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Decreased food intake rather than zinc deficiency is associated with changes in plasma leptin, metabolic rate, and activity levels in zinc deficient rats☆

Lisa M. Gaetke[a,*], Robert C. Frederich[b], Helieh S. Oz[c], Craig J. McClain[c]

[a]*Department of Nutrition and Food Science, University of Kentucky and the Lexington Veterans Administration Medical Center, Lexington, Kentucky 40506, USA*

[b]*Research and Medical Service VAMC and Department of Internal Medicine and Graduate Program in Nutritional Sciences, University of Kentucky and the Lexington Veterans Administration Medical Center, Lexington, Kentucky 40506, USA*

[c]*Departments of Medicine and Pharmacology and Toxicology, University of Louisville and Louisville Veterans Administration Medical Center, Louisville, Kentucky 40292, USA*

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Abstract

This study investigated the hypothesis that the reduced food intake and poor weight gain in zinc deficient rats is due to: increased plasma leptin concentration, increased physical activity and/or increased metabolic rate. Weanling rats were assigned to three groups: controls fed *ad libitum* (C), zinc deficient (ZD), and pair-fed controls (PF), and tested in a metabolic chamber and activity monitor at baseline and weekly for four weeks. At the end of the study, all groups were compared for differences in plasma leptin concentrations. ZD and PF animals had markedly reduced food intake and weight gain. ZD had reduced stereotypic and locomotor activity compared to PF animals and both groups demonstrated an abolished peri-nocturnal activity spike and were much less active than controls. This was associated with a reduced total metabolic rate by day 30: ZD (0.73 ± 0.07 kcal/hr, $p = 0.0001$) and PF (0.83 ± 0.06 kcal/hr, $p = 0.0001$) groups vs. controls (1.82 ± 0.09 kcal/hr). Plasma leptin concentrations in ZD (1.55 ± 0.06 μg/L) were lower than controls (2.01 ± 0.18 μg/L, $p < 0.03$), but neither ZD nor controls were statistically different from PF (1.68 ± 0.05 μg/L). Both low leptin concentrations and low metabolic rates in the ZD and PF rats were associated with decreased food intake rather than zinc deficiency. The reduced food intake and poor weight gain observed in zinc deficient rats could not be explained by elevated leptin concentrations, hypermetabolism, or increased activity. Low serum leptin concentrations, hypometabolism, and decreased activity are more likely the result of the anorexia of zinc deficiency.

Keywords: Zinc deficiency; Leptin; Metabolic rate; Activity level; Zinc; Anorexia

1. Introduction

Zinc is an essential trace element required for normal growth, protein metabolism, the functioning of many zinc metalloenzymes, membrane integrity, gene expression, wound healing, and immune function [1]. Zinc deficiency in animals causes anorexia, weight loss, poor food efficiency, and growth retardation ([2], reviewed in ref. [3]). The mechanisms for these signs of zinc deficiency are complex and still undefined. The anorexia of zinc deficiency consistently results in decreased food intake with subsequent poor weight gain. Zinc sufficient rats pair fed the same amounts of food as the zinc deficient rats always weigh somewhat more and exhibit better food efficiency than their zinc deficient counterparts [2]. Thus, other factors besides anorexia must be involved. We hypothesized that altered leptin concentration leading to increased metabolic rate and/or increased physical activity as well as the reduced food intake may lead to the poor weight gain observed in zinc deficient rats.

Leptin is a cytokine hormone secreted by the adipocyte, which plays a central role in the maintenance of body weight and energy balance. The serum leptin concentration

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* Corresponding author. Tel.: +1-859-257-1031; fax: +1-859-257-3707.

E-mail address: lgaetke@uky.edu (L.M. Gaetke).

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is linearly related to fat mass over a wide range in both *ad libitum* fed mice [4] and humans [5]. In the setting of unrestricted food intake, the serum leptin concentration accurately communicates body fat mass to the hypothalamic centers that control food intake and metabolism. Serum leptin rapidly declines with fasting and is elevated with refeeding [6–7]. Studies show that lipopolysaccharide (LPS) and the cytokines, interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and leukemia inhibitory factor (LIF), elevate serum leptin, which induced anorexia [8–9]. Although one study has reported low plasma leptin concentration in relation to reduced body fat in zinc deficient rats [10], we measured plasma leptin concentration to further investigate the possibility that elevated leptin would induce the anorexia observed in zinc deficient animals.

