EFFECTS OF MARGINAL ZINC DEFICIENCY AND ZINC SUPPLEMENTATION ON LEAD TOXICITY, METALLOTHIONEIN AND SKELETAL DEVELOPMENT IN GROWING RATS

By

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A thesis submitted to the Department of Human Nutritional Sciences
In partial fulfillment of the requirements for the degree of Master of Science

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Effects of Marginal Zinc Deficiency and Zinc Supplementation on Lead Toxicity, Metallothionein and Skeletal Development in Growing Rats

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ABSTRACT

EFFECTS OF MARGINAL ZINC DEFICIENCY AND ZINC SUPPLEMENTATION ON LEAD TOXICITY, METALLOTHIONEIN & SKELETAL DEVELOPMENT IN GROWING RATS

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Zinc deficiency and lead exposure adversely affect growth and development. The molecular mechanisms of lead toxicity remain unknown. It is proposed that metallothionein (MT), a metal binding protein regulated by dietary zinc, may be a common binding protein for zinc and lead, and therefore may also regulate lead absorption and toxicity.

The objective of this study was to investigate the effects of marginal and supplemental zinc intakes on lead accumulation, toxicity, skeletal development, and MT in growing rats exposed to lead. In a 4x2 factorial design, weanling rats were assigned to marginal zinc (MZ; 8 ppm), zinc-adequate control (C; 30 ppm), zinc-adequate pair-weighed (PW; 30 ppm) or supplemental zinc (SZ; 300 ppm) groups, with and without lead-treated drinking water (200 ppm) for three weeks. Tissues were analyzed for lead and zinc concentrations, and MT by immunostaining and mRNA levels. Excised femurs were analyzed by dual-energy x-ray absorptiometry, morphometric analysis, and serum markers of bone formation and resorption were assessed.

The MZ-fed rats had higher tissue lead and lower zinc concentrations than C and SZ rats, and impaired skeletal growth, mineralization, and rates of bone formation. MT immunohisto-chemical staining in the intestine and kidney was weak to moderate in MZ rats, but strong in the other treatments. These results were supported by similar findings with intestinal MT mRNA analysis. PW treatment also inhibited growth, but did not result in higher tissue lead concentrations than C rats. Skeletal growth inhibition in PW rats was associated with impaired bone resorption,

rather than formation. SZ-fed rats had higher tissue zinc and lower tissue lead concentrations than other treatments. PW and SZ rats had impaired bone mineral density (BMD) versus C and MZ rats. Lead also retarded skeletal and ponderal growth and impaired BMD, but the effects of lead and zinc were generally not additive. Lead treatment had no effect on MT expression.

In summary, the results of this study indicate that while MZ deficiency exacerbated tissue lead deposition, it generally did not intensify lead toxicity. SZ was protective against tissue lead deposition, but did not appear to directly involve MT. The detrimental effects of lead and MZ deficiency on bone development appeared to operate through different mechanisms. SZ was detrimental to BMD in the femur, suggesting that the optimal level of SZ to reduce lead absorption, while supporting growth and bone development, requires further investigation.

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LIST OF ABBREVIATIONS

AAS.....Atomic Absorption Spectrophotometry
AE.....Acrodermatits Enteropathica
ALA.....δ-Aminolevulinic Acid
ALAD.....δ-Aminolevulinic Acid dehydratase
ALAS.....δ-Aminolevulinic acid synthase
ALP.....Alkaline Phosphatase
ATSDR .....Agency for Toxic Substances and Disease Registry
BMA.....Bone Mineral Area
BMC.....Bone Mineral Content
BMD.....Bone Mineral Density
BMP.....Bone Morphogenic Proteins
Cd.....Cadmium
CD.....Charles River, Sprague Dawley-derived strain of rat
CDF.....Cation Diffusion Facilitator (Zinc Transport Protein)
CRIP.....Cysteine-Rich Intestinal Protein
CT.....Cycle Threshold
DEXA.....Dual Energy X-ray Absorptiometry
DMAB..... Dimethylaminobenzaldehyde
DMT1.....Divalent Metal Transporter 1
FEP.....Free Erythrocyte Protoporphyrin
FGF-2.....Fibroblast Growth Factor 2
GPC-Th.....Growth Plate Cartilage Thickness
ICP-OES.....Inductively-Coupled Plasma Optical Emission Spectrophotometry
IGF-1.....Insulin-like Growth Factor-1
IZiNCG.....The International Zinc Nutrition Consultative Group
LIM.....Cysteine-rich motif for the lin-11, isl-1, and mec-3 genes
MRE.....Metal Responsive Element
MT.....Metallothionein
MTF-1.....Metal Transcription Factor-1
Pb.....Lead
PbAc.....Lead Acetate
PbB.....Lead Blood Concentrations
Pb-BP.....Lead Binding Protein
PBG.....Porphobilinogen
PPM.....Parts Per Million
PTH.....Parathyroid Hormone
PTHrP.....Parathyroid-related Peptide
PW.....Pair Weighed
RT-PCR.....Reverse Transcriptase Polymerase Chain Reaction
SD.....Sprague Dawley
TGF-β1.....Transforming Growth Factor-Beta 1
WHO.....World Health Organization
ZIP.....ZRT, IRT-like Protein (Zinc Transport Protein)
Zn.....Zinc
ZPP.....Zinc Protoporphyrin

I. LITERATURE REVIEW

Introduction

Lead is one of the most serious environmental toxicants in the world today because of its ubiquitous nature and plethora of toxicological effects. Chronic exposure to environmental lead is a significant public health problem among the populations of developing countries, as well as low income sub-groups in industrialized nations. Poor nutritional status is thought to exacerbate the health effects of lead exposure and also has a high prevalence in these populations. Children are especially vulnerable to lead toxicity, and the damage is often irreversible and persistent throughout life.

Optimal nutritional status of the essential dietary minerals may be important in reducing lead absorption and toxicity during periods of exposure. Clinically significant interactions with lead have been characterized for calcium, iron, and zinc. Of these, the interactions between lead and zinc are the least well defined.

Increasing dietary zinc is known to reduce lead absorption, tissue accumulation, and cellular toxicity in animal models, whereas zinc deficiency enhances these processes. However, the impact of a marginal zinc intake is less clear. Additionally, the mechanisms of zinc and lead interactions at both absorptive and intracellular sites are unknown.

The zinc-binding proteins, metallothionein (MT) and cysteine-rich intestinal protein (CRIP) have been implicated in heavy metal detoxification processes, although never fully investigated. MT synthesis is induced by dietary zinc in the gastrointestinal tract, liver, and kidneys, but there is limited evidence of lead-induced MT synthesis. MT has demonstrated high affinity binding with lead *in vitro* and although it has also shown *in vivo* binding properties, data on the mechanisms of

interaction is lacking from such studies. MT may modify the cellular toxicity of lead in a manner similar to its more well-established role in cadmium toxicity. CRIP has been studied for its role in zinc absorption across the gastrointestinal tract. Interestingly, another potential role has been suggested for this protein, as a possible route of elimination for heavy metals. These hypotheses both require further investigation.

This chapter will explore the interrelationships between dietary zinc, lead, MT, and CRIP by reviewing the current knowledge around these interactions.

LEAD (Pb)

Environmental Lead Exposure

Lead is a member of the group IV A metals and shares many common properties of the alkaline earth metals (Nolan and Shaikh, 1992). While it is a ubiquitous, naturally-occurring metal, lead has become increasingly distributed and mobilized in the environment largely due to anthropogenic activities (ATSDR, 1999; Tong et al. 2000). Environmental lead contamination rose significantly during the industrial revolution and with the advent of large-scale mining operations (Tong et a., 2000). Later, the appearance of leaded gasoline led to another significant increase in the 1920s (Health Canada, 2003). Since the 1970s, lead exposure has been significantly decreasing in Canada, as leaded gasoline and lead-based consumer products have been phased-out (Health Canada, 2003). However, global lead contamination remains a significant public health problem as consumption by the lead industry has continued to increase, particularly in developing countries (Tong et al. 2000).

Lead exposure to the general population occurs through air, various foods, drinking water, soil, and dust (ATSDR, 1999). The primary source of lead contamination is through atmospheric

deposition, which results mainly from automobile emissions, incineration of fossil fuels, and industrial emissions from smelters and refineries (Nolan and Shaikh, 1992; ATSDR, 1999; Health Canada, 2003). Lead is not readily degraded in the environment, but rather, cycles through air, water, and soil continuously (ATSDR, 1999). Atmospheric residence times are limited to an average of only 10 days, with soil and sediment appearing to be important lead sinks (ATSDR, 1999). Lead deposited in soil enters the human food chain through uptake by fruits and vegetables. In addition, food stored in lead-soldered cans may become contaminated, a practice no longer allowed in Canada. Highly acidic foods and beverages that have come in contact with lead-laden pottery or crystal may also contain dangerous levels of lead (Health Canada, 2003).

Daily dietary intake of lead has been estimated at about 0.2-0.3 mg/day in the United States, which represents about 75% of total intake (Nolan and Shaikh, 1992). Lead is also a concern in water sources, due to its very persistent nature (ATSDR, 1999). Generally, drinking water in developed countries has lead concentrations below 0.01 mg/L, but in areas with soft water or lead-soldered pipes, levels may be as high as 1 mg/L (Nolan and Shaikh, 1992). Rural areas generally have ambient, airborne lead concentrations of less than 0.2 $\mu g/m^3$, with more remote areas having concentrations one or two orders of magnitude below this (Kazantzis, 1989). In contrast, urban areas with high traffic densities tend to have airborne lead concentrations closer to 10 $\mu g/m^3$ (Kazantis, 1989). Therefore, lead inhalation may account for between 0.004 mg/day to more than 0.2 mg/day, depending upon locale (WHO, 1995).

Lead exposure in developed countries is more likely to occur in urban environments, in residences located near lead emission sources, as a result of occupational exposure and

secondary occupational exposure of lead worker's families, upon contact with interior lead paint dust, and as a result of smoking and wine consumption (ATSDR, 1999).

Sources and pathways of lead exposure in developing countries are considerably more variable (Tong et al. 2000). Lead exposure from lead mining, smelting, and battery factories tend to be significant contributors to environmental contamination in developing nations (Tong et al. 2000). Countries undergoing rapid industrialization, which rely upon the use of leaded petrol, such as China, have documented rising blood lead levels in children (Tong et al. 2000). Childhood lead poisoning has also become a pervasive health issue throughout most countries in Africa, where gasoline lead levels are among the highest in the world (0.5-0.8 g/L). In Latin America, lead-glazed ceramics are a major source of lead ingestion (Tong et al. 2000).

Environmental lead exposure is clearly a global public health issue (Tong et al. 2000). Most developed countries have reduced lead emissions through the elimination of leaded gasoline, paints, and consumer products, as well as stricter industrial emission regulations (Tong et al. 2000). However, due to the persistent nature of lead in the environment and the rapid trend of industrialization in developing nations, this issue is likely to remain a significant global problem for many years to come (Tong et al. 2000).

Health Effects of Lead

Lead is arguably the most important environmental toxicant in the world today due to its ubiquitous and cumulative nature, as well as the wide range of toxicological effects that it produces (Qu et al. 2002).

The health effects of lead exposure are dependent upon both the level and duration of exposure. Acute exposure to high levels of lead is uncommon in developed countries, but occurs during accidental occupational exposure and ingestion of lead-contaminated substances by children. The introduction of more stringent safety regulations and improved processing methods has decreased occupational lead poisoning in highly industrialized nations (Tong et al. 2000). However, this is not always the case in developing countries, where industrial operations may not be regulated and the exposure level of workers may not be monitored (Tong et al. 2000).

Symptoms of acute lead intoxication include vomiting, diarrhea, convulsions, coma, and fatality (Health Canada, 2003). At high levels of exposure there is damage to almost all organ systems in the body, most critically the nervous, renal, hematopoetic, skeletal, and reproductive (Goyer, 1997; Tong et al. 2000). Lead blood concentrations (PbB) of 60 to 80 µg/dL will present as overt anaemia, while concentrations in the range of 80 to 120 µg/dL produce severe central nervous system effects, with rising severity until death (Tong et al. 2000). Lead has also been shown to be carcinogenic in rats and mice, although these findings can not be applied to humans at this time (WHO, 1995).

While the consequences of overt lead intoxication have been well-documented, critical information on the long term and immediate health effects of chronic, low level lead exposure remains inadequate (ATSDR, 1999). Chronic exposure to even minimal levels of lead can be very serious, especially in young children and pregnant women (Health Canada, 2003). Measurable effects can be seen with PbB in the range of 10 to 15 µg/dL (Goyer, 1997). In the United States, over one million children, aged 1 to 5, have lead blood levels within this range, or higher (Goyer, 1997). At these concentrations there are a wide range of effects, including the impairment of heme

synthesis and central nervous system functioning (Tong et al. 2000). In Canada, data on the risk of lead exposure is lacking but it has been estimated that 1 in 20 children have higher than acceptable PbB (Lead Environmental Awareness and Detection, 2004). The 2006 Canadian Health Measures Survey will be the first national survey to assess blood lead concentrations in the general public since 1978.

Clinical symptoms of chronic lead exposure may include anaemia, nervous system damage leading to impaired mental function, loss of appetite, abdominal pain, constipation, fatigue, insomnia, headaches, and irritability (Health Canada, 2003). However, critical data on the level and length of lead exposure in relation to biochemical, behavioural, and clinical manifestations, during various stages of growth and development is lacking (Miller et al. 1990). Although there may be a minimal threshold level at which toxicity can be detected, there may be no tolerable level at which no adverse molecular effects occur (Goyer, 1997). Specifically, determining the relationship between biochemical or molecular changes and clinical symptoms and health outcomes is fundamental.

Lead in Growth & Development

Young children have a heightened risk for adverse health effects to lead exposure as they absorb lead more readily and are more susceptible to its toxic effects than adults (Health Canada, 2003). Socially and economically impoverished children are particularly vulnerable, as they are more likely to live in poor housing conditions, be located near lead emitting facilities, have compromised nutritional health, or have an occupationally exposed parent (Tong et al. 2000). In

addition, children tend to have a higher lead intake as a result of normal hand to mouth activities and the close proximity of their breathing zone to the floor (Tong et al. 2000).

Elevated PbB in children have been associated with growth retardation in various epidemiological studies (ATSDR, 1999). The strongest associations have been found between PbB and height, as children with higher PbB were found to have reduced growth rates and stature, as compared to low blood lead controls (ATSDR, 1999). However, all of these studies are weakened by their failure to account for one or more confounding variables which may have affected height (ATSDR, 1999). New, well-controlled studies to assess the relationship between lead intoxication and growth retardation in children are needed. These interventions should include measures of bone lead accumulation in addition to blood lead analysis.

The fetus is also extremely vulnerable to the harmful effects of lead, as the blood-brain barrier is not yet present. Lead can readily cross the placenta, especially in the final trimester of pregnancy (Health Canada, 2003). Women exposed to high levels of lead have an increased incidence of miscarriage and stillbirth (Health Canada, 2003). Chronic, low level, perinatal lead exposure has been associated with developmental effects in children, including low birth weight, decreased gestational age, and neurobehavioural delays or deficits (ATSDR, 1999). There is considerable evidence that PbB of only 10 to 15 µg/dL in the developing young may result in developmental deficits (Grant and Davis, 1989) and concentrations of only 25 µg/dL have been associated with decreases in IQ (Tong et al. 2000). PbB less than 25 µg/dL resulted in a reduction of 1 to 3 IQ points for each 10 µg/dL increment in PbB in children assessed at 3 years of age or older (Tong et al. 2000). In addition, there was no definitive evidence of a threshold for this effect (Tong et al. 2000).

Lead Absorption

Lead absorption occurs primarily through inhalation and ingestion in humans and animals, as percutaneous lead absorption is minimal (WHO, 1995). As much as 50% of inhaled lead compounds may be absorbed, although this is dependent on several factors, including chemical speciation, size of the particles, and solubility in body fluids (WHO, 1995). Dietary lead absorption approximates 10% in adult humans and up to 50% in children, although absorption from lead in soil, dust, and paint chips tends to be lower, depending upon bioavailability (WHO, 1995). Factors affecting absorption of ingested lead include the physio-chemical nature of the ingested substance, nutritional status, and dietary patterns (WHO, 1995). Lead absorption increases during periods of fasting and dietary deficiency of calcium, selenium, phosphate, iron, or zinc (WHO, 1995). Conversely, the presence of calcium, iron, phosphate, and phytate has been shown to reduce lead absorption (Skerfving, 1995).

Gastrointestinal absorption of lead appears to occur principally in the duodenum (Diamond et al. 1997). Although definite mechanisms are not known at this time, they are thought to involve active transport and/or trans-cellular diffusion through intestinal epithelial cells or para-cellular diffusion between cells (Diamond et al. 1997). These processes likely involve ionized lead (Pb^{+2}) and/or inorganic and organic complexes of lead as well (Diamond et al. 1997).

