food intake is the primary symptom of AN, it is also seen in other conditions. Generalized anxiety disorder (GAD), as well as other anxiety-related disorders (depression, obsessive-compulsive disorder, and posttraumatic stress disorder), often has an anorexia component (DSM-IV). Similarly, conditions that activate an immune response, such as infection and cancer, and increase serum corticosteroids, as seen in zinc deficiency, can result in anorexia and metabolic disturbances leading to muscle wasting and cachexia (Rossi et al., 1999).

Conclusion

The regulation of food intake and metabolism is a complex system. When the system is disrupted, obesity or anorexia can result. It is clear that zinc deficiency results in anorexia and previous research suggests the existence of alterations in energy efficiency and metabolism. Zinc deficiency results in changes in neuropeptides that regulate energy intake and expenditure. Numerous diagnostic conditions also result in anorexia and wasting, similar to that of zinc deficiency. However, the mechanism underlying these abnormalities remains unknown, and the behavioral and metabolic effects of zinc deficiency have not been fully established. Therefore, the purpose of this work is to fully characterize the behavioral and metabolic consequences of zinc deficiency and its association with anxiety, and to suggest mechanisms underlying the anorexia associated with zinc deficiency and other clinical conditions.
CHAPTER 2
METHODS

Metabolic and Consummatory Experiments

Diets and Housing. Weanling male Sprague-Dawley rats were provided ad libitum access to commercially prepared powdered egg white-based zinc adequate (ZA: 28 ppm; n = 6) or zinc deficient (ZD: <1 ppm; n = 9) diets for 9 days, followed by 5 days of zinc re-feeding, where ZD animals were given zinc adequate food. Because zinc deficiency is known to cause anorexia, an additional group of rats was pair-fed (PF; n = 9). PF rats were provided the weighed amount of zinc adequate food eaten by the ZD rats the previous day. Rats were housed in shoebox cages (18 x 9.5 x 5 inches, 13L vol.) and provided ad libitum access to deionized water. Powdered food was contained within a stainless steel feeder to minimize spillage. The cages were placed within custom-constructed environmental chambers that provide computer control of temperature ($T_a = 23 \, ^\circ C$) and the 12:12-h light-dark schedule (lights on at 0700) (Williams et al., 2002). Body weight and food and water consumption were measured daily during the 12$^{th}$ hour of the light phase.

Metabolic Variables. MR and RQ were measured using indirect calorimetry. The shoebox cages were fitted with a custom-made polycarbonate lid providing a near air-tight seal for continuous determination of oxygen consumption (VO$_2$; mL/min) and carbon dioxide production (VCO$_2$; mL/min). A constant flow rate of fresh air into the chamber was set at 0.85 L/min. Mixed cage air was sampled for 30s every 4 min and dried and compressed prior to reaching gas analyzers (Williams et al., 2002). VO$_2$ and VCO$_2$ were determined by open circuit respirometry to isolate successive samples (Williams et al., 2000). Averages for MR and RQ data were calculated during 12 hours of dark-phase and 11 hours of light-phase activity. MR was described as VO$_2$ normalized to body weight. RQ was determined by dividing VCO$_2$ by VO$_2$. Basal MR (BMR) was determined by averaging VO$_2$ from the lowest 15 4-min bins.

Ingestive Behavior Monitoring. Feeding behavior was monitored by a photo beam sensor across the entrance to the feeder. The duration of photo beam breakage (50msec resolution) was monitored by computer and accumulated in 30s bins. The water bottle was positioned in a lick block modified to measure contact at the bottle’s spout. Drinking behavior was computer monitored by the number of licks, which was accumulated every 30s (Williams et al., 2002).
Averages were calculated for data during 12 hours of dark-phase and 11 hours of light-phase ingestive behaviors.

**Locomotor Activity Monitoring.** Locomotor activity was measured using a custom-designed force platform that has a pivot under its center. The shoebox cage was positioned on this platform to obtain quantification of locomotor activity. Stiff strain-gauge load-beam transducers attached under two adjacent corners of the platform prevented the platform from swaying on the pivot. The transducers measured changes in the cage’s center of gravity, allowing localization of the animal’s position in two dimensions. The cumulated distance of locomotor activity in meters was saved every 30s (Williams et al., 2002). Averages were calculated for data during 12 hours of dark-phase and 11 hours of light-phase activity.