A second possible mechanism for the wasting observed in zinc deficiency may be a hypermetabolic state independent of leptin. Zinc deficient rats have reduced weight gain and food efficiency compared to pair-fed controls. This has been conjectured to be due to elevated basal metabolic rate [2,11]. Humans, however, fed a marginally zinc deficient diet, had lower metabolic rates assessed by metabolic changes [12].

A third possible mechanism may be hyperactivity with resultant weight loss independent of leptin. Despite the appearance of hyperactivity observed in zinc deficient rats [2], other studies have reported low activity levels when zinc deficient rats were evaluated by specific testing devices such as an open field test [13–14]. In this study, activity levels were measured throughout the progression from mild to severe zinc deficiency using computer linked activity holders equipped with multiple light beams to determine both ambulatory and stereotypic activity without removing the rats from their regular plastic laboratory cages.

Thus, the purpose of this study was to evaluate the hypotheses that 1) elevated plasma leptin concentrations, 2) increased metabolic rate, and 3) increased physical activity contribute to the anorexia, reduced weight gain, and poor food efficiency observed during zinc deficiency in rats.

2. Methods and materials

2.1. General procedure

Twenty-seven male Sprague-Dawley weanling rats (Harlan Sprague-Dawley Inc. Indianapolis, IN) were individually housed in stainless steel wire bottom cages in an environmentally temperature controlled room 22 ± 0.5°C with alternate 12-hr light and dark cycles. The rats were randomly divided into three equal groups (n = 9): zinc sufficient controls fed *ad libitum* (C), zinc deficient (ZD) and pair-fed, zinc sufficient controls (PF). At baseline, the mean body weight for all of the rats was 85 ± 2 g. This study was approved and performed in accordance with the guidelines for the care and use of laboratory animals with both the Internal Animal Care and Use Committee (IACUC) and the Veterans Administration Medical Center in Lexington, KY.

Food consumption and weight gain for all groups were recorded throughout the experiment. Diet spillage common with zinc deficient animals was measured and accounted for in determining food consumption for both the zinc deficient and pair fed animals. Each rat was tested in an Oxymax System metabolic chamber (Columbus Instruments International Corporation, Columbus, OH) and Omni-Scan System activity monitor (Model OSB-48, Omnitech Electronics, Inc. Columbus, OH) at days 0, 10, 20, and 30 ± 1 day of the study.

2.2. Experimental diets

All of the animals ate a modified AIN-76 zinc deficient diet[1] that contains <1 mg Zn/kg. This commercially prepared semi purified diet (ICN Biomedicals Inc., Cleveland, OH) had 18% egg white solids as the protein source. The normal controls (C) and zinc deficient (ZD) rats were given free access to food. The pair-fed (PF) rats were given an amount of diet equivalent to that consumed by a paired zinc deficient rat. C and PF groups received deionized water containing 35 mg Zn/L as zinc acetate $Zn(C_2H_3O_2)_2$.

The zinc deficient diet was stored at 4.5 ± 0.5°C in plastic containers and handled with plastic gloves and appropriate utensils to avoid contamination. The diet was placed in shallow glass food cups with stainless steel follow-through disks to reduce food spills. All rats had free access to water with or without zinc from plastic bottles with silicone stoppers.

[1] Composition of zinc deficient diet[1]

Ingredient	g/100 g
Corn Starch	44.30
Sucrose	20.00
Egg White	18.00
Corn Oil	10.00
AIN-76 Mineral Mix[2]	3.50
Alphacel hydrolyzed	3.00
AIN-76 Vitamin Mix[3]	1.00
Choline Bitartrate	0.20
Biotin	0.002

[1] ICN Nutritional Biochemicals, Cleveland, OH, By analysis <1 ppm Zn.