It has been suggested that lead uptake may accompany iron absorption through a common gastrointestinal pathway (Tandon et al. 1994). Cell culture studies have reported that the major intestinal ferrous iron transport protein, divalent metal transporter (DMT1) transports both iron and lead with similar affinity in yeast cells (Bannon, 2002). In addition, the transport of lead was completely inhibited in the presence of 25-fold iron. Lead transport by DMT1 may also explain the

correlation between low dietary iron intake and increased lead absorption in animal models, as DMT1 mRNA is sharply up-regulated when an iron-deficient diet is provided (Bannon et al. 2002). Increased lead absorption may be the result of increased DMT1 expression or the lack of dietary iron to compete with lead for transport (Bannon et al. 2002).

Intracellular Lead

Lead is known to accumulate intranuclearly in the cell and bind to acidic, non-histone proteins within the nucleus (Cherian & Nordberg, 1983). However, the biological and toxicological significance of this binding is not understood. Morphologically distinct nuclear inclusion bodies are formed upon continuous exposure to acute or chronic doses of lead in the liver, kidney, and other organs (Cherian & Nordberg, 1983). Intranuclear binding and inclusion body formation may function as detoxification mechanisms, which protect sensitive cytoplasmic processes (Cherian & Nordberg, 1983). Inclusion body formation is thought to play an important role in the transport, intracellular partitioning, and toxicity of cellular lead (Qu et al. 2002). The nuclear translocation of lead may be mediated by cytosolic lead binding proteins (Mistry et al. 1984), including MT (Qu et al. 2002).

Divalent lead compounds target zinc and calcium binding proteins at the molecular level (Marsden, 2003). For example, the zinc-dependent enzyme δ-aminolevulinic acid dehydratase (ALAD), present in erythrocytes, binds 99% of blood lead (Marsden, 2003). ALAD is involved in heme synthesis and differs from most zinc enzymes in that it has a unique zinc-binding site with three cysteine residues, rather than an assortment of histidine, cysteine, and carboxylate residues (Godwin, 2001). The inhibition of ALAD activity by lead is thought to contribute to the anemia often

present in lead-intoxicated (≥40 µg/dL PbB) children and adults (Godwin, 2001). Lead also impedes the ability of calcium to activate the exocytosis of neurotransmitters in neuronal cells, suggesting that proteins involved in calcium-mediated signal transduction may be general targets of lead toxicity (Godwin, 2001). This hypothesis may explain many of the lead-induced neurological problems seen with lead intoxication (Godwill, 2001). Lead has been shown to bind to the C2 domain of calcium-dependent proteins, although with many orders of magnitude less tightly than it binds a cysteine-rich zinc site (Godwill, 2001).

Lead Metabolism

Distribution of lead within the body is not homogenous (WHO, 1995). Following lead absorption there is rapid uptake into blood and soft tissue, which is eventually redistributed to bone (WHO, 1995). The majority of lead in blood is bound to erythrocyte intracellular proteins (Mugahi et al. 2003). Traditionally, attempts to model the toxicokinetics of lead have compartmentalized the body into three distinct pools for lead disposition (Mushak, 1989). The most kinetically labile pool, the central blood compartment, contains only a small fraction of whole body lead (Mushak, 1989). The soft tissues, the second compartment, also carry a small fraction of the lead burden (Mushak, 1989). The half-life of lead in blood and soft tissues is generally within the range of 28-36 days (WHO, 1995). The third and largest compartment, the skeletal system, is kinetically slow and capable of storing more than 200 mg of lead in humans (Mushak, 1989). The half-life of lead in bone is estimated to be 9 to 12 years, with about a one year shorter span in trabecular bone than cortical bone (Skerfving et al. 1995). The accumulation of lead in bone occurs throughout most of

the life span, and the percentage retention of lead within bone tends to be higher in children than adults (WHO, 1995).

There have been very few attempts to model the biokinetics of lead in the developing animal and human, despite the fact that children are the key population at risk for the adverse health effects of lead (Mushak, 1989). This may be attributed to the highly labile nature of lead toxicokinetics in the young (Mushak, 1989).

Lead Elimination and Excretion

Lead is eliminated from the body in urine and feces, in both humans and experimental animals (WHO, 1995). Blood lead not stored in bone is removed through biliary excretion into feces and urinary excretion (WHO, 1995). The total amount of lead excreted in each route is species-dependent and influenced by age and exposure characteristics (WHO, 1995). Renal clearance of blood lead is thought to increase, in accordance with rising levels of plasma lead (WHO, 1995).

Bone Metabolism and Lead

Accumulation of lead in bone is of scientific and clinical interest for several reasons. Primarily, skeletal lead is the main reservoir for lead in the body, the best measure of cumulative lead exposure, and the most accurate predictor of lead-induced deficits in neurobehavioural outcomes (Pounds et al. 1991). In addition, skeletal lead may be mobilized during physiological and pathological conditions such as pregnancy, lactation, and osteoporosis (Pounds et al. 1991). Mobilized lead becomes toxic to other tissues, including the fetus. Bone is now also being

considered as an important target organ for lead toxicity (Pounds et al. 1991).

Bone metabolism is critical to the fate of lead introduced to the body (Peraza et al. 1998). Lead accumulation in the skeleton begins with the fetus and continues throughout adulthood (Hamilton & O'Flaherty, 1995), with the developing skeletal system being much more sensitive to toxicity than the adult (Pounds et al. 1991). The skeletal system stores over 95% of the lead body burden, which has serious implications for development, as skeletal growth is the principal stimulator and a central component of somatic growth (Ronis et al. 2001). Lead accumulation is a function of both age and exposure (Mushak, 1989) with deposition occurring in areas of bone mineralization and growth, displacing calcium within the bone matrix (Ronis et al. 2001).

Low-level lead exposure has recently been implicated in disorders of bone metabolism, including osteoporosis (Campbell et al. 2004; Escribano et al. 1997). Postnatal impairments in skeletal development, including reduced stature and chest circumference, have been documented with PbB less than 10 μg/dL (Pounds et al. 1991). Lead exposure during pregnancy also has been associated with low birth weight and PbB levels are strongly correlated with reduced growth rates in preadolescence (Ronis et al. 2001). In rats and mice, lead exposure has been shown to produce skeletal malformations in the fetus (Kennedy et al. 1975).

Exposure to lead can directly and indirectly affect many features of bone cell function (Pounds et al. 1991). Lead may indirectly modify bone cell function through alterations in the circulating concentrations of systemic hormones which regulate bone cell function, such as 1,25-dihydroxyvitamin D_3 and parathyroid hormone (PTH) (Pounds et al. 1991). Such lead-induced hormonal changes have been well documented with clinical evidence, although their functional significance has yet to be shown (Pounds et al. 1991). Alternately, lead may directly affect the capacity of bone cells to respond to hormonal cues (Pounds et al. 1991). For example, the

synthesis of osteocalcin, a calcium binding protein made by osteoblasts, is stimulated by 1,25-dihydroxyvitamin D_3 and this stimulation is inhibited by low levels of lead (Pounds et al. 1991). Diminished production of osteocalcin may impede novel bone formation and may also hinder the functional coupling of osteoblasts and osteoclasts (Pounds et al. 1991). Additionally, lead may reduce the synthesis and/or secretion of various bone matrix components, such as collagen, by bone cells (Pounds et al. 1991).

Osteoblasts are derived from mesenchymal stem cells and secrete many of the major constituents of the organic bone matrix (the osteoid), induce the process of mineralization, and have a critical role in the regulation of bone formation (Pounds et al. 1991). Osteoclasts function to resorb bone in the development, maintenance, and repair of the skeleton (Pounds et al. 1991). Osteoclasts also expand on paracrine factors that affect osteoblastic proliferation, migration, differentiation, synthesis of the matrix, and synthesis termination (Pounds et al. 1991). These cells are large, multinucleated, and found on the bone surface near vascular channels (Pounds et al. 1991). Lead may target several processes involved in bone resorption as lead inclusion bodies are formed in the cytoplasm and nuclei of osteoclasts, but not osteoblasts or osteocytes (Pounds et al.1991). These inclusion bodies are indistinguishable, ultra-structurally, from renal and hepatic lead inclusion bodies (Pounds et al. 1991).

Skeletal lead accumulation is primarily mediated by the activity of osteoblasts whereas the release of lead from bone is mostly due to osteoclast activity (Pounds et al. 1991). Additionally, lead has been found to readily displace Ca^{2+} in both binding sites of the hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$) crystal lattice (Pounds et al. 1991). However, the mechanisms of lead toxicity in bone are not well understood.

Osteoblasts may be a direct target of lead, as their function is analogous to developmental

systems in that cell division and differentiation are key processes, and developing organ systems are generally more sensitive to lead toxicity (Pounds et al. 1991). Lead has been found to inhibit osteoblastic activity through reduced osteocalcin synthesis (Escribano et al. 1997). Osteocalcin is a non-collagenous bone protein (Escribano et al. 1997). Serum osteocalcin concentration is useful as a biomarker of osteoblast activity and is also a more general indication of the rate of bone formation (Guity et al. 2002). Decreased concentrations of plasma and serum osteocalcin have been reported in lead intoxicated children, although it is not known whether this is a cause or an effect of impaired bone formation (Pounds et al. 1991). This effect may be the result of reduced circulating levels of 1,25-dihydroxyvitamin D_3, increased breakdown of osteocalcin, or reduced synthesis by osteoblasts (Pounds et al. 1991).

The synthesis of osteocalcin has been shown to be stimulated by 1,25-dihydroxyvitamin D_3 both *in vivo* and *in vitro* (Skjodt et al. 1985). It has also been reported that lead decreases vitamin D_3-induced osteocalcin synthesis in rat osteosarcoma (ROS 17/2.8) cells (Long et al. 1990). Although the mechanism of this effect is not known, protein kinase C as a potential mediator has recently been ruled out (Guity et al. 2002).

Impaired bone resorption has been consistently reported in the limited number of studies that have examined skeletal lead toxicity, suggesting an inhibition of osteoclast function (Pounds et al.1991). Potential mechanisms for this inhibition include effects on 1,25-dihydroxyvitamin D_3 or other systemic regulators of bone resorption, overt toxicity on osteoclasts resulting in cell death, specific effects on local factors that regulate osteoclast function, including the uncoupling of osteoblastic regulation, and some combination of these actions (Pounds et al. 1991).

Lead has been found to act disparately on the axial and appendicular skeletons in animal models (Escribano et al. 1997). A study by Escribano et al. (1997) exposed 50 day old female

Wistar rats to a low-level of dietary lead (17 ppm) for 50 days and found a significant decrease in the length of the fifth lumbar vertebrae but no difference in femur length in the lead-treated rats versus controls. These differences may be related to bone composition and the proportion of cortical to trabecular bone in the axial and appendicular skeletons (Escribano et al. 1997). Cortical and trabecular bone vary in their compositions, remodeling times, rates of metabolic activity, and responses to medications and physical activity (Escribano et al. 1997). It should also be noted that lead exposure at 17 ppm may have been too low to show an effect on long bone growth.

Escribano et al (1997) also reported increased bone resorption (decreased trabecular number and thickness), increased trabecular spaces, and decreased trabecular bone volume in rats on the lead-supplemented diet. The authors concluded that lead had effects on osteoblast, as well as osteoclast, activity. Changes in bone remodeling appeared to cause a reduction in bone mass, as measured by histomorphometry. However, measures of densitometry (bone weight and mineral content) indicated an increase in bone mass in the lead-treated animals. While the authors attributed these findings to increased lead deposition in bone or cortical bone thickening, these hypotheses were not further investigated.

The same research group also investigated the effects of low-level lead exposure (17 ppm) on the longitudinal development and cartilage growth plate of the femur in 50 day old female Wister rats (Gonzalez-Riola et al., 1997). Surprisingly, after the 50 day treatment, the lead-exposed rats weighed more than the control rats. Although femur length did not differ, growth plate cartilage thickness (GPC-Th) was significantly lower in the lead-treated group. These findings suggest that the growth plate is a key target in the toxic effects of lead on skeletal development (Gonzalez-Riola et al. 1997).

A study by Hamilton & O'Flaherty (1994) looked at the effects of continuous lead exposure

(250 ppm and 10000 ppm) on female rats and their offspring. The authors found tail length to be shorter in the pre- and post-natally lead-exposed offspring, suggesting growth inhibition of the distal vertebrae. This finding may explain the reduced stature reported in children living in lead-contaminated areas (ATSDR, 1999). The lead-exposed weanlings also showed increased growth plate width with disruption of chondrocyte organization, and widened metaphyseal trabeculae. Lead appeared to affect growth plate chondrogenesis and mineralization of the metaphysis, although the mechanisms of these effects are not known (Hamilton & O'Flaherty, 1994).

Another study by Hamilton & O'Flaherty (1995) looked at the effects of lead on mineralization in growing rats. They suggested that the adverse effects of lead on bone development during growth were mediated through interruption of mineralization in an ectopic bone (plaque) induction model with male, weanling Long Evans rats. Ectopic bone plaques have been found to follow the same developmental sequence as seen in early bone growth (Hamilton & O'Flaherty, 1995). Lead added directly to plaques (200 μg/g plaque) completely disrupted the mineralization process, as evidenced by the absence of alkaline phosphatase (ALP) activity and cartilage mineralization. These plaques also had increased calcium deposition, although this likely precipitated with phosphorus and lead to form lead hydroxyapatite (Hamilton & O'Flaherty, 1995). In contrast, rats exposed to lead in drinking water (1000 ppm PbAc) showed reduced ALP activity with increased cartilage calcification. The authors speculated that lead may have accelerated calcification through enhanced cartilage lysosomal activity, as this has been demonstrated *in vivo* in several other studies (Hamilton & O'Flaherty, 1995). Lead may exert its adverse effects on skeletal development during chondrogenesis or during earlier events that will ultimately impact upon mineralization (Hamilton & O'Flaherty, 1995).

As previously mentioned, chondrocytes appear to be an important target for lead toxicity.

Chondrocytes have a critical role in endochondral ossification, a complex and multi-tiered process in which the axial and appendicular skeletons are formed (Zuscik et al. 2002). Briefly, a cartilaginous template is formed during embryogenesis by aggregation of mesenchymal cells which differentiate into chondrocytes (Zuscik et al. 2002). Chondrocytes undergo proliferation, maturation, and hypertrophy within the cartilage, followed by specific physical and biochemical changes, including increased ALP expression, *de novo* collagen X expression (a key marker of maturation), and a 10-fold expansion in volume (Zuscik et al. 2002). The chondrocytes undergo apoptosis as the surrounding matrix calcifies and bone is deposited as blood vessels enter the newly calcified cartilage (Zuscik et al. 2002).

Local expression of several growth factors regulates growth plate chondrocyte proliferation and maturation, as well as the mineralization process (Zuscik et al. 2002). Chondrocyte proliferation and the early steps in differentiation are regulated by IGF-1 (insulin-like growth factor 1), transforming growth factor-beta1 (TGF-β1), and fibroblast growth factor 2 (FGF-2) (Zuscik et al. 2002). Terminal differentiation is directed by bone morphogenetic proteins (BMPs), thyroid hormone, and retinoic acid (Zuscik et al. 2002). Parathyroid hormone-related peptide (PTHrP) and TGF-β1 are local regulators of chondrocyte maturation (Zuscik et al. 2002).

PTHrP is a key determinant in the rate of chondrocyte terminal differentiation, as well as a potent suppressor of maturation (Zuscik et al. 2002). TGF-β1 is a powerful suppressor of chondrocyte hypertrophy in rat, chick, and chicken models (Zuscik et al. 2002). Interestingly, these factors may also be linked, as some effects of TGF-β1 appear to be due to the upregulation of PTHrP in chondrocytes (Zuscik et al. 2002). Interruption of these regulatory processes greatly disrupts skeletal growth and development (Zuscik et al. 2002).

In vitro studies with isolated chicken growth plate and sternal chondrocytes have reported

that lead accelerates chondrocyte maturation toward hypertrophy at doses equivalent to 8-56 μg Pb / dL blood in lead-intoxicated children (Zuscik et al. 2002). Lead affects growth factor responses, specifically inhibiting type X collagen expression and mildly inhibiting the capacity for PTHrP and TGF-β1 to induce proliferation. Lead also was found to alter the normal transmission of various second messenger signals. These findings have implications in endochondral ossification, as well as fracture healing (Zuscik et al. 2002).

Lead exposure during growth and development may reduce peak bone mineral density (BMD), thereby predisposing an individual to osteoporosis later in life (Campbell et al. 2004). Lead exposure has been associated with reduced BMD (Campbell et al. 2004) and bone strength in rat models (Ronis et al. 2001), but this association has limited and equivocal evidence in humans (Campbell et al. 2004). However, human studies have been relatively short in duration (18-47 months) and relied upon blood lead, rather than bone lead, as an indicator of body lead burden (Campbell et al. 2004).