**Behavioral Experiments**

**Novelty-seeking Behavior.** After 2 weeks of the ZA (n = 7), ZD (n = 8), or PF diets (n = 8), novelty-seeking behavior was measured using a 20x16x8 in Plexiglass apparatus. The apparatus was equally divided into two compartments. One compartment was completely covered while the other remained open and illuminated. A divider, with a 4x4 opening in the center allowing free movement between compartments, separated the dark and light sides of the apparatus. Each rat was placed in the dark side of the apparatus. The number of times the rat entered into the light side (head and both front limbs) and time spent in the light side was measured for 5 min.

**Two-bottle Saccharin Test.** Weanling male Sprague-Dawley rats were provided *ad libitum* access to zinc deficient (-Zn: <1 ppm) diet for 2 weeks. Each was housed separately in steel hanging cages. Rats were randomly separated into two groups. One group of rats was given free access to deionized water (n = 5). The other group was given free access to 0.05% saccharin solution (n = 10). To study the effect of the choice model, after one week the group receiving saccharin was given an additional bottle containing deionized water for the remainder of the study. Food, water, and saccharin intake and body weights were measured every 48 hours.

**Statistical Analysis**

Data are presented as mean ± SEM. Each experiment was analyzed with a two-way analysis of variance (ANOVA). Significant main effects were analyzed for differences between groups using Tukey’s post hoc test with significance set at P < 0.05.
CHAPTER 3
RESULTS

Food Intake and Body Weight

The effect of zinc deficiency on caloric intake over the 2-week experimental period is shown in Figure 1. At the beginning of the experiment caloric intake (kcals) was similar for all groups. As expected, food intake increased in the ZA group with time (Figure 1A). A decline in caloric intake in ZD and PF animals was observed by day 3 and became significant by day 4 of dietary treatment when compared to ZA ($P < 0.05$). A characteristic peak in caloric intake in ZD and PF rats occurred by day 6. Significant differences remained until day 12, 3 days following the initiation of zinc re-feeding. However, increased caloric intake could be seen in ZD and PF rats immediately following zinc restoration.

The reduction in food intake by ZD and PF rats led to a significant reduction in body weight in these groups compared to ZA (Figure 1B). By day 6, body weights of ZA rats was significantly greater than ZD or PF rats ($P < 0.05$). By day 9, body weights of ZD and PF animals were 60% of ZA ($ZD=66.5\pm2.2$ and $PF=65.2\pm2.4$ vs $ZA=104.6\pm8.5$ g, $P < 0.01$). During the re-feeding period body weights of ZD and PF rats increased at the same rate as ZA, but remained significantly less. No significant differences were observed between ZD and PF rats on any day.

There were no significant differences in water consumption between ZD and PF rats (data not shown). Water consumption patterns in all groups followed that of food consumption.

No significant differences in the number of feeding events or feeding time were observed between groups throughout the study (Figures 2A and 2B).
Figure 1. Effect of zinc deficiency on (A) body weight and (B) caloric intake. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. Body weight for each day is reported as mean ± SEM.
Figure 2. Effect of zinc deficient diet on dark-phase (top, blue) and light-phase (bottom, yellow) food events. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. Food events for each day are reported as mean ± SEM.
Figure 3. Effect of zinc deficient diet on dark-phase (top, blue) and light-phase (bottom, yellow) feeding time. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. Feeding time for each day is reported as mean ± SEM.
Metabolic Variables and Locomotor Activity

MR. MR is expressed as VO$_2$ normalized to body weight (mL/min/kg$^{0.75}$). At the beginning of the study there were no differences in dark phase or light phase MR between groups (Figure 4). Both ZD and PF rats had significantly lower MR by day 4 in the dark phase and day 3 in the light phase. Significant differences remained in the dark phase until day 12, 3 days following the initiation of zinc re-feeding, and day 10 in the light phase. However, increased MR could be seen in both phases in ZD and PF rats immediately following zinc restoration.

RQ. RQ was determined by dividing VCO$_2$ by VO$_2$. RQ was not different between groups at the beginning of the study (Figure 5). RQ tended to follow caloric intake patterns. RQ began to decline by day 3 and became significantly lower in ZD and PF animals compared to ZA on day 5 (P<0.05). On day 6, when food intake increased in both groups, ZD and PF RQ returned to ZA levels, but quickly dropped thereafter and remained significantly less until zinc was restored to ZD. Following zinc restoration, RQ of all groups was not different.