[2] Mineral Mixture (gms/kg of mixture): Calcium Phosphate Dibasic 500.00, Potassium Citrate Monohydrate 220.00, Sodium Chloride 74.00, Potassium Sulfate 52.00, Magnesium Oxide 24.00, Ferric Citrate (16–17% Fe) 6.00 Manganous Carbonate (43–48% Mn) 3.50, Zinc Carbonate (70% ZnO) 1.60, Chromium Potassium Sulfate 0.55, Cupric Carbonate 53–55% Cu) 0.30, Potassium Iodate 0.01, Sodium Selenite 0.01, Sucrose, finely powdered 118.00.

[3] Vitamin Mixture (per kg of mixture): Pyridoxine Hydrochloride 700.00 mg, Thiamine Hydrochloride 600.0 mg, Riboflavin 600.0 mg, Cholecalciferol 250.0 mg, Folic Acid 200.0 mg, D-Biotin 20.0 mg, Menaquinone 5.0 mg, Cyanocobalamin 1.0 mg, DL-alpha-Tocopherol Acetate (250 IU/gm) 20.0 gm, Nicotinic Acid 3.0 gm, D-Calcium Pantothenate 1.6 gm, Retinyl Palmitate 1.6 gm, Sucrose, finely powdered 972.9 gm.

2.3. Metabolic and activity testing

All of the rats in this study were tested at baseline in a metabolic chamber and an activity monitor. Several rats from each group were started on the study over consecutive days to allow for metabolic and activity testing when ZD rats were at the bottom (lowest food intake days) of their cyclical eating pattern with a cycle interval of approximately 3 to 4 days. Zinc deficient rats at the peak of their eating cycle (highest food intake days) tend to act more like control rats. Thus, the low point of the feeding cycle was used to be consistent and to control for the zinc deficient state.

For metabolic testing, each rat was placed individually in the metabolic cage for 40 min on days 0, 10, 20, and 30 ± 1 day. Overall, the rats were measured during the light cycle between the hours of 0900 to 1500 with each rat measured during the same hour on each measurement day. The Oxymax System metabolic chamber determines metabolic rate by indirect calorimetry. The value provided by the Oxymax System is the metabolic rate of the animal in kcal/hr. The volume of O_2 and CO_2 are measured in units of mL kg^{-1} h^{-1}. A mass correction factor was used to normalize the data to body weight (per kg) of the animal. The information was calculated and recorded by a microcomputer through the Oxymax System Software, version 3.5. The instruments were calibrated with standardized gas samples (20.36% O_2, 0.628% CO_2, 79.01% N_2; Central Welding Supples, Inc. Lexington, KY).

Physical activity levels for all of the animals were measured in the Omni-Scan System activity monitor. Each rat, in its regular clear plastic cage with free access to food and water, was placed in the activity monitor cage holder for a 14-hr test period over the nocturnal cycle between the hours of 1800 to 0800. The animal's activity in number of movements was recorded and interpreted by the Omni-scan Analyzer and a microcomputer. Activity was counted as the total number of movements per hour for both ambulatory activity (AAM-back and forth movement from one location of the cage to another) and stereotypic activity (SAM-up and down movement, such as grooming).

2.4. Biochemical measurements

On day 32 of the study, all rats were starved for 20 hr to equalize the starvation state because pair-fed animals generally consume all their allotted feed in the first few hours. The animals were anesthetized with sodium pentobarbital and exsanguinated. Heparinized blood was collected for leptin and zinc analysis, and leg bones were collected for zinc analysis. Mean plasma zinc (μg/ml) and mean bone zinc (μg/g) concentrations were used as an index of zinc status for each group [15]. Tissue samples were prepared using a nitric acid-hydrogen peroxide digest as previously described [16]. Zinc concentrations for plasma and tissue (tibia) were determined by flame atomic absorption spectrophotometer (Perkin Elmer Model 5000, Norwalk, CT). Integration time was 2 s with an air-acetylene flame. Wavelength was set at 213.9 nm and spectral band width was 0.7nm. Plasma concentrations of leptin were measured using a leptin RIA kit purchased from Linco Research, Inc. (St. Louis, MO). The lower limit of detection was 0.2 ng/ml, the interassay co-efficient of variation was 4% and the interassay co-efficient was 6%.