Campbell et al. (2004) measured BMD in 35 African American children with known cumulative lead exposure over four age groups (13-24, 25-36, 37-48, and 49-60 months of age). There were no statistical differences in age, sex, body mass index, socioeconomic status, physical activity, or calcium intake. The children were categorized as having low (mean, 6.5 μg/dL blood) or high (mean, 23.6 μg/dL blood) cumulative lead exposure with a cut-off of 15 μg/dL. BMD was measured by a dual-energy X-ray absorptiometry (DEXA) scanner. Contrary to their hypothesis, the authors found that children with high lead exposure had significantly increased BMD compared to the low lead exposure group in four of seventeen measured site, including the head, and the third and fourth lumbar vertebrae. The increased BMD was not thought to be a false reading due to the deposition of lead in bone. Also, these results appear to be clinically relevant as a 7%

increase in lumbar vertebrae BMD is equivalent to about 2 years of bone growth (Campbell et al. 2004).

The mechanism of a lead-induced increase in childhood BMD is not known. However, lead inhibits PTHrP *in vitro* leading to premature chondrocyte maturation (Zuscik et al. 2002), which could result in elevated BMD (Campbell et al. 2004). In addition, the higher BMD associated with the inhibition of PTHrP is likely to be transient, based on studies in mice. PTHrP acts on bone remodeling in adults, rather than endochondral ossification, by promoting osteoblast differentiation and impeding apoptosis, which may lead to increased bone loss in adults (Campbell et al. 2004). Alternatively, early lead exposure may result in the attainment of a lower peak bone mass in young adulthood, which would predispose one to osteoporosis later in life (Campbell et al. 2004).

The effects of calcium, phosphorus, and vitamin D on lead absorption and deposition in bone have been well studied (Peraza et al. 1998). These effects are complex and interrelated. In general, adequate intakes of calcium and phosphorus inhibit lead absorption and retention in bone (Peraza et al. 1998). Vitamin D, however, not only enhances calcium and phosphorus absorption but also stimulates the uptake of essential trace minerals and toxic metals, including lead (Peraza et al. 1998). During calcium deficiency, vitamin D may contribute to increased lead absorption (Peraza et al. 1998), although this effect is dependent upon the duration of exposure, as well as the magnitude of body lead stores (WHO, 1995). Inadequate calcium intake intensifies lead toxicity and affects the hormonal regulation of calcium absorption (Mahaffey, 1981). Furthermore, lead exposure appears to suppress pituitary growth hormone, IGF-1, vitamin D metabolites, and sex steroids, all of which regulate the quality and rate of skeletal growth (Ronis et al. 2001). Thus, the interaction between lead and calcium status are thought to have clinically relevant effects.

Lead in Blood

Measuring PbB is the most common method used in biological monitoring of lead exposure and health risks in humans, as well as experimental animals (Mushak, 1989). There is a curvilinear relationship between the lead concentration of an exposure source and PbB (WHO, 1995). For example, a four-fold increase in dietary lead intake has reportedly led to a doubling of the PbB in infants (WHO, 1995). PbB is most reflective of recent exposure, as well as the kinetically active portion of soft tissue lead burden (Mushak, 1989). The degree to which PbB is indicative of lead burden stored in bone, which may have become toxicologically active, is dependent upon the exposure history of the subject, the status of mineral metabolism within the subject, and/or age, as PbB tends to be more labile in infancy, becoming more stable with increasing age (Mushak, 1989). PbB is most consistent with external exposure sources when bone mobilization is minimal (WHO, 1995). Lead in blood is overwhelmingly bound within erythrocytes (99%), with only 1% remaining in the plasma to be conveyed to other tissues (Mugahi et al. 2003).

Lead and Hematopoeisis: Inhibition of δ-Aminolevulinic Acid Dehydratase (ALAD)

The adverse effects of lead on heme biosynthesis are well known (ATSDR, 1999). Lead induces the inhibition of ALAD and ferrochelatase activity, two enzymes required for heme biosynthesis, resulting in a reduction in heme synthesis and subsequent accumulation of δ-aminolevulinic acid (ALA) in the cytosol (ATSDR, 1999). ALAD is a cytosolic enzyme that catalyzes the second step of heme biosynthesis, and is reflective of intracellular lead toxicity with a high degree of sensitivity and specificity, including low level exposures (Goering and Fowler, 1985). Low level lead exposure markedly inhibits ALAD activity in the liver and erythrocytes (Goering and Fowler, 1985). Significant reductions in erythrocyte ALAD activity have been documented at PbB

as low as 5 µg/dL (Sakai & Morita, 1996). However, renal ALAD inhibition occurs only after overt lead intoxication (Goering and Fowler, 1985). This protective effect may be mediated by tissue-specific factors, such as the lead binding proteins, thymosin β4 and acyl-CoA binding protein, which are present in the kidney (Goering and Fowler, 1987a; Smith et al. 1998).

The first step of heme synthesis is the condensation of glycine and succinyl CoA by δ-aminolevulinic acid synthase (ALAS) to form ALA (Figure 1; ATSDR, 1999). ALAD catalyzes porphobilinogen synthesis from 2 molecules of ALA (Goering and Fowler, 1985). Therefore, during lead intoxication porphobilinogen synthesis is reduced, subsequently impairing heme synthesis. The activity of ALAS, which is regulated through feedback inhibition by heme, is thereby enhanced during lead exposure, further contributing to the ALA build-up (ATSDR, 1999). Consequently, elevated levels of porphyrins and ALA are seen in the urine, as well as increased blood and plasma ALA concentrations (ATSDR, 1999).

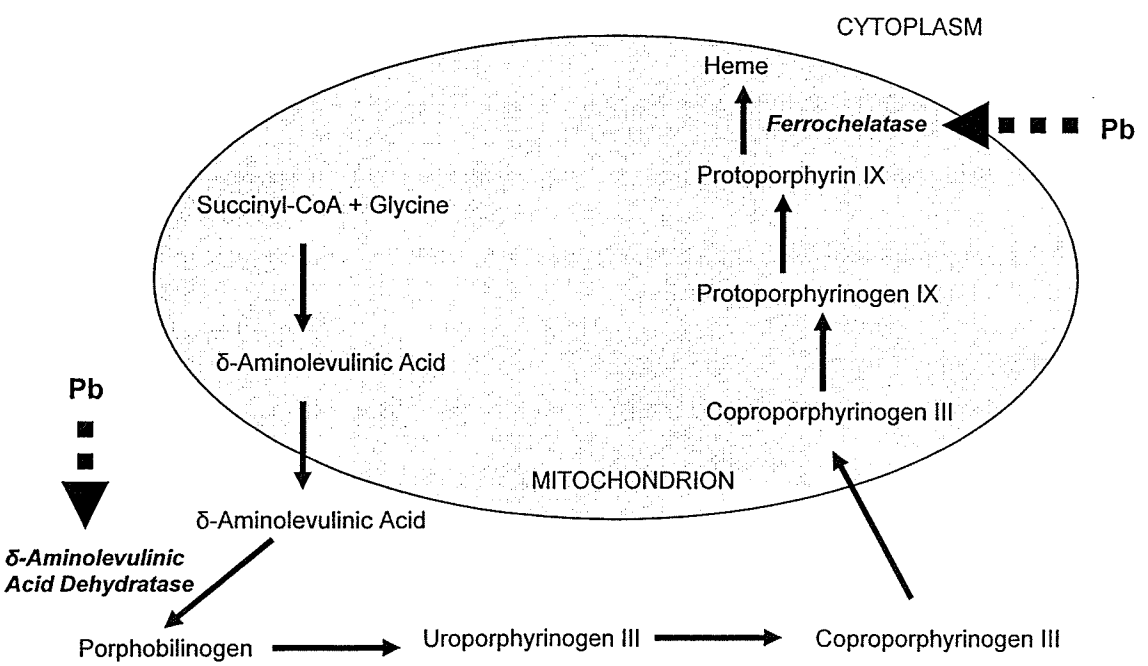


Figure 1. The heme biosynthetic pathway. Enzymes subject to inhibition by lead (ALAD and ferrochelatase) are indicated by a dashed arrow (adapted from King, 2005).

There is also an elevation in erythrocyte zinc protoporphyrin (ZPP) and free erythrocyte protoporphyrin (FEP) as a result of lead poisoning (ATSDR, 1999). This is due to the inhibition of the enzyme ferrochelatase (Figure 1), which is responsible for insertion of the iron atom into the ring structure of protoporphyrin to form heme (ATSDR, 1999). Zinc is incorporated into protoporphyrin rather than iron to form ZPP, which results in a microcytic hypochromic anemia as seen in iron deficiency (ATSDR, 1999). FEP is a product of ZPP and is therefore also increased during lead exposure (ATSDR, 1999).

The physiological significance of decreased ALAD at low lead blood levels, in the absence of observable changes in hemoglobin concentration and erythrocyte function or survival rate are debatable (ATSDR, 1999). Minimal blood lead levels required to see a decrease in hemoglobin levels have been estimated at 50 µg/dL in occupationally exposed adults (ATSDR, 1999). However, lead-induced inhibition of ALAD at blood lead levels below 10 µg/dL appears to cause immediate elevations in plasma-ALA and blood-ALA, indicating the detectable threshold of lead (Murata et al. 2003). The Centre for Disease Control in the United States has proposed that the level of concern for blood lead levels in children be set at 10 µg/dL (Tong et al. 2000). However, chelation therapy for treatment of lead intoxication is only recommended at PbB greater than 40 µg/dL (ATSDR, 1999).

Lead and Renal Effects

The kidneys are the primary site for the initial accumulation of lead following oral or inhalation exposure (Nolan and Shaikh, 1992). Although lead targets several organ systems, renal effects may be the most insidious (Nolan and Shaikh, 1992). In general, lead-induced nephrotoxicity results in nephromegaly, cellular inclusion body formation, and renal tubular

dysfunction (Qu et al. 2002). The kidney is vulnerable to the toxic effects of lead as urinary excretion is a main elimination route for lead from the body (Madden & Fowler, 2000). In addition, the proximal convoluted tubules are especially susceptible as a result of their high reabsorptive activity (Madden & Fowler, 2000).

There are considerable differences between acute and chronic lead-induced renal damage. Acute, lead-induced nephropathy in humans is characterized by nuclear inclusion bodies, mitochondrial effects, proximal tubular epithelial cell cytomegaly, elevated sodium excretion, decreased uric acid excretion, and proximal tubule dysfunction (Fanconi's Syndrome), which presents as aminoaciduria, glucosuria, and phosphaturia with accompanying hypophosphatemia (ATSDR, 1999). All of these symptoms appear to be reversible (ATSDR, 1999). In contrast, the renal effects of chronic lead-induced nephropathy are irreversible. Features include progressive interstitial fibrosis, tubule dilation and atrophy or tubular epithelium hyperplasia, no or few inclusion bodies, azotemia, and a decreased rate of glomerular filtration (ATSDR, 1999). Additionally, lead compounds have produced renal tumours in rodents and are considered a possible carcinogen in adults (Qu et al. 2002). The potential for childhood lead poisoning to produce renal failure later in life has also been examined, although results have been conflicting (Nolan and Shaikh, 1992).

Secondary to the effects on renal function, lead may also be involved in the pathogenesis of gout and hypertension (Nolan and Shaikh, 1992). There is a much greater incidence of gout in cases of chronic lead nephropathy, compared to other renal diseases (Nolan & Shaikh, 1992). Although critical evidence is lacking, several hypotheses have been put forward, including changes in urate metabolism, the impairment of purine metabolism, and the result of an increased turnover of nucleoproteins (Nolan & Shaikh, 1992). Hypertension may be the result of lead-induced alterations in the vasculature of the kidney, the renin-angiotensin system, or various renal ion

transport processes (Nolan & Sheikh, 1992). Many population-based studies have emerged in recent years examining the association between environmental lead exposure, chronic renal insufficiency, and hypertension (Marsden, 2003). However, whether an increased body lead burden is a cause or consequence of renal disease remains unclear.

The molecular mechanisms underlying renal lead toxicity are not well defined (Smith et al. 1998). Specifically, the identification of the molecular binding sites for lead, under chronic, low-level, environmental lead exposure is critical (Smith et al. 1998). The previously mentioned lead binding proteins thymosin β4 and acyl-CoA binding protein are likely candidates for this function (Smith et al. 1998). However, the zinc binding protein, MT, may also contribute to the cellular metabolism and toxicity of this metal (Goering and Fowler, 1987a).

Lead and the Nervous System

The nervous system is extremely sensitive to lead intoxication, especially in developmental stages (Mahaffey, 1981). In adults, the principal neurological effects are manifested in the peripheral nervous system and may result in peripheral motor neuropathy, axon degeneration, and impaired nerve conduction velocity (Mahaffey, 1981). Conversely, children with acute lead intoxication are at increased risk of more serious central nervous system damage, which can be fatal in severe cases (Mahaffey, 1981). Lower but chronic levels of exposure can affect psychological, behavioural, and intellectual functioning in children (Mahaffey, 1981).

While precise mechanisms of lead toxicity in the nervous system are incompletely defined, they may involve calcium-mediated transduction signals, as previously discussed (Godwill, 2001).

Nutrition and Lead Toxicity

While remediation is always the top priority in cases of lead intoxication in children, there may be circumstances in which this is not possible. Poor nutritional status, while not a causative agent in toxicity, can increase the risk of adverse health effects from environmental lead exposure (Mahaffey, 1981). Nutrients can interfere with metal absorption and toxicity by interacting at the primary sites of absorption and action, as well as modifying the metabolism and transport of a toxic metal (Flora, 2002). Thus, supplementation with essential minerals may compete for lead during intestinal uptake and transport, thereby decreasing absorption and/or toxicity.

The main function of essential dietary minerals is to participate in vital biochemical or enzymatic reactions (Goyer, 1997). However, several metabolic interactions between these minerals and toxic heavy metals also may play a role in cellular detoxification processes (Goyer, 1997). In fact, nutritional deficiency of several essential dietary minerals is known to enhance the harmful effects of lead exposure through increased lead absorption and toxicity in animals (Goyer, 1997). In particular, calcium, iron, and zinc have the most clinically significant interactions with lead (Goyer, 1997). Dietary deficiency of calcium, iron, and zinc enhance lead absorption and calcium deficiency is known to increase bone lead retention (Peraza et al. 1998). Iron deficiency renders the heme biosynthetic enzyme ferrochelatase more sensitive to the inhibitory effects of lead (Peraza et al. 1998). Of these minerals, the interactions between lead and zinc have been the least well defined (Goyer, 1997).

While the mechanisms of cellular lead toxicity remain unknown, it is thought that they may be mediated by nutritional factors, rather than by lead itself (Miller et al. 1990). For example, the neurological and haematological symptoms of lead poisoning may be manifestations of essential trace element deficiencies, as both conditions produce similar effects. Lead intoxication may impair

mineral availability within the body and/or increase target organ sensitivity to lead during inadequate mineral supply (Miller et al. 1990). Studies in rats have demonstrated reduced tissue levels of zinc and copper in lead-exposed rats, although it is unclear whether this was the result of decreased feed intake or excessive lead ingestion (Miller et al. 1990).

ZINC (Zn)

Zinc is a member of the group II B transition metals and is a nutritionally essential trace mineral (Tapiero and Tew, 2003). Zinc is among the most abundant essential minerals found in the human body and is present in all tissues (Tapiero and Tew, 2003).

There are at least three fundamental functions for zinc in biological systems: catalytic, structural, and regulatory (Blanchard and Cousins, 1996). Zinc is required as a cofactor or structural component in more than 300 enzymatic reactions, including 70 metalloenzymes, as well as for zinc-containing DNA binding proteins (Basha et al. 2003; Tapiero and Tew, 2003). Regulatory functions are exerted through gene promoters with metal response elements (MREs) (Blanchard and Cousins, 1996). Consequently, zinc has a critical role in a diverse array of biological processes, including the regulation of cell growth and differentiation (Basha et al. 2003; Miller et al. 1990).

Zinc Deficiency

Due to its broad range of functions, inadequate zinc intake has an equally diverse set of clinical manifestations (Miller et al. 1990). Zinc deficiency states affect the epidermal, gastrointestinal, central nervous, immune, skeletal, and reproductive systems (Tapiero and Tew,

2003). Severe zinc deficiency can result in dermatitis, failure to thrive in children, anorexia, pica, irritability, neuropathy, hypotension, hypothermia, alopecia, and frequent loose stools (Aggett et al. 1995; Tapiero and Tew, 2003). In contrast, excess zinc is highly toxic to cells, thus strict regulatory mechanisms for cellular zinc concentration and distribution are essential (Kambe et al. 2004).

Mild zinc deficiency states are much more pervasive than severe deficiency and create non-specific effects such as growth retardation (Aggett et al. 1995). However, it is difficult to know how significant a health problem mild zinc deficiency syndromes are in various populations or individuals, as there is currently no single assay available that can measure and reflect the entire spectrum of zinc status, from deficiency to excess (Aggett et al. 1995).