Locomotor Activity. Locomotor activity was not significantly different between groups at the beginning of the study (Figure 6). Dark-phase activity was significantly higher in ZD and PF rats than ZA on days 3 and 4, but by day 5 ZD and ZA were not different and PF remained higher throughout the rest of the study, even after food intake was restored to ZA levels following zinc re-feeding. On day 9 PF locomotor activity was 6-times that of ZA and ZD (316 ± 43 vs 53 ± 6 and 59 ± 5 m). Light phase locomotor activity was significantly lower in all animals compared to dark phase. By day 5, PF animals exhibited 2-times as much light phase locomotor activity as ZA and ZD (PF=93.5 ± 33.9, ZA=40.6 ± 4.4, ZD=41.8 ± 3.3 m). No differences in light phase locomotor activity were observed between ZA and ZD rats. Light phase activity in PF animals declined and was restored to that of ZA and ZD when caloric intake was restored (day 12).

BMR. BMR was expressed as the average VO$_2$ from the lowest 15 4-min bins. There were no differences between groups in dark phase or light phase BMR at the beginning of the study (Figure 7). As expected, BMR increased in ZA rats with time and body weight. ZD and PF BMRs were significantly lower than ZA by day 5 and remained lower throughout the study (P<0.05). ZD and PF BMR increased following zinc re-feeding at the same rate as ZA. There were no significant differences between dark phase or light phase BMR in any group.
Figure 4. Effect of zinc deficient diet on dark-phase (blue) and light-phase (yellow) MR. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. MR for each day is reported as mean ± SEM.
Figure 5. Effect of zinc deficient diet on dark-phase (top, blue) and light-phase (bottom, yellow) RQ. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. RQ for each day is reported as mean ± SEM.
Figure 6. Effect of zinc deficient diet on dark-phase (top, blue) and light-phase (bottom, yellow) locomotor activity. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. Locomotor activity for each day is reported as mean ± SEM.
Figure 7. Effect of zinc deficient diet on dark-phase (blue) and light-phase (yellow) BMR. Weanling male rats were fed zinc adequate (ZA, black squares, n = 6) or zinc deficient (ZD, red triangles, n = 9) diet for 9 days. An additional group was pair fed to the ZD animals (PF, green circles, n = 9). On day 9 ZD rats were given zinc adequate food for the remainder of the study. BMR for each day is reported as mean ± SEM.
Novelty-seeking Behavior

Novelty-seeking behavior was tested in ZD, ZA, and PF rats using the light-dark test of anxiety for 5 minutes. Figure 8A shows the mean number of entries into the novel environment for each group. ZD rats explored the novel (light) environment significantly fewer times than ZA, and ZA explorations were significantly less than PF (ZD = 4.0 ± 0.7, ZA = 8.4 ± 2.0, PF = 15.4 ± 3.0, P<0.05). The same pattern was observed for the amount of time the rats spent in the novel environment (Figure 8B). However, only PF time was significantly different (ZD = 74.6 ± 25.8, ZA = 115.3 ± 31.0, PF = 225.0 ± 36.3 s, P<0.05).

Saccharin and Choice

Figure 9 shows the effect of choice on total fluid intake in zinc deficient rats. Fluid intake in zinc deficient rats that received only deionized water (ZDW) remained constant throughout the study (~25g/2d). In zinc deficient rats that received 0.05% saccharin solution as their sole fluid source (ZDS), 2-day fluid intake declined 50% by day 7. Following the initiation of choice (additional bottle of deionized water for ZDS rats on day 8), total fluid intake increased 50% from day 1 and 300% from day 7. By day 13, ZDS rats were consuming significantly more fluid than ZDW (ZDS=41.7 ± 7.2 vs ZDW=20.5 ± 5.3 g, P<0.05). Consumption of water accounted for nearly half of total fluid consumption following the initiation of water as a fluid choice (20.5 ± 6.3 of 36.8 ± 4.7 g/2d) and nearly 1/3 by the end of the study (Figure 10).