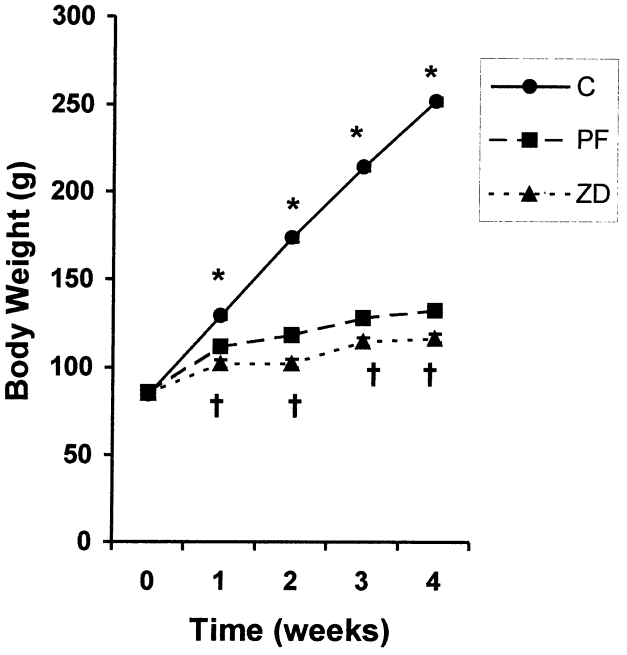

Fig. 1. Decreased body weight in ZD rats. Mean body weight for each group (n = 9) at each week. Data expressed as mean ± SE. * = $p < 0.0001$ vs. ZD and PF, † = $p < 0.0001$ vs. PF.

2.5. Statistical analysis

All results are expressed as the mean ± SEM. Mean responses, such as body weight, food efficiency ratios, metabolic rate, and activity level, were compared by using a repeated measures analysis of variance (ANOVA) adjusting for the animal pairings in the ZD and PF groups. Means, such as plasma leptin, were compared between groups by two sample independent t-tests. Post hoc comparison of means was based on Fisher's protected least significant difference procedure. Statistical significance was determined by a $p < 0.5$.

3. Results

3.1. Body weight and weight gain

Body weight was measured weekly (Fig. 1). By the end of the 4th week, ZD had the lowest body weight (116.6 ±

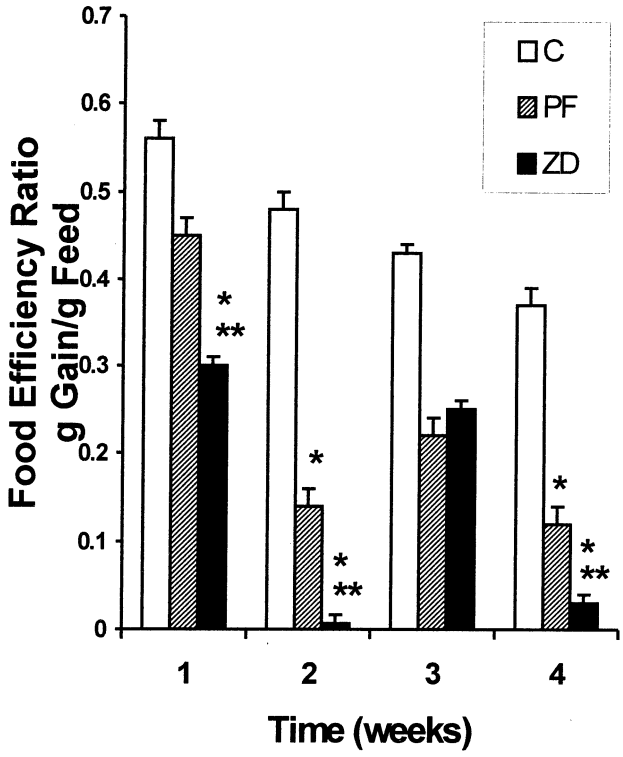

Fig. 2. Decreased food efficiency ratio (FER) in ZD rats. The efficiency of food utilization was assessed by the ratios of changes in body weight to food consumption in the same period for each group (n = 9): zinc deficient (ZD), pair-fed (PF) and controls (C). Data expressed as mean ± SE. * = $p < 0.0001$ vs. C, ** = $p < 0.03$ vs. PF.