Zinc deficiency in weanling rodents also presents, primarily, as growth inhibition (O'Dell and Reeves, 1989; Dorup et al. 1991; Prescod, 1998). All growing species, subjected to rapid reductions in dietary zinc, invariably reduce their feed intake and display growth failure (O'Dell and Reeves, 1989). In the zinc deficient state, feed intake follows a cyclical pattern of three to four day intervals (O'Dell and Reeves, 1989). In general, weanling animals receiving zinc deficient diets demonstrate appetite depression within three to five days, resulting in the selective catabolism of zinc-containing tissues. This response is unusually rapid and readily reversible, in comparison to other nutrient deficiencies (O'Dell and Reeves, 1989). The release of endogenous zinc during this phase supports critical metabolic functions (O'Dell and Reeves, 1989). At the same time, zinc is taken up by various tissues and retained very efficiently, contributing to the apparent reduction in endogenous zinc losses across the gastrointestinal tract (King et al. 2000). Zinc concentrations tend to be relatively constant in hair, skin, heart and skeletal muscle (Tapiero and Tew, 2003). In contrast, liver, bone, and testes zinc concentrations may fall considerably during periods of reduced

zinc intake (Tapiero and Tew, 2003). Plasma and serum zinc concentrations decline during moderate to severe zinc deficiency, but not with a mild deficiency (King et al. 2000).

Although the AIN-93G diet for growing laboratory rodents provides 30 ppm zinc (Reeves, et al. 1993), the minimal zinc requirement for weanling rats appears to be 12.5-13 ppm for both males and females (Williams & Mills, 1970). Diets containing 9, 6, 3, and 1 ppm zinc result in increasingly slower rates of weight gain. As well, the onset of the characteristic cyclic pattern of feed intake was seen at 6 ppm zinc (Williams & Mills, 1970).

In comparison with control groups, zinc deficient rats and mice have demonstrated significant reductions in body weight and weight gain (O'Dell and Reeves, 1989; Dorup et al, 1991; Prescod, 1998), as well as reduced linear growth (Prescod, 1998). Furthermore, compensatory growth has been shown to occur during dietary zinc repletion in zinc-depleted rats (Prescod, 1998). However, it is always difficult to isolate the effects of zinc deficiency from those of anorexia as a consequence of the decline in feed intake seen in rodents upon the administration of a zinc deficient diet (O'Dell and Reeves, 1989; Prescod, 1998). This is critical, as a decline in feed consumption has considerable effects on many different physiological and biochemical parameters (O'Dell and Reeves, 1989). Dorup et al. (1991) demonstrated growth inhibition due to zinc deficiency, independent of caloric depletion by employing relative pair-feeding, rather than absolute pair-feeding (Dorup et al. 1991). In relative pair-feeding, controls are maintained by feeding equivalent amounts as provided to zinc restricted animals, but as a percentage of body weight (Dorup et al. 1991). According to Dorup et al. (1991), the effect of zinc deficiency on appetite depression appeared to be even larger than what had been suggested by absolute pair feeding studies.

Unfortunately, pair-weighing and pair-feeding can not adequately separate the effects of zinc deficiency from the effects of reduced feed intake (O'Dell & Reeves, 1989). Pair-fed and pair-weighed animals rapidly become meal-feeders and fast for most of the day when restricted. Force feeding is an acceptable alternative for short term studies but can not be done for more than 7 days, as prolonged force feeding is fatal (Dorup et al. 1991). New approaches to control for the effect of deficiency-induced anorexia are required.

Zinc Status

Human requirements for zinc are estimated at 5 mg/day for children aged 4 to 8 years, 8 mg/day for women and 11 mg/day for men (National Health Institute, 2002). Zinc intakes of most populations around the globe are known to be less than amounts currently considered to be adequate (King et al. 2000). In addition, dietary inhibitors of zinc absorption are thought to contribute significantly to marginal zinc deficiency (Lönnerdal, 2000). Although the estimation of zinc status is very complicated, zinc supplementation and fortification has been shown to increase linear growth, improve immune function, reduce diarrheal disease, and improve outcomes of pregnancy (Lönnerdal, 2000).

At the population level, the risk of zinc deficiency can be estimated indirectly from the prevalence of conditions that are generally improved by zinc supplementation, such as stunted growth and low birth weight (IZiNCG, 2000). Plasma zinc concentration is also useful at the population level, however the interpretation of this measure can be complicated by infection and it is more reflective of severe, rather than mild, deficiency states (IZiNCG, 2000). New potential approaches to assess zinc status include the use of stable isotope techniques to measure rapidly

exchanging zinc pools, erythrocyte metallothionein concentrations, and monocyte metallothionein mRNA determination (IZiNCG, 2000).

An alternative approach to identify populations at risk of low zinc intake is to examine national food balance data (IZiNCG, 2000). Zinc availability in the global food supply is estimated at 9-12 mg/d for total zinc and 1-3 mg/d absorbable zinc. However, the amount of absorbable zinc in low-income populations is considered to be approximately one-third of that in developed countries (IZiNCG, 2000). This is a consequence of the greater dependence on plant sources of zinc, which contain much less bioavailable zinc than animal foods (IZiNCG, 2000). According to these estimations, 49% of the global population is at risk of having inadequate zinc in their food supply (IZiNCG, 2000). This is a great concern, as zinc deficiency is a key factor, not only in the impairment of the growth and development of children, but also in the high rates of morbidity and mortality among children living in poverty (IZiNCG, 2000).

Zinc Homeostasis

Zinc homeostasis is maintained by regulation of absorption and excretion in the gastrointestinal tract, but also by renal reabsorption and subsequent distribution, and intracellular storage (Kambe et al. 2004). Movement through these pathways is controlled by plasma membrane proteins, specifically zinc transporters (Kambe et al. 2004). Following uptake from the apical to basolateral membrane zinc is released into portal circulation (Kambe et al. 2004). However, the intracellular transport pathway is not known. Fecal zinc excretion is derived from excess dietary zinc as well as endogenous sources, including sloughed mucosal cells and pancreatic exocrine and biliary secretions (Kambe et al. 2004). Urinary zinc losses are minimal

under normal, nonpathological conditions, signifying extensive reabsorption in the renal tubules (Kambe et al. 2004).

There are thought to be four intracellular pools available to zinc (Figure 2; Kambe et al. 2004). Zinc binds to metalloproteins and metalloenzymes, as a structural component or cofactor, with high affinity (Kambe et al. 2004). MT binds zinc with low affinity, possibly providing a significant labile pool to act as a reservoir for cytosolic zinc (Kambe et al. 2004). Zinc is also compartmentalized within organelles, which supplies zinc-dependent proteins and may play a part in zinc storage and detoxification (Kambe et al. 2004). Free cytosolic zinc concentrations are extremely small, thought to be at less than a nanomolar level (Kambe et al. 2004).

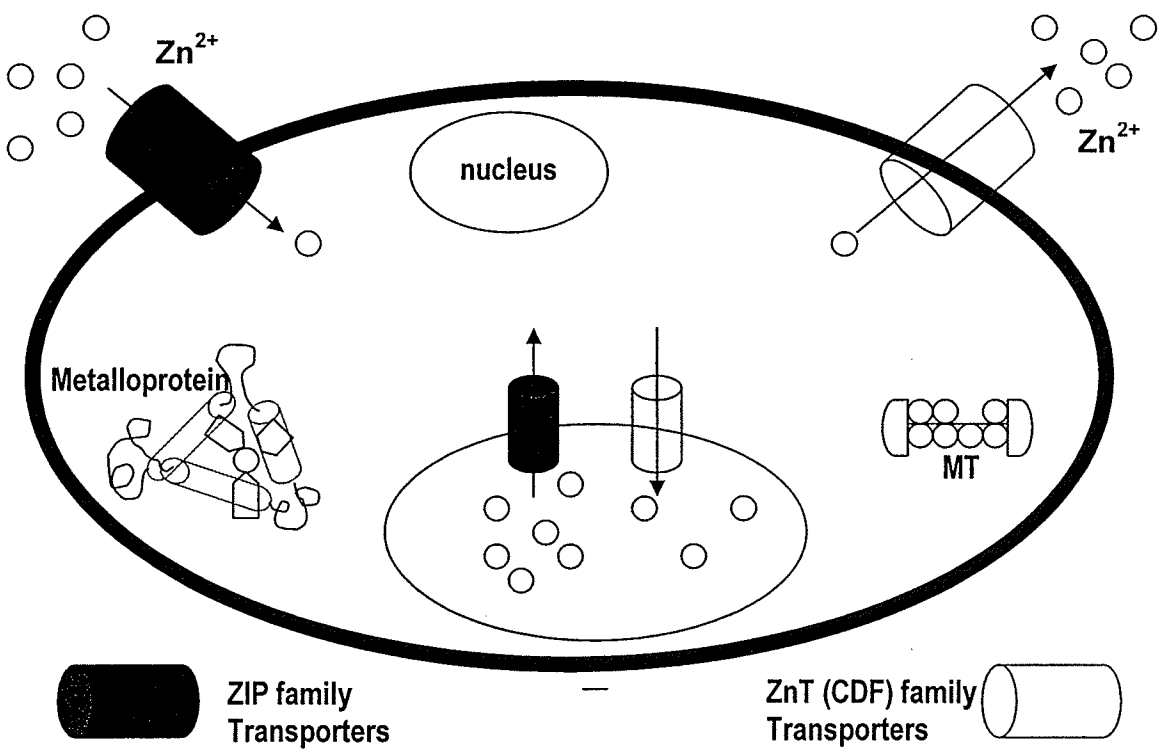


Figure 2. Zinc transport, storage and function in the mammalian cell (adapted from Kambe et al. 2004).

Several zinc transporters have recently been cloned from a variety of organisms. Discovering the functional mechanisms of these transporters will greatly add to the understanding of how zinc homeostasis is maintained. There are two metal-transporter families: ZIP and CDF (Kambe et al. 2004). ZIP-family transporters (ZRT, IRT-like Protein) operate in zinc influx to the cytosol, from outside of the cell or from within intracellular compartments (Figure 2). CDF-family transporters (Cation Diffusion Facilitator) function in zinc efflux from the cytosol to either move zinc into intracellular compartments or outside of the cell (Figure 2). In humans, there are 14 members of the ZIP family and 7 members of the CDF family (Kambe et al. 2004).

In the enterocyte, transporter ZIP4 is the primary importer of dietary zinc (Figure 3; Kambe et al. 2004). It is found on the apical membrane of enterocytes, mainly lining the intestinal villi (Kambe et al. 2004). Mutations in the gene for this protein result in the rare, autosomal recessive disorder, acrodermatitis enteropathica (AE) (Kambe et al. 2004). In AE there is inadequate zinc absorption resulting in zinc deficiency, which can be fatal without supplemental zinc intake (Kambe et al. 2004). Extracellular zinc concentrations are thought to regulate the synthesis of ZIP transporters (Kambe et al. 2004).

CDF transporter ZnT-1 is located in the basolateral membrane of enterocytes lining the intestinal villi and is responsible for zinc efflux (Figure 3; Kambe et al. 2004). ZnT-1 is transcriptionally and post-translationally regulated by zinc. ZnT-1 expression is induced by high zinc conditions and suppressed by zinc deficiency, supporting the proposed role of CDF transporters in cellular zinc detoxification (Kambe et al. 2004). As with MT, transcription is under the control of the metal response element (MRE)-binding, metal transcription factor-1 (MTF-1). MTF-1 binds to two MRE consensus sequences on the ZnT-1 promoter (Kambe et al. 2004).

Notably, ZnT-1 gene deletion is fatal in the mouse embryo indicating an essential role in normal development (Kambe et al. 2004).

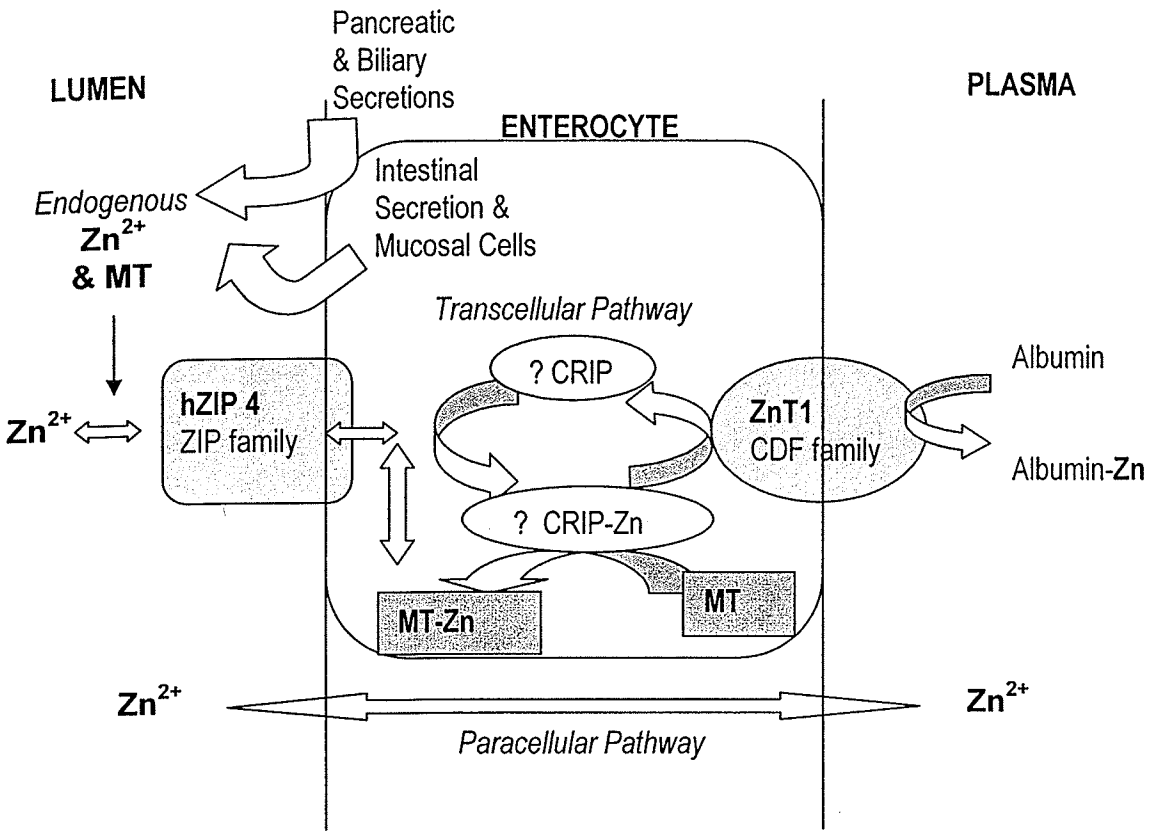


Figure 3. The pathway of zinc absorption and transport in the enterocyte (adapted from Hempe and Cousins, 1992; Kambe et al. 2004).

Zinc Absorption

Dietary zinc absorption, which can not be directly measured in animals and humans, is estimated at about 20-40% and is absorbed mainly as a complex in the small intestine (Tapiero and Tew, 2003). Fractional absorption rates are influenced by the amount of zinc, physicochemical

interactions of binding ligands in liquid and food, localized intestinal pH, glycocalyx retention, gastric emptying, and intestinal transit rate (Coyle et al. 2000).

Zinc absorption is dependent on two transport processes (Figure 3). One is a non-specific, unsaturable, and diffusion-mediated process, not regulated by dietary zinc intake. However, this pathway is inefficient and may be negligible in humans (Hambidge, 2005; personal communication). Zinc is primarily absorbed by a specific, saturable, carrier-mediated transport process, stimulated by dietary zinc depletion (Tapiero and Tew, 2003). The rate of zinc absorption is influenced by the amount of zinc in a meal, in that as dietary zinc intake increases, fractional zinc absorption decreases (Lönnerdal, 2000).

Although data is limited, during periods of inadequate zinc intake, gastrointestinal regulation of zinc homeostasis appears to be maintained differently (King et al. 2000). At low levels of zinc intake, zinc intestinal absorption in the rat appears to be under the control of a carrier-mediated process, possibly CRIP (Hempe and Cousins, 1992). This is thought to be due to an increase in the rate of transfer of zinc on the carrier, across the mucosal membrane, as opposed to an increase in affinity of the carrier for zinc (King et al. 2000). Although the mechanism of this proposed increased transfer is not known, some authors have also implicated MT, secreted from the exocrine pancrease, as a potential candidate in this process (King et al. 2000; Tapiero and Tew, 2003). MT, or another ligand, unsaturated by zinc may bind dietary zinc in the lumen and facilitate its absorption (King et al. 2000). Further work in MT-null mice by Coyle et al. (2000) supports this hypothesis, as zinc-depleted mice absorb more zinc from an intragastric zinc solution by a MT-enhanced processed, than do zinc-depleted mice.