Food intake (Figure 11A) and body weights (Figure 11B) were not different in the first week of the study, when ZDS rats had only saccharin and ZDW rats had only water as their sole fluid source. Following the initiation of choice, food intake and body weight increased in ZDS rats. By the end of the study ZDS food intake was 100% higher than ZDW (29.3 ± 1.2 vs 12.8 ± 1.2 g/2d) and ZDS body weights rose 65% (117.2 ± 5.0 vs 75.0 ± 8.3 g).
Figure 8. Effect of zinc deficient diet on (A) entries into a novel environment and (B) time spent in a novel environment. Male weanling rats were fed zinc adequate (ZA, black, n = 7) or zinc deficient (ZD, red, n = 8) diet for 2 weeks. An additional group was pair fed to the ZD animals (PF, green, n = 8). Rats were then tested in the novelty-seeking test for anxiety for 5 minutes. Number of entries into and time spent in the novel (light) environment is reported as mean ± SEM. * Significance at P<0.05.
Figure 9. Effect of choice on total fluid intake of zinc deficient rats. Male weanling rats were fed zinc deficient diet for 16 days. ZDW rats received water as their sole fluid source (blue squares, n = 5) throughout the study. ZDS received 0.05% saccharin as their sole fluid source for 1 week and were given water in addition to saccharin for the remainder of the study (pink diamonds, n = 10).
Figure 10. Saccharin and water intake in zinc deficient rats. Male weanling rats were fed zinc deficient diet for 16 days. ZDS received 0.05% saccharin as their sole fluid source for 1 week and were given deionized water in addition to saccharin for the remainder of the study (n = 10). Saccharin solution intake (bottom, magenta) and water intake (top, lavender) was recorded.
Figure 11. Effect of choice on (A) food intake and (B) body weight in zinc deficient rats. Male weanling rats were fed zinc deficient diet for 16 days. ZDW rats received water as their sole fluid source (blue triangles, n = 5) throughout the study. ZDS received 0.05% saccharin as their sole fluid source for 1 week and were given deionized water in addition to saccharin for the remainder of the study (red squares, n = 10).
CHAPTER 4  
DISCUSSION

Zinc deficiency produces profound anorexia in animals. The mechanism by which this occurs is not well defined, nor do we have a full understanding of the metabolic and behavioral consequences of zinc deficiency. Thus, this study was designed to fully characterize the effects of dietary zinc deficiency on metabolism and behavior in male weanling rats. A further purpose of this work was to suggest, based on literature and these data, possible mechanisms by which this anorexia occurs and its association with other causes of anorexia, such as cancer, inflammation, and anxiety.

**Zinc Deficiency and Food Intake**

Food intake in rats declines ~50% within 5 days of the onset of a zinc deficient diet (Rains et al., 1998; Lee et al., 1998). Furthermore, when given a three-choice macronutrient self-selection paradigm, zinc deficient animals decrease nearly all their food intake by decreasing intake of carbohydrates (Rains et al., 1998). Following zinc repletion, carbohydrate intake is resorted to control levels, as is total food intake, within 24 hours. Studies using intra-peritoneal injections of 2 deoxyglucose (2DG), which stimulate carbohydrate consumption in control rats, found that zinc deficient animals did not respond to this stimulus (Cole et al., 2002). This suggests an impaired glucose sensing system in zinc deficiency, which may contribute to the decreases in carbohydrate intake. The current data are consistent with these reports. Food intake was 50% less than ZA by day 5. Furthermore, 0.05% saccharin solution was reduced as zinc deficiency progressed, suggesting a possible attempt by the rat to reduce the consumption of carbohydrate. Though saccharin does not contribute to carbohydrate intake, studies have shown that its consumption will elicit similar insulin responses to that of sucrose consumption (Malaisse et al., 1998). What are different in the current study compared to previous reports are the feeding behaviors associated with zinc deficiency. Rains et al. reported a delayed onset of the first meal in the dark phase and a reduction in total meals in zinc deficient rats (1998). This is contradictory to the present study in which no differences were observed in dark phase or light phase food events or food time between ZD, ZA, or PF rats, suggesting no difference in the number of meals consumed, but a difference in the size of the meals. It is also noted that in the present study investigators observed a peculiar pattern in the ZD animals: the powdered food was
moved around in the food dish as if the rats were playing with their food rather than eating it. They were motivated to go to the food, but consumed less than controls while they were at the food dish. This suggests a decline in the consummatory, but not the appetitive behaviors in the rats.

Zinc deficiency also affects the regulation of the orexigenic neuropeptide NPY. Food deprivation increases hypothalamic NPY mRNA (Swart, et al., 2001). NPY is also increased in the PVN and ARC of zinc deficient animals (Lee et al., 1998). However, this endogenous increase in NPY does not stimulate feeding. Furthermore, injection of exogenous NPY into the PVN stimulated feeding in both ZD and ZA rats (Lee et al., 1998), but later studies revealed this response in ZD was not normalized to that of ZA controls (Williamson et al., 2002). This suggests that there must be other factors involved, which inhibit the function of endogenous NPY and impair exogenous NPY-induced feeding responses in ZD rats.