3.5 g) compared to controls (252.0 ± 4.3 g, $p < 0.0001$) and PF controls (132.2 ± 4.8 g, $p < 0.0001$). Overall, ZD gained the least amount of weight (31.3 ± 1.6 g) compared to controls (167.4 ± 4.4 g, $p < 0.0001$) and PF (49.6 ± 3.8 g, $p < 0.0001$). PF rats gained more weight with the same amount of food over the 4 weeks than their ZD counterparts.

3.2. Food consumption and food efficiency ratios

Food intake was measured daily, and overall, the ad libitum-fed controls (369.9 ± 2.0 g) consumed more diet than ZD (195.6 ± 1.8 g, $p < 0.0001$) and PF (196.3 ± 1.8 g, $p < 0.0001$). The food efficiency ratio (FER), which is the ratio of weight gain (g) to food eaten (g), assesses utilization of food consumed. Fig. 2 shows food efficiency ratios for each week of the study. By the end of week 4, both ZD (0.04 ± 0.04, $p < 0.0001$) and PF (0.08 ± 0.04, $p < 0.0001$) had lower food efficiency ratios compared to controls (0.37 ± 0.02). Moreover, ZD rats had decreased food efficiency at weeks 1, 2, and 4 compared to PF ($p < 0.03$).

3.3. Zinc status

As expected by the end of the study, ZD rats had lower plasma zinc concentrations (0.89 ± 0.2 μg/ml) than both controls (1.61 ± 0.2 μg/ml, $p < 0.0001$) and PF (1.59 ± 0.1 μg/ml, $p < 0.002$). There was also a pronounced decrease in bone zinc concentrations in the ZD group (77.9 ± 4.8 μg/g) compared to controls (169.9 ± 2.0 μg/g, $p < 0.0001$) and PF (236.5 ± 5.4 μg/g, $p < 0.0001$) (Table 1).

Table 1
Zinc and leptin concentrations of all groups at the end of 4 wks[a,b]

	Controls (C)	ZD	PF
Plasma zinc μg/ml	1.61 ± 0.2	0.89 ± 0.2**	1.59 ± 0.1
Bone zinc μg/g	169.9 ± 2.0	77.9 ± 4.8*	236.5 ± 5.4*
Plasma leptin μg/L	2.01 ± 0.18	1.55 ± 0.06†	1.68 ± 0.05

[a]Values are means ± SEM, $n = 9$ for each group.
[b]* = $p < 0.0001$ vs. C, ** = $p < 0.03$ vs. C and PF, †= $p < 0.03$ vs. C.

3.4. Plasma leptin concentrations

We hypothesized that plasma leptin concentrations would be elevated in the zinc deficient rats in relation to controls during the starved state. However, serum leptin concentrations were found to be lower in ZD (1.55 ± 0.06 μg/L) rats compared to controls (2.01 ± 0.18 μg/L, $p < 0.03$) (Table 1). The serum leptin in the PF (1.68 ± 0.05 μg/L) group was lower than controls, but not statistically different from ZD or controls.

3.5. Total metabolic rate

Metabolic rate (kcal/hr) was measured in a metabolic chamber at baseline, days 10, 20, and 30 (Fig. 3). First apparent at day 10, ZD (0.80 ± 0.07 kcal/hr) had a significantly lower metabolic rate than both controls (1.58 ± 0.08 kcal/hr, $p < 0.0001$) and PF (0. 97 ± 0.05 kcal/hr, $p < 0.05$). Testing at days 20 and 30 showed similar results, however, ZD and PF were no longer statistically different. By day 30, ZD (0.73 ± 0.07 kcal/hr, $p < 0.0001$) and PF (0.83 ± 0.06 kcal/hr, $p < 0.0001$) both had lower energy expenditures than controls (1.82 ± 0.09 kcal/hr).