Conversely, at higher doses of dietary zinc, zinc-induced MT in rat intestinal mucosal cells is also thought to mediate absorption (Hempe and Cousins, 1992). Intestinal MT binding with zinc

appears to reduce zinc absorption, possibly by preventing zinc-CRIP (or another transport protein) binding and subsequent movement to the basolateral membrane for transport to the plasma (Hempe and Cousins, 1992).

Zinc Metabolism and Excretion

Following absorption, albumin is the primary zinc carrier in the plasma (Tapiero and Tew, 2003). Plasma zinc concentrations in rats respond quickly to a zinc deficient diet, with an abrupt decline of 25% within the first 8 to 12 hours of initiation (Hurley et al. 1982). Plasma zinc concentration also appears to be under hormonal control and is responsive to stress (Tapiero and Tew, 2003).

Intracellular zinc is tightly regulated and primarily bound to metalloproteins, including metalloenzymes, gene regulatory molecules, and storage or carrier molecules (Tapiero and Tew, 2003). Endogenous fecal zinc excretion is the primary means by which zinc is excreted from the body and is directly related to total zinc absorption, as low dietary zinc intake is reflected by low dietary zinc excretion (Tapiero and Tew, 2003). In comparison, renal zinc losses are relatively small and tend to remain constant, irrespective of dietary intake (Tapiero and Tew, 2003).

Zinc and Bone Metabolism

Bone contains a large fraction of total body zinc content and is able to release zinc during periods of deficiency to support soft tissue metabolism (Zhou et al. 1993). Bone zinc has been shown to amass in the layer of osteoid prior to calcification, but is also deposited in the mineral component, most likely in hydroxyapatite (Yamaguchi, 1998). Marginally zinc deficient rats (6 ppm Zn) release zinc from bone primarily over the first week of zinc restriction (Zhou et al. 1993). This is

later followed by a decrease in growth rate in an attempt to maintain zinc homeostasis (Zhou et al. 1993). In contrast, the bones of zinc adequate, pair-fed controls continue to deposit zinc, at least until the fifth week of dietary restriction (Zhou et al. 1993).

Approximately 20% of skeletal zinc is thought to be mobile and able to significantly contribute to whole-body zinc turnover in the young-adult rat (Windisch et al. 2002). Windisch et al. (2002) examined the changes in bone mineral composition and biochemical markers of bone turnover during zinc deficiency (1.4 ppm zinc, 12 days) followed by repletion with a 21 ppm zinc diet. Approximately half of the mobilized skeletal zinc was replaced by the second day of repletion and fully restored by two weeks. Plasma osteocalcin concentrations and bone ash concentrations of calcium, phosphorus, and magnesium were unaffected. Femoral sections analyzed for ^{65}Zn by autoradiography revealed a marked exchange of zinc in the metaphyseal and epiphyseal regions. The authors concluded that the transient mobilization and restoration of skeletal zinc appears to take place primarily in trabecular bone and does not seem to include changes in bone mass, macro-mineral content, or bone tissue turnover in young-adult rats (Windisch, 2002).

Zinc is essential for the growth, development, and maintenance of healthy bones (Yamaguchi, 1998). Zinc deficiency is associated with retarded skeletal growth and decreased bone mass in a variety of animal models (Rossi et al. 2001) and zinc supplementation in children has been shown to stimulate both skeletal growth and maturation (Yamaguchi, 1998). Reported defects in skeletal development in zinc deficient rats have included various tetratogenic abnormalities (Hurley, 1981), smaller bones with reduced zinc content, decreased epiphyseal cartilage thickness in long bones, and impaired chondrocyte activity at the level of the growth plate (Rossi et al. 2001) . Flawed mineralization, delayed appearance of epiphyseal centres (versus pair-fed controls), and gross skeletal alterations, similar to what is seen in rickets, including

widened growth plates, indistinct zones of provisional calcification, bowed long bones, and thin cortices have been reported in marginally zinc deficient infant rhesus monkeys (Yamaguchi, 1998). In humans, zinc deficiency has been associated with delayed skeletal maturation, reduced pre-menopausal bone mass, and post-menopausal osteoporosis (Rossi et al. 2001). Zinc deficiency has been reported to reduce bone trabeculae in a variety of experimental models (Rossi et al. 2001) and affects the biosynthesis and degradation of all types of collagen (Rossi et al. 2001). Thus, zinc deficiency may be a risk factor in the development of osteoporosis (Eberle et al. 1999).

Zinc is thought to stimulate bone formation and inhibit resorption, although these physiological roles have not been fully explained (Yamaguchi, 1998). *In vitro* studies have shown zinc to stimulate bone formation (Hurley et al. 1969), bone growth and mineralization in weanling rats (Becker & Hoekstra, 1966), as well as to have a potent inhibitory effect on bone resorption (Holloway et al. 1996). As previously discussed, ALP is an enzyme involved in bone calcification (Yamaguchi, 1998) and IGF-I stimulates the proliferation and differentiation of bone cells, which supports longitudinal bone growth (Roughead and Lukaski, 2003). Zinc deficiency in rats has been associated with reduced ALP activity (Rossi et al. 2001) whereas zinc administration stimulates ALP activity (Yamaguchi, 1998). Dietary zinc deficiency and supplementation also influence serum IGF-I in both humans and rats (Roughead and Kukaski, 2003). For example, epidemiological evidence indicates that bone loss during osteoporosis may be at least partly due to decreased circulating IGF-1 concentrations, as a result of low zinc intake (Devine et al. 1998).

A study by Rossi et al. (2001) reported morphometric abnormalities of the growth plate in severely zinc deficient rats, which is likely reflective of the role of zinc in cell division, differentiation, and apoptosis. Of note, the growth plates of the zinc deficient animals were significantly more atrophic than both pair-fed and control animals and this effect was accompanied by a reduction in

serum IGF-I. Also of interest, an increase in bone resorption was related to inanition but was not a specific effect of zinc deficiency (Rossi et al. 2001). While zinc deficiency appeared to reduce bone mass through growth plate dysfunction, which led to decreased bone growth, the loss in bone mass associated with pair-fed animals was thought to be due to a less severe decrease in bone growth but enhanced bone remodeling (Rossi et al. 2001).

The effects of severe zinc deficiency on the structural and cellular parameters of cancellous and cortical bone were investigated by bone histomorphometry in growing, male Sprague Dawley rats fed 0.76 ppm zinc or 60 ppm zinc for 42 days (Eberle, et al. 1999). Zinc deficiency resulted in a 45% reduction in cancellous bone mass of the distal femoral metaphysis and a weakening of trabecular bone, as evidenced by fewer and thinner trabeculae. In addition, this was accompanied by reductions in both osteoblastic bone formation and osteoclastic bone resorption, suggesting a low turnover type of osteopenia. However, these results are hampered by the lack of a pair fed control to compare the zinc-deficient animals with, therefore these skeletal effects may be at least partly due to growth retardation and reduced food intake. Although, the authors refer to a study by Fernandez-Madrid et al. (1973) in which zinc deficient rats were compared to both pair fed and *ad libitum* controls and reported that the impaired protein and collagen synthesis seen with zinc depletion were in fact due to zinc deficiency and not caloric intake.

Zinc supplementation has been shown to have a stimulatory effect on skeletal growth. *In vitro* studies in tissue culture systems have shown zinc to stimulate bone formation, as evidenced by increased bone calcium in the presence of zinc and direct stimulation of protein synthesis at the translational level in bone cells (Yamaguchi, 1998). Zinc addition to cultured osteoblastic cells directly stimulates proliferation, as well as differentiation, and results in considerable increases in

many matrix protein components, as well as concentrations of osteocalcin, IGF-I, and TGF-β1 (Yamaguchi, 1998). Furthermore, the anabolic effect of IGF-I is further enhanced by zinc in osteoblastic cells (Yamaguchi, 1998).

Evidence from the many *in vitro* studies previously described is also supported by *in vivo* research. Oral zinc administration to weanling rats at 5 and 10 mg Zn/kg body weight for 3 days reportedly resulted in dose-dependent increases in femoral diaphysis zinc content, DNA content (a marker of the total number of bone cells), collagen, calcium, and ALP activity (Yamaguchi, 1998). In another study, growing male Wistar rats were fed 2, 47, or 60 mg zinc/kg diet for 4 weeks (Ovesen et al. 2001). A dose-dependent improvement in body weight and femur length was reported and zinc was found to increase bone strength in the femoral diaphysis and the femoral neck. Zinc exerted its main histomorphometric effects on the periosteal envelope, thereby increasing bone area, tissue area, and the axial moment of inertia (Ovesen et al. 2001). The authors concluded that zinc has a positive effect on bone metabolism in a manner which mimics that of growth hormone or IGF-I. In humans, zinc supplementation has shown positive linear growth responses in children at risk of malnutrition or zinc deficiency (Salgueiro et al. 1999).

Zinc is also thought to have an inhibitory effect on bone resorption, although this has only been reported in tissue culture studies (Yamaguchi, 1998). Adding zinc to culture inhibits the bone-resorbing factors present from decreasing the bone calcium content of weanling rat calvaria (Yamaguchi, 1998). Zinc also inhibits osteoclast-like cell formation in mouse marrow culture, but not osteoclast function (Yamaguchi, 1998). These findings have not been replicated *in vivo.*

Lead and Zinc Interactions

Lead and zinc interactions have been observed at both absorptive and enzymatic sites (Flora et al. 1991). Supplementation of dietary zinc, above the required level, reduces the absorption of lead and other trace metals in animal models (Cerklewski and Forbes, 1976; Flora, 2002). Zinc has been shown to affect lead tissue accumulation as well as susceptibility to lead toxicity in rats (Flora et al. 1991). Increased dietary zinc intake can reduce lead blood levels and restore lead-induced biochemical alterations, such as the inhibition of ALAD (Cerklewski and Forbes, 1976; Goering and Fowler, 1985; Flora et al. 1991). The specific studies that follow will describe the basis for these conclusions.

Cerklewski and Forbes (1976) measured the tissue lead accumulation at three and seven weeks in male, albino, growing (45 to 55 g), Sprague-Dawley (SD) rats fed 8 (low), 35 (adequate), or 200 ppm (excess) Zn, and 0, 50, or 200 ppm lead acetate (PbAc). Ten rats were assigned to each of nine treatment groups. Many of the toxic effects of lead were alleviated in animals fed excess zinc, but there were no significant differences between the third and seventh week (Cerklewski & Forbes, 1976). In general, as dietary zinc increased, lead toxicity decreased. Evidence of improvement included reduced blood, liver, tibia, and kidney lead concentrations, decreased rates of urinary ALA excretion, decreased accumulation of FEP, decreased inhibition of kidney ALAD, and decreased apparent lead absorption. The treatment with excess zinc produced overall effects more typical of rats fed 50 ppm lead with adequate Zn, even though they were fed 200 ppm lead. The authors attributed these results to a decrease in lead absorption due to the excess dietary zinc, based on the close agreement among the biochemical tests (kidney ALAD, urinary ALA, and erythrocyte protoporphyrin accumulation), lead excretion data, and tissue lead

concentrations. In addition, measures of lead toxicity were unaffected by two doses of injected zinc (100 µg as $ZnSO_4$, intraperiotoneally) 24 and 48 hours prior to termination, in a supplemental experiment where animals were maintained on a low zinc diet (8 ppm zinc) for one week. Furthermore, dietary zinc did not affect lead excretion in the principal study.

The inability of zinc to completely overcome the toxic effects of lead is not surprising, as lead toxicity is also modified by dietary calcium, iron, and copper, in rats, for example. However, zinc appears to be a nutritionally significant factor in lead metabolism (Cerklewski and Forbes, 1976).

Flora et al. (1991) studied lead and zinc interactions in growing (150 g), male albino rats consuming a standard laboratory diet (45 ppm Zn) and drinking water *ad libitum*. The animals were divided into three groups. Group I (Control) and group III had five animals, while group II had 15 animals. Over the next four weeks, group II and III received drinking water with 545 ppm PbAc, but group III was given a supplement of 1 mg/kg Zn (~ 0.15 mg Zn), as $ZnSO_4$ orally by gavage. All groups were given access to a standard laboratory chow *ad libitum*, with the recommended levels of zinc and all other nutrients. After four weeks, group I, group III, and five animals from group II were terminated. The remaining 10 animals were then separated into two additional groups and treated for six more days. Group IIa was given water (4 ml/kg) orally, by gastric intubation, while group IIb received 10 mg/kg Zn (~ 3 mg Zn) as $ZnSO_4$, orally by gavage once daily.

The lead-induced inhibition of erythrocyte ALAD activity, elevation of blood ZPP, and urinary excretion of ALA were significantly less affected in group III (1 mg/kg Zn; PbAc) than group II (PbAc). Treatment with zinc following the lead exposure period, however, did not create a significant improvement. The simultaneous administration of zinc and lead (group III) also significantly decreased the accumulation of lead in the blood, liver, and kidneys, but no differences

were observed in the pre-exposed groups (IIa & IIb). Finally, lead exposure decreased the zinc content of the blood and liver, but not the kidney. Simultaneous treatment with zinc (group III) significantly increased both blood and liver zinc content, while subsequent treatment with zinc (group IIb) only significantly increased liver zinc content. Flora et al. (1991) concluded that the protective effects of zinc against plumbism are likely due to a decrease in lead absorption across the gastrointestinal tract. Zinc and lead may compete for similar binding sites on a MT-like transport protein in the intestine (Flora et al. 1991). When zinc absorption is low, zinc associates with this MT-like protein. However, when zinc absorption is high, zinc associates with a chelating protein of low molecular weight (Flora et al. 1991). Although the mechanism of lead absorption in the presence of excess or minimal zinc is unknown, one theory proposes that a zinc binding protein stores and transports lead to soft tissues in the body, during oral lead exposure (Flora et al. 1991). Zinc-induced intestinal MT is a potential candidate in this mechanism. Oral zinc supplementation could therefore reduce lead absorption by inducing MT synthesis and effectively competing for and decreasing available binding sites for lead uptake (Flora et al. 1991). Additionally, increased dietary zinc intake also enhances renal and hepatic MT content, which may contribute to heavy metal detoxification in the cell. This hypothesis will be discussed in the following sections.

METALLOTHIONEIN (MT)

MT proteins are ubiquitous in human cells and characterized by their highly conserved cysteine content, low molecular weight (6-7 kDa), high metal content, and lack of aromatic amino acids (Romero-Isart and Vasak, 2002; Tapiero and Tew, 2003). MT synthesis is predominantly carried out in absorptive and excretory organs. Although concentration is variable with age and

tissue type, basal hepatic MT concentration is approximately 700 µg/g liver in humans and 2 to 10 µg/g liver in the rat (Tapiero and Tew, 2003). Cellularly, MT is primarily found in the cytoplasm, with a small amount localized within lysozymes and nuclei (Tapiero and Tew, 2003). Nuclear translocation of MT has been associated with zinc requirements (Tapiero and Tew, 2003).

MT is induced by essential trace elements, such as zinc and copper, several toxic metal ions, hormones, cytotoxic and inflammatory agents, and by cellular stress (Tapiero and Tew, 2003). MT has a high affinity for many metals, including cadmium, zinc, copper, silver, and mercury (Goering and Fowler, 1987b). This property can primarily be attributed to the great number of cysteine residues present and their cluster molecular arrangement, which maximizes contact with each cysteine residue (Georing and Fowler, 1987b).

Despite the great quantity of publications on MT over the past four decades, the primary biological function of this protein has yet to be resolved unequivocally (Davis and Cousins, 2000). The suggested functions of MT include intracellular metabolism and/or storage of metals, donation of metals to target apometalloproteins (especially zinc finger proteins and enzymes), detoxification of metals, and protection from oxidant species and electrophiles (Davis and Cousins, 2000). There is considerable evidence to support a role for MT in the regulation of zinc metabolism (Davis and Cousins, 2000). If MT functions in heavy metal cellular detoxification, this is more likely an adventitious effect rather than a primary function of the protein (Templeton and Cherian, 1991).

A study by Szczurek (2000) reported that the concentration and distribution of MT was affected by zinc status, in the small intestine, liver, and kidney. Briefly, weanling SD rats were randomly assigned to zinc deficient (<1 ppm Zn), pair-fed (diet restricted), and control (30 ppm Zn) treatments for 3 weeks. Half of the zinc deficient and pair-fed rats were then repleted with the control diet for a further 24 hours.

Immunohistochemical localization of MT revealed nuclear and cytoplasmic MT staining of surface intestinal epithelial cells, as well as strong staining in the Paneth Cells of the intestinal crypts, in all treatments except the zinc deficient rats. Notably, MT staining was present following the 24 hour dietary repletion in zinc depleted animals. MT distribution was primarily localized within the proliferative region of the intestinal epithelium, rather than the tip of the villi, supporting potential roles in heavy metal detoxification and gut immunity (Szczurek, 2000).