Giugliano and Millward measured protein turnover, RNA and DNA in thymus and skeletal muscle of zinc deficient rats and zinc deficient, adrenalectomized (ADX) rats (1987). Zinc deficiency induced a loss of DNA and protein in thymus, and a decrease in protein synthesis in thymus as well as in skeletal muscle. This effect was less severe in ZD-ADX rats, which had increased mortality and less cycling of food intake than ZD. They also reported inverse correlations between urinary corticosterone and cyclic food intake in ZD rats. Zinc deficiency activates the hypothalamus-pituitary-adrenocortical (HPA) axis and induces profound increases in glucocorticoid synthesis (DePasquale-Jardieu and Franker, 1980). This rise in corticosterone is similar to that seen in protein calorie malnutrition (PCM) (Alleyne and Young, 1967) and many have suggested that zinc deficiency in children produces similar muscle wasting and immune disturbances to that of PCM (Franker et al., 2000). The results of these studies suggest a role for corticosterone in the reduced protein synthesis and cyclic food intake, as well as the diminished immune response seen with zinc deficiency.

Zinc Deficiency and Metabolism

No differences in body weight between ZD and PF rats were observed in the present study, nor were there any differences in caloric intake, MR, or RQ. There were, however, differences in locomotor activity. PF rats had significantly greater dark phase and light phase activity than ZD or ZA rats, though ZD and PF rats consumed the same amount of food. This suggests that the mechanism which increases locomotor activity in food restricted animals is
zinc-dependent. Many have reported a decrease in food efficiency ratios (body weight gain to food intake) in zinc deficient animal models compared to pair-fed controls (Essatara et al., 1974; White, 1988). Others have failed to see these differences (Gaetke et al., 2002). That we see increases in locomotor activity in PF compared to ZD without increases in MR, suggests there may be metabolic differences between the two groups. We hypothesized that ZD rats would have higher BMR compared to PF, suggesting a maladaptive metabolic response to caloric restriction with zinc deficiency. However, this was not the case. There was no difference in BMR between ZD and PF. But there still remains the possibility of metabolic differences. Tobin et al. measured the effect of iron deficiency on VO$_2$max and food efficiency with and without exercise and found that exercise exacerbates the poor growth associated with iron deficiency (1993). It is possible that there is a higher metabolic cost of activity in ZD, which would explain the current data. Studies measuring the metabolic response to voluntary or treadmill exercise are needed to examine this further (Yancey and Overton, 1993).

**Anxiety-like Behavior**

Zinc deficiency produces anorexia, impaired immunity, and may cause anhedonia. These conditions are also seen in anxiety (Ninan, 1999). Furthermore, both zinc deficiency and anxiety activate the HPA axis and increase plasma corticosterone (DePasquale-Jardieu and Franker, 1980; Ninan, 1999). Based on these observations, we hypothesized that zinc deficiency would also produce anxiety-like behaviors in the rat, which may partially account for the anorexia it produces. Indeed, ZD animals displayed significantly fewer entries into the novel environment than either ZA or PF. These behaviors are consistent with anxiety in rodent models (Kabbaj et al., 2000). Studies using other methods of testing anxiety, such as the elevated plus maze, are needed to strengthen this conclusion. Zinc deficient rats also reduce their intake and preference of sweet solution, suggesting anhedonia consistent with depression. When these rats are given a fluid choice (between saccharin and water), food and total fluid intake increases to that of ZA rats. Choice abolishes the anorexia associated with zinc deficiency. It is possible that the choice of fluids elicits a reward response, which increases the synthesis of orexigenic peptides and stimulates food intake. The final section of this discussion will attempt to explain this phenomenon.

An increased level of corticosterone has been implicated in other causes of anorexia and wasting, including inflammation- and cancer-induced anorexia. These conditions have also been
known to induce depression- or anxiety-like behaviors in animals and humans. For instance, injection of lipopolysaccharide (LPS), which activates an inflammatory response, results in reduced food consumption, body weight, locomotor activity, and social interaction, as well as a decrease in free consumption of saccharin (Yirmiya, 1996). The anorexia-cachexia syndrome observed in cancer patients is associated with metabolic dysregulation resulting in anorexia, weight loss, negative nitrogen balance, and skeletal muscle wasting (Barber et al., 2000). Cancer patients also often have a component of distress and anxiety, which is believed to be associated with poor outcomes and quality of life (Gallagher et al., 2002). It is reasonable to hypothesize that similar mechanisms may be involved in the anorexia associated with zinc deficiency and these conditions.