3.6. Activity levels

To assess whether the change in total metabolism was related to work expended by activity, we measured ambulatory and stereotypic movements at days 0, 10, 20, and 30 ± 1 day (Figs. 4A and 4B). Overall, there was an increase in activity for the rats at about 1900 and 0700 at the photoperiod change. As expected at day 0, all of the groups had similar activity levels for both ambulatory and stereotypic movement. By day 10, ZD were less active in both ambulatory and stereotypic movement compared to controls and PF at 1900 and compared to controls at about 0700 ($p < 0.05$). By day 20, both ZD and PF demonstrated decreased ambulatory movements with an abolished peri-nocturnal activity spike compared to controls at 1900 ($p < 0.05$). By day

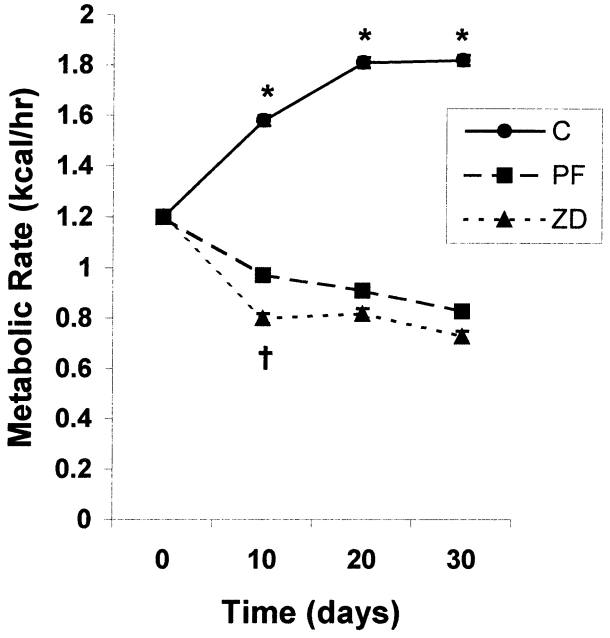

Fig. 3. Decreased metabolic rate in ZD and PF rats. All rats were tested in a metabolic chamber to determine metabolic rate (kcal/hr) by indirect calorimetry at baseline, days 10, 20, and 30. ZD were tested at the bottom (lowest food intake day) of their cyclical eating pattern. Data was normalized to the body weight (per kg) of the rat. Data expressed as mean ± SE. * = $p < 0.0001$ vs. ZD and PF, † = $p < 0.05$ vs. PF.

30, only the ZD rats demonstrated reduced ambulatory and stereotypic movements at 1900 and about 0700 compared to controls ($p < 0.05$) and at 0700 compared to PF ($p < 0.05$).

4. Discussion

This study investigated the hypothesis that elevated leptin concentrations, increased metabolic rate, and/or increased physical activity played a role in the anorexia, poor food efficiency, and poor weight gain of zinc deficient rats. Reduced food intake continued throughout the experiment and appeared to be the dominant contributor to the low weight gain of the ZD rats. ZD rats gained significantly less weight compared to the controls and their pair-fed counterparts. This did not appear to be due to elevated plasma leptin since plasma leptin concentrations in the ZD group were similar to PF, and lower than controls in the starved state at the end of the study. Leptin concentrations appeared to reflect the body mass of each respective group, because the reduced body weight of the ZD and PF groups had lower leptin concentrations than controls, as would be anticipated if leptin were merely reflecting the reduced fat mass of these animals. Although fat mass was not measured in this study, zinc deficient rats have a reduced proportion of carcass fat compared to controls [11]. Our data are consistent with an earlier study that reported low plasma leptin concentration in relation to reduced body fat in zinc deficient rats [10].

Plasma leptin concentrations were also decreased in humans consuming a marginally zinc deficient diet [17]. When subjects were supplemented with zinc during the zinc repletion period, their plasma leptin increased. It was proposed that increased circulating concentrations of TNF-α and interleukin-2 (IL-2) with zinc repletion may have caused the increased plasma leptin concentrations [17]. Studies in animals have shown that TNF-α, but not IL-2, elevates serum leptin which contributes to anorexia [8,9,18, 19,20]. Concentrations of TNF-α and IL-2 were not measured in this study. Here, leptin concentrations in both ZD and PF rats were low, commensurate with reduced food intake and decreased body/fat mass. We predict that the low plasma leptin concentrations in the ZD and PF groups were more a result of the reduced food intake and the low body/fat mass of these groups.