Immunolocalization of MT in the liver also was absent in the zinc deficient rats, but was not detected following 24 hour dietary repletion. Therefore, low dietary zinc intake appears to produce a decline in hepatic MT synthesis. Interestingly, the MT distribution of control animals showed a scatter pattern whereas it was concentrated around the central vein in pair-fed and pair-fed repleted rats. MT staining in control animals was weak, as expected. Pair-fed animals demonstrated strong MT staining, which was consistent with previous reports, possibly indicating a stress-induced response in MT synthesis (Szczurek, 2000).

Observations of the immunolocalization of MT in the kidney found strong staining in the epithelial cells of renal proximal convoluted tubules in the control, pair-fed, and pair-fed repleted groups, but only weak staining in the zinc deficient animals and moderate staining in the zinc deficient repletion group. Therefore, renal MT synthesis appears to be affected by zinc status. MT distribution was localized within the entire cortex, as well as the medulla, in the control and pair-fed groups. However, the zinc deficient groups only demonstrated staining in the outer rim of the cortex. Furthermore, all groups except the zinc deficient treatment showed some staining in the lumina of renal tubules and all groups had MT staining in the large collecting duct epithelia in the lower portion of the renal pelvis. This specific tubule staining supports the potential role of MT in metal detoxification and/or excretion (Szczurek, 2000).

Zinc, Metallothionein, and Lead-Induced Inhibition of ALAD

ALAD activity is dependent upon the continuous availability of zinc, as a cofactor (Goering and Fowler, 1987b). Lead intoxication results in the inactivation of ALAD, through the reversible replacement of zinc atoms by lead atoms (Kaya et al. 1994). The high content of sulfhydryl groups (-SH) on ALAD is thought to contribute to the susceptibility of this enzyme to inhibition by many heavy metals (Haeger-Aronsen et al. 1976). In fact, recent molecular studies have revealed that lead binds a unique catalytic zinc-binding site on ALAD, with three cysteine residues (Godwin, 2001). Zinc has been shown to activate ALAD and prevent ALAD inhibition by lead in both *in vitro* and *in vivo* studies (Finelli et al. 1975; Goering and Fowler, 1987b). However, the interaction of lead with MT has not received much attention, most likely due to the fact that lead is a relatively weak inducer of MT synthesis (Goering and Fowler, 1987b).

MT has demonstrated an ability to mediate lead and zinc interactions with ALAD, in a rat model (Goering and Fowler, 1987b; Goering and Fowler, 1987c). Goering and Fowler (1987b) investigated the ability of zinc-induced renal MT to attenuate the inhibition of renal ALAD by lead, the distribution of lead and zinc binding with renal MT, and the capacity for MT to regulate the availability of zinc to ALAD in adult (300-350 g), male CD rats (Charles River, Sprague Dawley-derived strain). To meet their first objective, the authors pre-treated rats with a subcutaneous injection of 200 µmol Zn/kg as $ZnCl_2$ (~4.25 mg Zn per injection) at 48 and 24 hours prior to the assay. Renal homogenates were prepared and 10 µL of 3 µM lead was added to the reaction mixtures. The presence of lead resulted in an inhibition of ALAD activity to 52% of the control value, while the zinc pre-treatment caused a partial reversal of this inhibition, with an ALAD activity of only 84% of the control. Notably, pre-treatment with zinc alone (no lead) elevated ALAD activity to a 55% increase over that of the control.

In order to explain the above results, the authors then examined the *in vivo* binding patterns of lead and zinc in the kidney cytosol. Control (pre-treated with saline, 2 mL/kg, subcutaneously) and zinc pre-treated animals received intraperitoneal injections with ^{203}Pb (170 µCi/kg), four hours prior to termination. Kidney homogenates were fractionated on a gel filtration column and analyzed by atomic absorption spectrophotometry (AAS). Although total kidney lead concentration was not different between treatment groups, induction of MT by the zinc pre-treatment markedly increased ^{203}Pb and zinc binding. Zinc-induced MT readily bound lead *in vivo* (Goering and Fowler, 1987b).

An *in vitro* ALAD assay was also carried out by incubating purified kidney MT (50 µg protein containing 2 µM Zn) with purified bovine liver ALAD (2 mg protein), in both the presence and absence of lead. Adding 10 µL of lead (1 µM) resulted in the inhibition of ALAD activity to a level of only 46% of that of the control. The combination of lead (1 µM) and MT, however, reduced ALAD activity to only 89% of that of the control. Also of interest, was the observation that the addition of MT alone led to an increase in ALAD activity of almost two times greater than the control, and 2 µM of free zinc ion more than tripled ALAD activity. This finding suggests that MT is able to release and donate some, but not all, of its bound zinc to metalloenzymes, possibly due to variation in the capacity of MT to release zinc from its metal clusters (Goering and Fowler, 1987b).

Finally, two further experiments were performed to investigate the potential mechanisms in the zinc protective effect on ALAD inhibition. MT was labelled with ^{65}Zn and added to ALAD reaction mixtures to explore the possibility of zinc donation to ALAD by MT, as a contributing factor in the reversal of ALAD inhibition. Under these experimental conditions, and assuming equilibration between zinc and ^{65}Zn, kidney MT appeared to release 3 to 4 µg-atoms zinc/µmol MT, following fractionation by gel filtration chromatography and AAS analysis. However, net transfer of

zinc from MT to ALAD was only about 1 to 2 µg-atoms zinc/µmol MT. The remaining zinc appeared as free zinc ion, perhaps due to low binding affinity between zinc and ALAD leading to dissociation (Goering and Fowler, 1987b).

To explore the alternative mechanism of lead chelation by MT, ALAD reaction mixtures were incubated with ^{203}Pb (10 µL, 1 µM lead) in the presence and absence of MT and fractionated, also with gel filtration chromatography. In the control reaction mixtures, 42% of lead was bound to ALAD and 58% eluted as free lead. When MT was added, 49% of ^{203}Pb bound MT, while lead-ALAD binding was reduced to only 24% and free lead ion made up only 27%. Therefore, assuming equilibration between lead and ^{203}Pb, approximately 1 µg-atom Pb/µmol MT was chelated by rat kidney MT, under these experimental conditions (Goering and Fowler, 1987b).

Goering and Fowler (1987b) concluded that rat kidney MT has demonstrated the capacity (*in vitro* and *in vivo)* to regulate zinc availability and also function in lead detoxification. Rat kidney MT readily bound lead, affecting the intracellular distribution and activity of lead toward ALAD and significantly preventing the inhibition of ALAD by lead (Goering and Fowler, 1987b).

The mechanism of zinc attenuation of lead-induced ALAD inhibition appears to involve both the donation of zinc to ALAD from MT, followed by enzyme activation, as well as lead chelation to this protein (Goering and Fowler, 1987b). Consequently, kidney MT may function as a pool to supply zinc for metalloenzymes, such as ALAD, whose activities depend upon continuous zinc availability (Goering and Fowler, 1987b). Therefore, when considering ALAD inhibition as an indicator of lead exposure, a role for MT in zinc availability and ALAD activity must be taken into account (Goering and Fowler, 1987b).

The findings described above have also been corroborated by zinc-induced hepatic MT on hepatic ALAD activity, by the same authors (Goering and Fowler, 1987c). However, *in vitro*

incubations of rat liver and kidney cytosol with ^{203}Pb have demonstrated renal ALAD to be 7.5 times more resistant to inhibition by lead than hepatic ALAD (Goering and Fowler, 1984).

Goering and Fowler (1984) also demonstrated the *in vitro* reversal of lead-inhibited hepatic ALAD activity by a low molecular weight, high-affinity renal lead-binding protein (Pb-BP) component. These Pb-BPs are not detectable in the liver and share many characteristics with MT (Goering and Fowler, 1987c). For example, they have a high zinc content, heat-stability, and similar elution profiles with gel-filtration analysis, anion-exchange, and hydrophobic-interaction chromatography (Goering and Fowler, 1987c). However, they differ in amino acid composition and mobility in electrophoretic fields (Goering and Fowler, 1987c). Renal Pb-BPs may serve as the primary cytosolic ligand for lead in the kidney and function in the translocation of lead to the nucleus (Goering and Fowler, 1987a). Additional *in vivo* studies have provided evidence of the capacity of rat renal Pb-BP, as well as MT, to mediate zinc and lead interactions with ALAD (Goering and Fowler, 1987a). The mechanism of this interaction appears to involve both zinc donation from Pb-BP to ALAD and lead chelation by Pb-BP, as has been suggested for the mechanism of MT action (Goering and Fowler, 1987a). Also of interest, lead does not effectively induce the synthesis of Pb-BPs or MT (Goering and Fowler, 1987a).

Metallothionein and Heavy Metals

Lead has a high affinity to bind MT *in vitro* and is capable of replacing zinc bound to MT (Waalkes et al. 1984). However, the ability of lead to induce MT protein synthesis *in vivo* is rather modest and lead also fails to displace zinc from MT *in vivo* (Waalkes et al. 1984). Previous studies in rats and mice have reported hepatic, but not renal, MT induction and low affinity binding of lead to this protein (Nolan & Shaikh, 1992). However, cadmium (Cd)-induced, renal MT has been

shown to bind lead in animal models (Nolan & Shaikh, 1992). Hepatic MT induction is strongest with intraperitoneal lead injection, followed by intravenous, and then subcutaneous injection (Nolan & Shaikh, 1992). Similar responses to oral lead administration have not been reported.

The importance of MT and other metal binding proteins has been clearly demonstrated in the regulation of the intracellular bioavailability of various heavy metals (Smith et al. 1998). For example, the modulation of Cd toxicity by MT is well known (Templeton and Cherian, 1991). Perhaps the most important mechanism of Cd detoxification is its incorporation in MT (Peraza et al. 1998). The presence of Cd in the cell induces MT synthesis, which has been demonstrated through rising levels of MT mRNA (Peraza et al. 1998). MT has a higher affinity for Cd than zinc, which is readily replaced by Cd on the protein (Peraza et al. 1998). Free cellular zinc may then induce additional MT synthesis or be excreted in the urine (Peraza et al. 1998). Cd-MT complexes are thought to be relatively non-toxic (Goyer, 1997). However, it should also be noted that Cd-bound MT is toxic to the renal tubule during urinary excretion in animal models (Goyer, 1997). Therefore, MT may be indirectly contributing to the toxic action of this metal on the kidney (Goyer, 1997). A function for MT in the cellular detoxification of lead has not been extensively studied.

CYSTEINE RICH INTESTINAL PROTEIN (CRIP)

CRIP is a 77-amino acid, 8.6-kDa protein containing seven cysteine residues (Hempe and Cousins, 1992). Although it is minimally expressed at birth, CRIP is developmentally regulated, with protein levels increasing to those of adults during the suckling period. CRIP is a LIM (cysteine-rich motif for the lin-11, isl-1, and mec-3 genes) protein family member, defined by the presence of a unique double zinc finger motif (Cousins and Lanningham-Foster, 2000). LIM domains have

frequently repeating, cysteine-rich sequences, thought to be involved in protein-protein or DNA binding interactions (Cousins and Lanningham-Foster, 2000). However, the LIM domain of CRIP is a homeodomain, making a DNA binding role unlikely (Fernandes et al. 1997). Most LIM-containing proteins have unknown functions, but they may participate in cellular proliferation, differentiation, or turnover (Khoo et al. 1996). Although the function of this protein remains unknown, CRIP appears to interact with intracellular zinc pools (Cousins and Lanningham-Foster, 2000) and binds ^{65}Zn isotope in the intestinal lumen (Fernandes et al. 1997). The CRIP molecule binds two zinc atoms to comprise the two zinc finger domains (Fernandes et al. 1997).

CRIP mRNA is abundant in rat and human intestine, monocytes, and macrophages, but is minimally expressed in liver and other tissues (Fernandes et al. 1997). CRIP is predominantly localized in the Paneth cells of the intestine, which are widely distributed in the base of the crypt of Lieberkuhn (Fernandes et al. 1997). The localization of CRIP may shed light on its, as yet undetermined, physiological function (Fernandes et al. 1997). Paneth cells have been implicated as functioning in mucosal host defense through the formation of lysozymes, cryptidins (defensins), tumour necrosis factor-α, phospholipase-A2, epidermal growth factor, and antitrypsin (Morita et al. 2001; Kelly et al. 2004). CRIP seems to be localized intracellularly within cytoplasmic vesicles rather than the nuclei of Paneth cells, confirming the hypothesis that CRIP is not a DNA binding protein (Fernandes et al. 1997).

It is well established that Paneth cells have a high concentration of zinc (Elmes, 1974; Fernandes et al. 1997). This property may be related to zinc storage for functional use or possibly as a route for elimination of heavy metals, although these hypotheses are lacking in critical evidence (Erlandsen and Chase, 1972; Fernandes et al. 1997). It is known that Paneth cell counts, distribution, and granule morphology do appear to be modified by zinc status (Kelly et al. 2004).

Paneth cell granule abnormalities and invasion of these cells by intestinal bacteria have both been documented in zinc-deficient animals (Kelly et al. 2004). Additionally, zinc chelation treatment in rats results in Paneth cell apoptosis (Kelly et al. 2004).

A Paneth cell specific, zinc-binding protein has been identified in the rat, although its function is currently unknown (Sawada et al. 1993). Intravenous injection of a zinc chelator, dithizone, selectively and rapidly kills Paneth cells and results in the loss of this zinc binding protein (Sawada et al. 1993; Morita et al. 2001).

Cysteine-rich Intestinal Protein and Metallothionein

Hempe and Cousins (1992) investigated the relationship between CRIP and intestinal MT as a model for zinc absorption in the rat. Male, growing (4-5 week old) SD rats were fed either a low zinc diet (1 ppm zinc), control diet (70 ppm zinc) or high zinc diet (180 ppm zinc) for four days. Prior to the absorption studies (20 hours), some of the rats receiving the low zinc diet were administered an intraperitoneal injection of 25 µmol zinc (~ 1.63 mg zinc). Zinc absorption and intestinal accumulation were measured by introducing ^{65}Zn into ligated 10-cm loops of the small intestine of anesthetized rats for a period of 15 minutes. Zinc absorption was significantly greater in the low zinc group than in either the high zinc or parenteral zinc groups. Zinc status clearly affected ^{65}Zn distribution in the cytosol of intestinal cells, as determined by gel filtration HPLC. When fed a low zinc diet, 4% of ^{65}Zn associated with MT-containing fractions, while 40% of ^{65}Zn eluted with CRIP-containing fractions. In comparison, the high zinc diet and parenteral zinc treatment both resulted in more than 50% of the cytosolic ^{65}Zn associating with MT and only 14% or less eluting with CRIP. These results suggest that CRIP and MT competitively bind zinc (Hempe and Cousins, 1992).

CRIP was also isolated by gel filtration HPLC and protein abundance was determined with Western blotting and radiolabeling techniques. Abundance of CRIP was not significantly different among the three treatments, indicating that CRIP synthesis was not affected by short-term changes in zinc status (Hempe & Cousins, 1992). Intestinal MT concentration was significantly increased by the high zinc diet and parenteral zinc treatment, as determined by a ^{109}-Cd binding assay. Also of interest was the observation that ^{65}Zn absorption increased as the luminal zinc concentration increased but the total amount of zinc bound to CRIP was not affected. However, as luminal zinc concentration rose from 5 to 200 μM, the proportion of cytosolic ^{65}Zn bound to CRIP in the intestinal cells progressively dropped from 42% to 25%. As CRIP binding dropped, a concurrent increase was seen in ^{65}Zn binding to higher molecular weight proteins. Notably, luminal zinc concentration did not change total cytosolic zinc or the proportion bound to MT.

This study by Hempe and Cousins (1992) supports the hypothesis that dietary zinc regulates intestinal zinc absorption at least in part by intestinal MT concentration, which in turn modifies zinc binding by CRIP. The authors propose that CRIP is involved in transcellular zinc absorption, functioning as a diffusible, saturable, intracellular zinc carrier in the transcellular pathway. MT is then thought to modulate zinc absorption through the competitive binding of zinc to prevent zinc-CRIP binding and diffusion across the cell (Hempe and Cousins, 1992). However, the localization of CRIP within the intestinal crypt, rather than along the villi, would suggest that this protein is more likely involved in zinc storage or gut immunity than the transcellular zinc absorption pathway.

Cysteine-rich Intestinal Protein and Lead

A potential role for CRIP in zinc and lead metabolism has not been investigated to date. Although much remains to be learned about the biochemistry and function of this protein, there is considerable evidence to support a role for CRIP in zinc metabolism and the possibility of an involvement in heavy metal toxicology is worthy of consideration. The study by Szczurek (2000), previously described, reported strong MT staining in Paneth cells, which diminished in response to zinc deficiency. The localization of MT within the intestinal crypt and proliferative region of the villi supports a potential role for zinc and MT in heavy metal detoxification, including lead. The localization of CRIP within Paneth cells and high zinc content of Paneth cells, suggests a possible interaction in the function of MT and CRIP.