**Reward: Dopamine or Opioids?**

It is clear that zinc deficiency, as well as anxiety, inflammation, and cancer produce anorexia. What is unclear is the mechanism by which this anorexia occurs. Activation of the HPA axis, and subsequent increase in corticosterone, has been implicated. However, it is not clear why zinc deficiency activates this system. This work has shown that zinc deficiency reduces food intake by reducing the consummatory phase (size of the meal), without interfering with the appetitive phase (motivation to eat) of the meal. Furthermore, a reward response initiated by fluid choice abolishes the anorexia associated with zinc deficiency. This suggests that the cause of the anorexia is associated with peptides or neurotransmitters associated with reward. This implicates the mesocorticolimbic dopaminergic system or opioids.

**Dopamine.** A number of behavioral and neurochemical methods have been employed to investigate the role of DA in reward. Mesocorticolimbic DA neurons in the ventral tegmental area (VTA) project to nucleus accumbens (NAc), amygdala, prefrontal cortex, and other forebrain regions (Kelley and Berdidge, 2002). Studies using the DA-selective neurotoxin 6-hydroxydopamine (6-OHDA) reveal the importance of this neurotransmitter in reward motivation. Rats become aphagic and adipsic after 6-OHDA lesions (Zigmond and Stricker, 1972). They also lose motivation to seek other rewarding stimuli, such as sex, drugs, and electrical stimulation (Berridge and Robinson, 1998). DA impacts the ‘wanting’ of rewarding stimuli without impacting the ‘liking’ of the reward. This has been shown using paradigms that involved a measure of an instrumental behavior required to obtain a reward, such as bar pressing, and those which use affective facial expressions to measure immediate ‘liking’ or hedonic
impact of a sweet solution. A food deprived rat will press a bar numerous times for a pellet of food and will continue pressing for additional pellets. DA depletion within the ventrolateral neostratum (VLS) produces severe initial bar pressing deficits, which reflects the loss of motivation or ‘wanting’ of the food reward (Cousins and Salamone, 1996). DA does not, however, impact the facial expression reactions, ‘liking’ or ‘disliking,’ to either sucrose or quinine (Pecina et al., 1997). These studies support a role for mesocorticolimbic DA in the initiation and motivational aspect of food reward. In the present work, ZD rats appear to be motivated to eat (no difference in food events or food time), but decrease the amount of food consumed, suggesting that the system which is involved in zinc deficiency-induced anorexia is not a result of DA, rather a result of the orosensory value, or ‘liking,’ of the food. Furthermore, preliminary studies in our lab have not measured differences in NAc DA between ZDW rats who were anorexic and ZDS rats who increased their food intake following fluid choice.

**Opioids.** Studies similar to those mentioned above have been used to examine the opioid peptide neurotransmission on reward. Whereas DA impacts bar pressing for the initial food pellet, reflecting motivational deficits, opioid antagonism using naloxone or naltrexone does not reduce bar presses for the first pellet, but affects presses for subsequent pellets (Kirkham and Blundell, 1984). Furthermore, naltrexone had little effect on the speed at which an animal would attain a food reward in a maze, but decreased the amount of food eaten after contact with the food (Kirkham and Blundell, 1986). Moreover, b- enkephalin knockout mice have selective attenuation of operant responding to food reward under nondeprived, but not deprived, feeding states, suggesting the reward deficit is related to the hedonic value of feeding and not energy homeostasis (Hayward et al., 2002). Opioid antagonism has similar effects on fluid consumption. Naltrexone has been shown to significantly decrease the maintenance phase of fluid consumption in water-deprived rats, without altering the early portion of drinking (Beczkowska et al., 1992). These studies appear to be consistent with the idea that opioid antagonists have an effect after a substantial amount of food has been consumed (Glass et al., 1999), suggesting a relationship between opioids and post-ingestive processes.