By the end of the study, both the ZD and PF rats had significantly lower metabolic rates as measured by indirect calorimetry compared to normal controls. Earlier work by Keys et al. [21] and Grande et al. [22], established that reduced food intake results in a decrease in basal metabolic rate. The metabolic rates of both ZD and PF decreased over the course of the study as food intake decreased and the ZD became more severely zinc deficient. Thus, the decreased metabolic rate of both groups correlated with food intake and reduced leptin rather than zinc deficiency.

This finding of reduced metabolic rate with zinc deficiency is supported by an earlier study in humans. Wada and King reported low basal metabolic rates in six normal volunteers consuming a marginally zinc restricted diet, suggesting zinc deficiency is related to hypometabolism [12]. They also concluded that a decrease in basal metabolic rate might be secondary to changes in thyroid hormone levels and/or protein utilization. Only free thyroxine (T_4) decreased, however, during the zinc depletion period of the normal volunteers and increased with a zinc adequate diet. Various measures of protein status, including urinary urea nitrogen, serum prealbumin, albumin and retinol-binding protein, decreased with a low zinc diet, but did not increase when subjects returned to a zinc adequate diet. Morley et al. showed that zinc deficient rats and their pair-fed controls had lower levels of triiodothyronine (T_3) and thyroxine compared to zinc sufficient controls [23]. Only T_3 levels were significantly lower in the zinc deficient rats as compared to pair-fed controls. More recently, it was shown that growth failure due to dietary zinc deficiency is not due to impaired T_3 activity [24]. Thyroid hormones were not measured in this study.

A further purpose of this study was to determine if hyperactivity caused the poor weight gain and poor food efficiency. We noted that the zinc deficient animals were less active for both ambulatory (back and forth) movements and stereotypic (up and down) movements during various time points through the night, with the difference first apparent at week 2. Differences in movements were most obvious at 1900 with the ZD rats demonstrating a reduced