II. STUDY RATIONALE

Despite the global public health concern of chronic lead exposure, the molecular mechanisms of toxicity for this metal have not been well defined. In addition, those populations at greatest risk of the adverse health effects of lead intoxication may also be at risk of inadequate zinc intake. Therefore, the *in vivo* interactions of these two metals have potentially significant health implications.

It has been well documented that zinc supplementation reduces lead absorption, tissue accumulation, and cellular toxicity, while low zinc intakes enhance these processes in rats (Cerklewski and Forbes, 1976; Flora et al. 1991; Flora, 2002). Furthermore, as dietary zinc supply declines, the rate of zinc absorption is known to increase (Miller et al. 1990). Thus, if lead and zinc are absorbed by a common mechanism, zinc deficiency may increase lead absorption. This is a potentially significant health concern, as marginal or sub-clinical zinc intakes in human populations may be more common than previously thought. American children between the ages of one and three years have been reported to consume only 77% of the RDA for zinc, on average, according to the nationwide Food Consumption Survey of 1985 (Miller et al. 1990). Additionally, zinc intakes of children in developing countries are expected to be even lower.

The skeleton is an important target organ for the toxic effects of lead and also is compromised by inadequate zinc supply. Therefore, skeletal growth outcomes should be considered when investigating the effects of lead exposure with various levels of zinc.

The hypothesis of the present study is that lead absorption, accumulation, and toxicity will change in response to the level of dietary zinc, and that these changes will be reflected in MT and CRIP levels and distribution, the inhibition of ALAD, as well as measures of bone growth and

development. Marginal dietary zinc intake is expected to reduce the amount and alter the distribution of MT and CRIP in the intestine and MT in the liver and kidney, while supplemental dietary zinc is expected to induce MT synthesis. Lead exposure is expected to alter the distribution of MT and CRIP in the small intestine, and MT in the liver and kidney. Hepatic and renal ALAD activity is expected to decline in the marginally zinc deficient rats exposed to lead, with a greater inhibition seen in hepatic tissue, and improve with supplemental zinc treatment. It should also be noted that the supplemental zinc treatment is not expected to restore these indices to the full level of control animals, as lead metabolism is affected by additional factors other than zinc status. Skeletal growth and development are expected to be compromised by lead exposure and the effects should be more detrimental during marginal zinc deficiency than when an adequate or excess amount of zinc is given.

The mechanism of zinc protection through decreased lead absorption at the gastrointestinal tract is thought to be through competition for binding on an intestinal, zinc-binding transport protein, such as MT and/or CRIP. The protective effects of reduced tissue accumulation of lead and tissue ALAD activity may be due to both decreased lead absorption and/or MT-lead binding to protect sensitive cellular processes from the toxic effects of lead. CRIP-lead interactions may also be important in the intestine. CRIP-lead binding and the localization of lead within the Paneth cell could lead to detoxification through mucosal turnover and fecal excretion. Alternatively, MT-lead binding could increase the amount of zinc available for transcellular zinc absorption. Lead bound to MT could then be excreted during mucosal turnover.

In terms of skeletal effects, marginal zinc deficiency is expected to increase bone lead accumulation and impair skeletal growth and development. Zinc supplementation should be protective against lead deposition in the skeleton and stimulate skeletal growth. In general, lead

treatment is expected to be more detrimental to skeletal development when a marginal zinc diet is fed than when an adequate or supplemental zinc diet is fed.

The objective of this study is to investigate the effects of marginal and supplemental zinc intakes on lead absorption and distribution by examining lead and zinc concentrations in the small intestine, liver, kidney, serum, and femur, MT immunohistochemical localization in the small intestine, liver and kidney, MT and CRIP mRNA levels in the small intestine, renal and hepatic ALAD activity, skeletal densitometry, femoral morphometric analysis, and serum markers of bone formation and resorption.

To meet this objective, weanling SD rats were randomly assigned to treatment groups of 8 ppm zinc, 30 ppm zinc (fed *ad libitum* or pair-weighed to the low zinc group), or 300 ppm dietary zinc and simultaneously exposed to 0 or 200 ppm lead acetate in the drinking water for 3 weeks. SD rats are the most commonly studied rat in lead and zinc toxicological research (Miller et al. 1990). Rats will be in the weanling stage of life, as the young undergo rapid growth and development, potentiating treatment effects. Additionally, growing animals are also more susceptible to the adverse effects of lead toxicity, in both humans and rats (Miller et al. 1990). The rat model is thought to be somewhat insensitive to the effects of lead compared to human studies (Ronis et al. 2001). For example, a cohort study with infants predicted significant decreases in stature with blood lead concentrations of 10 µg/dL or more (Ronis et al. 2001). In contrast, blood lead values of more than 60 µg/dL are required to produce impairments in rat somatic and skeletal growth (Ronis et al. 2001).

The rat is considered a good model of human osteopenias, as the fundamental biological mechanisms responsible for bone mass accumulation and loss during normal growth, as well as adult life, are the same in humans and rats (Frost & Jee, 1992). Linear growth of long bones in the

Sprague Dawley rat is rapid until 6 months of age, declines to insignificant levels by 12 months, and ceases by 18 months (Frost & Jee, 1992). In growing rats, both longitudinal growth of cortical bone and the thickening of trabeculae occur through the same mechanisms as seen in children (Frost & Jee, 1992). However, rats do have less intracortical bone remodeling and a higher rate of bone turnover at many skeletal sites than humans, due to the presence of hemopoietic marrow throughout life (Mosekilde, 1995). Therefore, dietary interventions and treatments that are deemed effective in a small animal model must also be demonstrated in a larger animal model with known intracortical remodeling (Mosekilde, 1995).

In a previous study, diets with 8 ppm, 35 ppm, and 200 ppm zinc, with or without 50 or 200 ppm lead did not result in significant differences in food intake or weight gain among treatment groups of growing, male, SD rats after three or seven weeks (Cerklewski & Forbes, 1976). However, severely restricted zinc diets and diets with up to 5 ppm zinc result in significant reductions in weight gain in growing rats (El Gazzar et al. 1978; O'Dell and Reeves, 1989). Therefore, a pair-weighed group for both 0 ppm lead and 200 ppm lead will be included and matched with the lowest weighing group, in order to control for calorie depletion due to anorexia. Rats were pair-weighed as pair-feeding often results in a higher body weight than achieved by the zinc deficient rats, making the interpretation of effects due to feed restriction versus effects due to zinc deficiency more difficult.

Lead exposure at 200 ppm has been reported to produce sub-clinical toxicity in rats fed standard laboratory chow (Six and Goyer, 1970; Victery et al. 1987). A 12 week study in 72 male, growing CD rats reported blood lead concentrations of 40-60 µg/dL in rats exposed to 200 ppm lead as PbAc in drinking water. These numbers are comparable to humans with sub-clinical or low-level Pb toxicity (Victery et al. 1987). In comparison, rats exposed to 500 or 1000 ppm lead

developed blood lead concentrations of 100-150 µg/dL, which correspond to levels of severe, overt lead toxicity in humans (Victery et al. 1987). Another 10 week study in 30 male, SD rats administered 200 ppm lead, as PbAc in distilled drinking water, did not result in significant changes in hematopoiesis or renal histology, function, and size, when consuming a standard laboratory chow (Six and Goyer, 1970). Additionally, water consumption was not found to be significantly different between the control and the lead-exposed animals, as determined over three day periods at week three, six, and ten (Six and Goyer, 1970).

This study investigated tissue-specific effects in the duodenum, jejunum, liver, kidney, femur, and serum. The duodenum was critical as this is the primary site of lead absorption and the protective effects of zinc against lead toxicity have been mainly attributed to a decrease in lead absorption at the gastrointestinal level, in previous studies. The jejunum was also included as it is the primary site for zinc absorption, as well as for comparison purposes to previous studies. The kidney was essential as it is the principal site of initial lead accumulation following exposure and the effects of lead toxicity are thought to be most insidious in this organ. The liver was important due to its critical role in the detoxification processes of the body. Hepatic ALAD also was assessed as an indicator of lead toxicity, as this enzyme is highly sensitive and specific to inhibition by lead. The femur was selected as a typical long bone to study the effects of lead and dietary zinc on bone growth and quality. Finally, serum was required for mineral status assessment, as well as biochemical analysis.

For the above reasons, zinc and lead concentrations were analyzed in the serum, liver, kidney, intestine, and femur, as indirect indicators of lead absorption. MT immunohistochemistry was conducted on liver, kidney, duodenum, and jejunum tissues, as MT is involved in zinc absorption and metabolism, and may also participate in the absorption and distribution of lead.

Unfortunately, it was not possible to complete the hepatic MT immunostaining, due to technical problems with the technique. MT and CRIP RT-PCR analysis was performed solely on the duodenum and jejunum, as CRIP is not present in significant amounts in other tissues. ALAD activity was determined in the liver. Due to the lack of a lead effect on hepatic ALAD activity, renal ALAD activity was not assessed.

Although the effects of zinc deficiency and supplemental zinc treatment on lead absorption and toxicity, and the ability of zinc to attenuate the lead-induced inhibition of ALAD have been previously reported, the mechanisms of interactions between zinc and lead at the absorptive and intracellular levels have not been fully elucidated. Lead absorption may be enhanced during periods of low zinc intake if lead and zinc share a common absorptive mechanism. At high dietary zinc intakes, absorption is thought to be mediated by zinc-induced MT. MT may be involved in the competition for absorption between lead and zinc, in the gastrointestinal tract. Zinc-induced MT may bind lead, thereby enhancing zinc absorption in the presence of lead. In the absence of MT induction by zinc lead may be preferentially absorbed over zinc.

While several authors have speculated a role for CRIP in heavy metal detoxification, this proposition has not been investigated. CRIP is predominantly localized within the Paneth cells of intestinal epithelium, which are known to have high concentrations of zinc. CRIP, within Paneth cells, may also be functioning as an intestinal storage reservoir for zinc, supporting the detoxifying action of MT, or as an elimination route for lead in the enterocyte.

At the cellular level, the mechanism of the protective effect of zinc against renal and hepatic lead intoxication is also not clear. Specifically, a role for MT in tissue lead detoxification has not been fully investigated. Zinc-induced MT may reduce histological alterations resulting from lead exposure, by binding lead to form non-toxic, insoluble complexes for storage and/or excretion.

Zinc also is known to attenuate the inhibitory effect of lead on the heme biosynthetic enzyme, ALAD. MT may contribute to this attenuation through donation of zinc to ALAD for enzyme activation, or by the direct chelation of lead.

III. MATERIALS AND METHODS

Materials

Hematoxylin, bovine serum albumin, lead (II) acetate, sucrose, trizma-acetate, reduced glutathione, and ρ-dimethylaminobenzaldehyde were purchased from Sigma (St. Louis, MO). Sodium chloride, $Na_2HPO_4 \cdot 7H_20$, $NaH_2PO \cdot H_2O$, reagent alcohol, hydrogen peroxide, formalin, xylene, eosin, permount, nitric acid (70%, trace element grade), sodium acetate, trichloracetic acid, mercuric chloride, perchloric acid, and glacial acetic acid were purchased from Fisher (Fair Lawn, NJ). Sources for all other reagents and materials are specified in the text.

Animals and Diets

Sixty-four weanling male Sprague Dawley (SD) rats were obtained from Charles River Laboratories (St. Constant, PQ). Animals were housed in a temperature (21-23°C) and humidity (55%) controlled room with a 14:10 hour light:dark cycle. Following a 5 day acclimatization period to a nutritionally complete control diet, rats were randomly assigned to treatment groups (n=8 per group). Treatment groups included marginal zinc intake (MZ group; 8 ppm Zn, 0 ppm Pb), lead exposed and marginal zinc intake (MZ+Pb group; 8 ppm Zn, 200 ppm lead), supplemental zinc intake (SZ group; 300 ppm Zn, 0 ppm Pb), lead exposed and supplemental zinc intake (SZ+Pb group; 300 ppm Zn, 200 ppm lead), a control group (C group; 30 ppm Zn, 0 ppm lead), a lead exposed control group (C+Pb group; 30 ppm Zn, 200 ppm Pb), a group pair-weighed to the MZ group (PW group; 30 ppm Zn, 0 ppm Pb), and a lead-exposed group pair-weighed to the MZ+Pb group (PW+Pb group; 30 ppm Zn, 200 ppm Pb) (see Figure 1). All dietary treatments, except PW

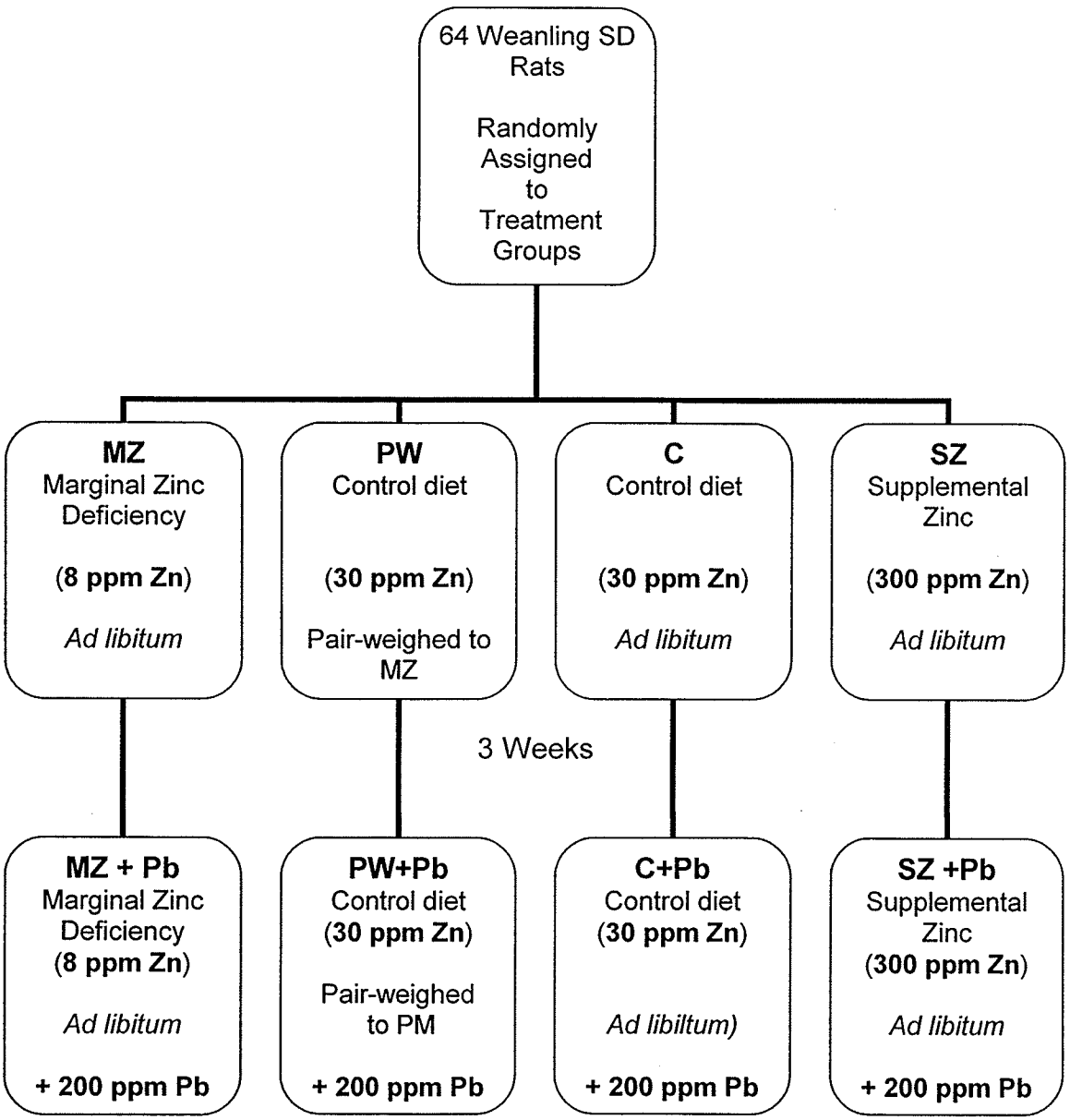


Figure 4. Experimental Design. Sixty-four rats were randomly assigned to eight treatment groups in a 4x2 factorial design. Rats were fed a marginally zinc deficient diet with Pb (MZ+Pb group) and without Pb (MZ group); Zn-adequate diet *ad libitum* with Pb (C+Pb group) and without Pb (C group); Zn-adequate diet pair-weighed to MZ with Pb (PW+Pb group) and without Pb (PW group); or a supplemental zinc diet with Pb (SZ+Pb group) or without Pb (SZ group) for 3 weeks. Each treatment group had 8 rats and all rats were terminated on day 21 of the experiment.

and PW+Pb were provided *ad libitum*. Diets were based on the AIN-93G formulation (Reeves, 1993). The diet formulation is provided in Table 1. The dietary treatment phase of the study was carried out for 3 weeks.