Neural systems controlling food intake include the NTS, PVN, and CeA (discussed previously). Opioid peptides and receptors are present within these areas (Mansour et al., 1995). Through a series of experiments using cannulae into these areas, Giraudo et al. provided
There is also a plethora of evidence showing that opioid antagonism can block the effects of orexigenic peptides. NPY is an orexigenic peptide that has diverse biological activities. For instance, intracerebroventricular injection of NPY has anxiolytic effects in the conflict test (Britton et al., 1997), which can be blocked by naloxone (Britton and Southerland, 2001). Li et al. demonstrated that NPY injected into the NAc of rats increased pain threshold, which was dose-dependently attenuated with injections of naloxone (2002). The same attenuation was seen in NPY-induced feeding. Rudski et al demonstrated that NPY increased pellets obtained in a bar pressing paradigm where 80 presses were needed for the first pellet and 3 presses for each additional pellet (1996). Naloxone reduced the total amount of pellets received, but failed to affect the time to obtain the first pellet. Similar effects were seen with AgRP-induced feeding. AgRP is a potent orexigenic peptide with long lasting effects, which is thought to induce feeding through antagonism of MCRs and inhibition of α-MSH signaling, as well as interactions with NPY (Grill et al., 1998). Simultaneous injections of naloxone with AgRP blocked AgRP-induced feeding in rats. These data further implicate opioids in the maintenance, but not the initiation of the meal and suggest that they do so by attenuating the effects of NPY and AgRP on feeding.

A Link Between ZD and Opioids?

The results of this work and review of literature bring up many parallels between the effects of zinc deficiency and opioid systems:

1. Food motivated behaviors seen in zinc deficiency resemble those of opioid antagonism: unaltered motivation to attain food, but decreased food consumption.
2. Like zinc deficiency, opioid antagonism increases hypothalamic NPY without stimulating food intake (Kotz et al., 1996).
3. Both zinc deficiency and naloxone reduce consumption of saccharin solution in a two-bottle choice paradigm (Cooper, 1983).
4. Like zinc deficiency, opioid antagonism and opiate withdrawal activate the HPA axis. Naloxone and opiate withdrawal increase plasma corticosterone and increase CRH in the amygdala and PVN (McNally and Akil, 2002)
5. Both zinc status and opioid tone are negatively correlated with depression. Depressed patients have decreased opioid tone compared to healthy subjects, as measured by ACTH and cortisol response to naloxone (Burnett et al., 1999). Because of these similarities between zinc deficiency and opioid antagonism, and the normalized food intake observed in ZD rats presented with a fluid choice paradigm, this work has led to the hypothesis that deficiency produces anorexia through modulation of the opioid reward system. Consumption of saccharin alone did not attenuate the decline in food intake of ZD rats. However, rats given a two-bottle choice between saccharin and water, either from the initiation of ZD diet or following anorexia, consumed food amounts no different than that of ZA. Hyperphagia induced by consumption of a palatable food increases dynorphin mRNA and peptide in the hypothalamus (Welch et al., 1996). This suggests that hedonic value of food may induce changes in opioid expression in areas known to control food intake. It is reasonable, then, to suggest that zinc deficiency decreases opioid expression, leading to activation of the HPA axis and alterations in orexigenic peptide activity, and that these effects can be reversed by a stimulus that increases opioid synthesis (i.e., fluid choice).

**Future Studies**

Alterations in metabolism were not directly observed in the current report, however, the differences in activity between ZD and PF animals without differences in MR or BMR suggests there may be differences in the metabolic cost of activity. Studies examining VO2max during voluntary wheel running or treadmill exercise are needed to confirm or rule out this possibility. This work suggests that there may be alterations in opioids systems within the brain. Future studies using microdialysis and naloxone in the choice paradigm are needed to examine the role of zinc deficiency, if any, on opioid peptides within the PVN, NTS, and CeA. Additionally, utilization of the elevated plus maze and other rodent anxiety tests are needed to strengthen the association between zinc deficiency and anxiety-like behaviors in the rat.

**Conclusion**

The data presented in this study characterizes the effects of zinc deficiency on feeding behavior and metabolism. Contrary to previous studies, this work shows a decrease in consummatory food intake with zinc deficiency without evidence of alterations in appetitive motivational behaviors. This suggests that zinc deficiency alters the hedonic impact of food reward, but not the motivation to seek food. The data presented here also suggests an anxiogenic
effect associated with zinc deficiency, which may be involved in the hedonic changes in food intake. Furthermore, this work suggests that alterations of the opioid reward system may be involved in the anorexia and anxiety-like behaviors produced by zinc deficiency.
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