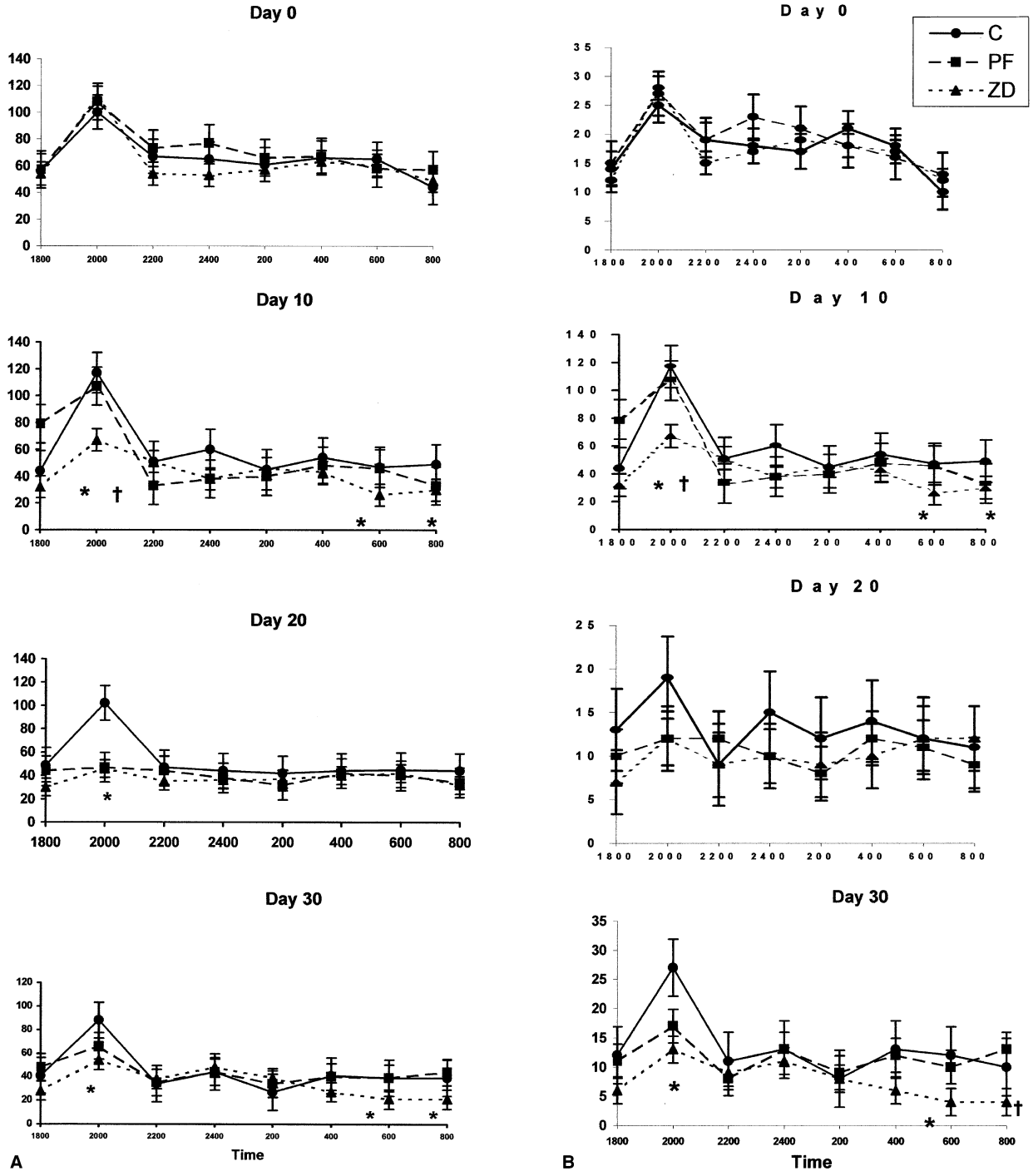

Fig. 4. Fewer ambulatory activity movements (AAM) and stereotypic activity movements (SAM) for ZD rats. All rat groups (n = 9) were tested for (A) ambulatory movements (back and forth) and (B) stereotypic movements (up and down, such as grooming) for each 2 hr period over 14 hr (nocturnal cycle) at days 0, 10, 20, and 30. ZD rats were tested during the bottom of their feeding cycle. Data expressed as mean ± SE. * = $p < 0.05$ vs. C, † = $p < 0.05$ vs. PF.

or abolished early nocturnal activity spike. This is the first reporting of a decreased peri-nocturnal activity spike in zinc deficient rats.

In zinc deficient animals, perhaps the lethargy and reduced activity are due to a compensatory mechanism for the reduced food intake. Keys et al. reported that starvation decreases metabolic rate and total energy expenditure [21]. These changes in metabolic rate are manifested by decreased activity, increased sleep, and decreased body temperature. The ZD rats were less active than controls sug-

gesting that the decrease in total metabolic rate may be related in part to the abolishment of the early nocturnal spike in activity.

In the study reported here, the PF rats (mildly starved and low metabolic rates) tended not to show overall low activity levels like the ZD rats except on day 20, when both PF and ZD showed an abolished peri-nocturnal activity spike for ambulatory movements. Ironically in this third week, both groups had slightly improved food efficiency ratios compared to weeks 2 and 4. The ZD and, therefore, the PF groups may have had proportionately more high food intake days and consumed more food, and thus, gained more weight to explain the slightly improved food efficiency ratios. This was not followed by increased activity on day 20 for either group, however, and PF was more inactive than on either day 10 and 30.

Earlier rodent studies reported a marked lethargy and low activity levels in zinc deficient rats as measured by various open field-testing devices [13,14,25,26]. Wallwork and Sandstead concluded that zinc is essential for brain development and function and that zinc deficiency during brain maturation is evident by subsequent deficits in behavioral functions [27]. The PF (mildly starved) rats in this experiment did not show overall decreased activity. Therefore, the decreased activity of zinc deficient rats may be related more to the effect of zinc deficiency *per se* than to the reduced food intake.

In conclusion, our data show that elevated serum leptin, increased metabolic rate and increased physical activity are not factors contributing to the anorexia, poor food efficiency and poor weight gain observed in zinc deficient rats. Anorexia is documented to be the major component, and our study validates that this anorexia is not related to elevated leptin concentrations. It appears more likely that the decreased leptin and reduced locomotor and stereotypic motion with associated reductions in metabolic rate are in fact a result of the zinc deficient state of the animals and resultant decreased food intake. Low serum leptin concentrations, hypometabolism, and decreased activity are intrinsically related to the signs and symptoms of zinc deficiency.

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