Lead (200 ppm) was administered as PbAc in the drinking water and was provided *ad libitum*. Non-lead treated animals received double deionized water *ad libitum*. Feed intake was recorded three times per week, while water intake was recorded weekly. Body weight was recorded weekly for all groups, except after Day 10, when it was recorded daily for the purpose of restricting the feed intake of the PW and PW+Pb groups. Feed restriction varied from approximately 10 to 18 g/day for the final 10 days of the study and was adjusted individually for each animal in the PW and PW+Pb treatments in order to match the weight of the MZ and MZ+Pb treatments.

Special precautions were taken to avoid zinc and lead recycling and contamination throughout the experimental period. Animals were housed individually in stainless steel hanging cages with wire bottoms. Double deionized water or lead acetate prepared in double deionized water was provided in plastic bottles with stainless steel sipper tubes. The MZ and MZ+Pb groups were placed on the top racks of the cages and the SZ and SZ+Pb groups were kept on the bottom racks. All lead-treated animals were housed on a separate cage. As well, special attention was made to prevent cross-contamination of the various diets during diet preparation and feeding. Dietary zinc content was verified as described in the method for mineral analysis. Lead cross-contamination during water bottle changes was also avoided and lead acetate-filled bottles were not re-used.

Table 1. Diet Formulation (g/kg)

Ingredients[1]	Marginal Zinc Deficient Diet (8 ppm zinc)	Control Diet (30 ppm zinc)	Zinc Supplemented Diet (300 ppm)
Dextrose (Cerelose)	602.3	594.6	504.6
Egg White	212.5	212.5	212.5
Fibre (Cellulose)	50	50	50
Mineral Mix (AIN-93M; zinc-free)	35	35	35
Potassium Phosphate	5.4	5.4	5.4
Vitamin Mix (AIN-93)	10	10	10
Choline Bitartate	2.5	2.5	2.5
Biotin Mix[2]	10	10	10
Zinc Pre-mix[3]	2.3	10	100
Soybean Oil	70	70	70

[1]Diet ingredients were purchased from Harlan Teklad (Madison, WI), except for dextrose (Moonshiners, Winnipeg, MB)
[2]Biotin mix (200 mg biotin/kg dextrose)
[3]Zinc premix (5.775 g zinc carbonate/kg dextrose)

Tissue Collection and Storage

All animals were terminated by CO_2 asphyxiation in accordance with the guidelines of the Canadian Council on Animal Care (1993). Body weights were recorded and trunk blood collected following decapitation. Blood samples were stored on ice until centrifuged (Beckman TJ-6R centrifuge) at 1290 x g for 15 minutes to obtain serum. Blood was also centrifuged in micro-hematocrit capillary tubes (75 mm; Fisher, Fair Lawn, NJ) for 10 minutes to obtain hematocrit. The liver, kidney, small intestine, and epididymal fat pads were dissected. The organs were weighed and intestinal length was measured from pylorus to cecum. Sections of the liver, kidney, duodenum (1 cm distal to the pylorus), and jejunum (mid-section) were excised and briefly rinsed with phosphate buffered saline (PBS, 0.15 M NaCl, 10 mM phosphate; pH 7.4) to remove superficial blood and intestinal contents. Tissue samples were then fixed in 10% phosphate buffered formalin for 24 to 48 hours in preparation for immunohistochemical studies. The remaining tissues were immediately frozen in liquid nitrogen and later stored at -80°C, along with the serum samples.

Rat carcasses were stored at -20°C for high resolution scanning, as described in the method for skeletal densitometry analysis. Carcasses were thawed prior to all *in vivo* scans. Right femurs were then excised, cleaned of soft tissue and re-scanned.

Mineral Analysis

Serum, liver, kidney, duodenum, jejunum, femur, and animal diets were analyzed for zinc and lead concentration. Tissue mineral concentration was determined in order to assess zinc and lead absorption, tissue retention, and whole body mineral status. In addition, femurs were

analyzed for calcium and phosphorus concentration to evaluate bone mineralization and livers were analyzed for iron and copper concentration to assess micronutrient status. Serum samples (200 µL) underwent a 10-fold dilution in double deionized water and were analyzed directly for zinc. These samples were processed along with human serum reference (BioRad Laboratories, Irvine, CA) and deionized water blanks in duplicate by atomic absorption (Varian Spectra AA, Varian, Victoria, Australia).

The organs and diets were subjected to acid digestion based on the method of Clegg et al (1981). Organ samples were weighed and dried in an oven for 48 hours at 85°C. Dry weight was recorded immediately following removal from the oven and placed in trace element-free, disposable DigiPrep tubes (SCP Science, Baie d'Urfé, Quebec). Concentrated nitric acid was added to each tube (1 mL for duodenum and jejunum; 2 mL for kidney; 3 mL for liver and femur; 4 mL for 0.5 g of each diet) and permitted to digest the tissues at room temperature for 1 hour, followed by 2 hours of heating at 85°C in a DigiPrep HP heater (SCP Science, 24 well). Digests were diluted with double deionized water and dilution factors varied depending on the dietary treatment group and dry weight of the sample. Bovine liver reference (National Institute of Standards and Technology, Gaithersburg, MD) was processed in triplicate as a quality control and three nitric acid blanks were included with each tissue and diet sample. Zinc and lead concentrations of diluted samples were measured with inductively coupled plasma optical emission spectroscopy or ICP-OES (Varian Liberty 200, Varian, Canada).

Mineral Concentration Calculations (*Zinc, Lead, Iron, Copper, Calcium, Phosphorus*) :

Tissues and diets:

$$\text{Mineral (µg/g dry weight)} = \frac{\text{Sample mineral concentration x dilution factor}}{\text{Sample dry weight}}$$

$$\text{Mineral (µmol/g dry weight)} = \frac{\text{µg mineral / g dry weight}}{\text{mineral molecular weight}}$$

Serum:

Mineral (µmol/L) = Sample mineral concentration x dilution factor

Immunohistochemical Localization of Metallothionein

MT localization was assessed with the indirect immunoperoxidase technique, in the kidney, duodenum, and jejunum. This technique provides data not only on the relative concentration of MT but also the cellular distribution.

Tissue samples were fixed in formalin and embedded in paraffin by the Pathology Lab, Health Sciences Centre (Winnipeg, MB). Five micrometre sections were cut and mounted on fixative-coated slides. Slides were deparaffinized in xylene and rehydrated in a graded series of ethanol to water, followed by immersion in 3% hydrogen peroxide for 10 minutes to inhibit the endogenous peroxidase activity in the tissues. The reaction was stopped by placing the slides in a bath of PBS (0.15 M NaCl, 10 mM phosphate; pH 7.2) for 5 minutes. Sections were then treated with 20% normal goat serum (DAKO Corporation, Carpinteria, CA) for 1 hour at room temperature to block nonspecific binding sites. Following blocking, monoclonal anti-MT antibody (clone E9; DAKO), diluted in PBS (1:25 for duodenum and jejunum, and 1:50 for kidney) was applied to the sections and incubated at room temperature for 1 hour (duodenum and jejunum) or at 4°C overnight (kidney) in a humid tray chamber. Sections were then layered with goat anti-mouse/anti-rabbit peroxidase labelled polymer (DAKO Envision System, DAKO, Carpinteria, CA) diluted in PBS (1:10; 250 µL solution per slide) and incubated at room temperature for 30 (duodenum and jejunum), or 60 (kidney) minutes, in a humid tray chamber. Following the incubation with the

primary and secondary antibodies, the sections were placed in a 5 minute PBS bath and then washed with fresh PBS. Visualization of the reaction product was possible after treatment with 250 µL (5 mg) of 3,3'-diaminobenzidine tetrahydrochloride (DAB·4HCl; Polysciences, Inc., Warrington, PA) in 10 mL PBS containing 30 µL of 3% hydrogen peroxide for 5 minutes at room temperature. Excess DAB was removed with deionized water and tissues were counterstained with hematoxylin for 15 seconds. Tissues were rinsed with water, dehydrated in graded concentrations of ethanol, and cleared in xylene, prior to mounting cover slips. Duodenum and jejunum sections were also counterstained with eosin for detection of Paneth cells. The specificity of the reaction was confirmed by omission of anti-MT antibody from the procedure. Using a light microscope, the intensity of MT staining was estimated subjectively at 4 levels: nil, defined as the absence of staining; weak, defined as the staining just visible above the background at lower magnification (10x objective); moderate, defined as the staining easily visible at lower magnification; and strong, defined as the staining easily visible at lowest magnification (2.5x objective). The evaluator was blinded to the animal number and treatment group during the evaluation process. Computer images of immunostained sections were obtained with Northern Eclipse software version 6.0 (Empix Imaging Inc., Toronto, ON).

MT & CRIP mRNA Levels

Total RNA Isolation & Primer Design

Total RNA extraction was carried out on 200 mg of intestinal tissue using TRIzol (Invitrogen Corporation, California, USA). Intestinal tissues stored at -80°C were frozen in liquid nitrogen immediately prior to extraction to prevent RNA degradation. The RNA was chloroform extracted,

isopropanol precipitated, and washed with 75% ethanol. The concentration and purity of samples were assessed spectrophotometrically by absorbance at 260 nm and the A_{260}/A_{280} absorbance ratio, respectively, and verified by ethidium bromide staining on a 1% agarose gel. Custom primers of 100-150 bp fragments were designed using the internet-based NCBI nucleotide database (http://www.ncbi.nlm.nih.gov.proxy2.lib.umanitoba.ca/entrez/query.fcgi?db=PubMed) for MT-(BC 058442) and CRIP (AA 925488). Oligonucleotide sequences were as follows: 5'-GCC TTC TTG TCG CTT ACA CC-3' MT sense, 5'-CTT CTT GCA GGA GGT GCA TT-3' MT anti-sense, 5'-CCC TGC TGT CTA GGG ACA AG-3' CRIP sense, and 5'-ACT GCA ACC ATC CCT GCT AC-3' CRIP anti-sense. The housekeeping gene L32 (X0 6483) was included in the analysis as a control, with an oligonucleotide sequence of: 5'-AAG ATT CAA GGG CCA GAT CC-3' L32 sense and 5'-GTT GCA CAT CAG CAG CAC TT-3' L32 anti-sense.

Real-Time Quantitative RT-PCR (RT-Q PCR)

RT-Q PCR reactions were performed with SYBR-green using RT-PCR reactions on a Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA) sequence detection system. Specificity and identity of the RT-PCR products were verified by melting curve analysis, according to the manufacturer's instructions. Isolated RNA was treated with DNAse I (Invitrogen Corporation) prior to analysis. Template RNA (500 ng) was then added to 12.5 µL QuantiTect® SYBR® Green, 2 µL sense primer, 2 µL anti-sense primer, 0.25 µL QuantiTect RT Mix, and diluted to 25 µL with RNase-free water (QuantiTect® SYBR® Green RT-PCR kit, Qiagen, Mississauga, Canada). Reverse transcription was induced by a 30 min incubation at 50°C, followed by a 15 min incubation at 95°C to initiate PCR activation. The following incubation cycle was then followed to produce

denaturation, annealing, and extension: 15 s at 94°C, 30 s at 55°C, 30 s at 72°C. Amplification was then allowed to proceed for 40 cycles. Relative amounts of mRNA were determined by comparing cycle threshold (CT) values for equal amounts of amplified RNA. The difference between control and treatment mRNA expression was calculated from the difference in CT values, using the formula $2^{\Delta CT}$. Of note, the housekeeping gene L32 was affected by dietary treatment when analyzed by 2-way ANOVA. Additionally, the incorporation of L32 mRNA levels as a statistical covariate did not alter the MT or CRIP mRNA results.

ALAD Assay

The enzymatic activity of ALAD was measured according to the methods of Gibson et al. (1955) and Baron and Tephly (1969), using modifications as described by Goering and Fowler (1984, 1985). The inhibition of ALAD is a highly sensitive and specific indicator of intracellular lead toxicity (Goering and Fowler, 1985).

ALAD activity was determined as the rate of product formation, porphobilinogen (PBG). ALAD (also called PBG Synthase) synthesizes PBG from 2 molecules of ALA (Figure 5). ALAD was determined in post-mitochondrial supernatant fractions (13, 000 x g) of liver homogenates (0.5 – 1.5 mg protein/mL), prepared in sucrose (Goering and Fowler, 1985). Reaction mixtures consisted of 0.3 mL of tissue supernatant in 0.25 M sucrose with 10 mM Tris-acetate buffer and 0.7 mL of 0.1 M sodium acetate with 3.3 mM ALA and 3.3 mM reduced glutathione (GSH), pH 6.8 (Goering and Fowler, 1985). A pH of 6.8 has been determined to be the optimum pH for this assay (Coleman, 1966). Reaction mixtures were incubated at 37°C for 90 minutes and terminated by the

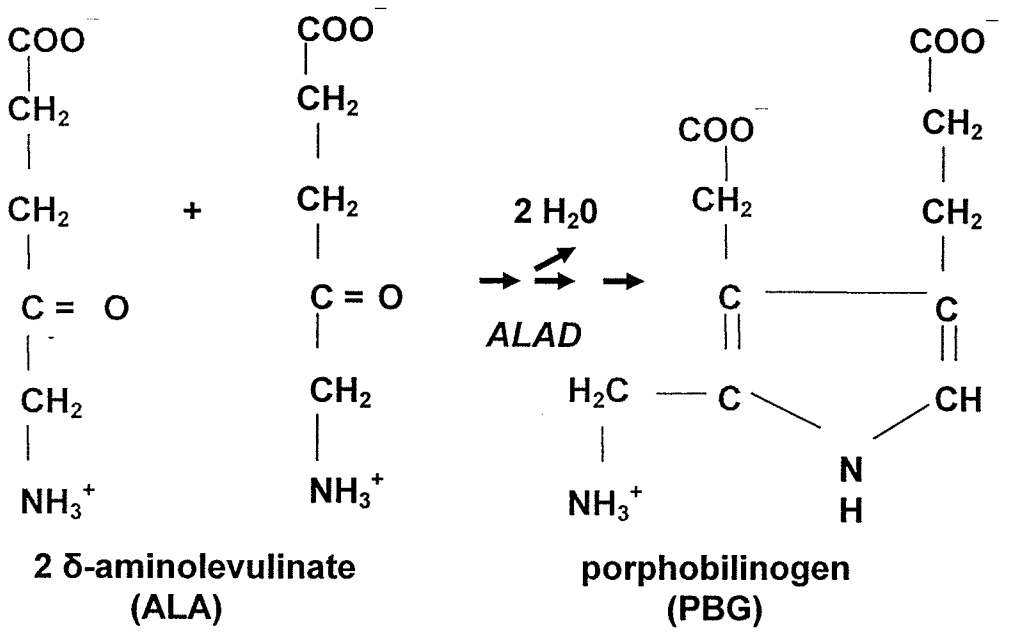


Figure 5. Porphobilinogen synthesis (adapted from King, 2005).

addition of 0.3 mL of 10% trichloracetic acid solution containing 0.1 M mercuric chloride (Baron & Tephly, 1969). Following removal of the precipitate by centrifugation (3250 rpm, 5 minutes), PBG levels were determined with a modified Ehrlich reagent, as described by Mauzerall and Granick (1956). Ehrlich's reagent or p-dimethylaminobenzaldehyde (DMAB) reacts with PBG to form a red compound in acid solution. Ehrlich's reagent contains 2% (w/v) DMAB in 6 N HCl (Mauzerall and Granick, 1956). Modified Erhlich's reagent contains 2N perchloric acid, which allows for more intense and stable colour development, and is prepared with 1 g of DMAB and 9.3 mL of 70% perchloric acid in 50 mL of glacial acetic acid. Equal volumes of modified Ehrlich's reagent (0.75 mL) were combined with the reaction mixture (0.75 mL) and the intensity of the coloured solution was read at 553 nm, following a 15 minute incubation period, with an Ultrospec 4000 Spectrophotometer (Pharmacia Biotech, Cambridge, England). See Appendix B for complete assay protocol.

Skeletal Densitometric Analysis

Femur, tibia, humerus, spine, and the whole body of rat carcasses were analyzed for bone area (BA), bone mineral content (BMC), and bone mineral density (BMD) *in situ* by dual energy x-ray absorptiometry (DEXA, 4500A; Hologic Inc., Bedford, MA; small animal software high resolution option). Animals were placed dorsally, in an anterior-posterior position. DEXA has demonstrated good precision and accuracy in measuring BMC and BMD in small animals, *in situ* (Lochmuller et al. 2001), as well as in isolated small animal bones (Kastl et al. 2002). The precision error (as CV%) for BA, BMC, and BMD was 2.7, 4.1, 2.3%, respectively, for the humerus; 4.0, 2.9, 1.5 %, respectively, for the spine; 3.8, 6.2, 6.3%, respectively, for the femur; 7.6, 3.2, 9.4%, respectively, for the tibia; 4.9, 2.5, 2.4%, respectively, for the femur and tibia combined; and 1.8, 1.5, 0.4%, respectively, for the whole body.

Excised femurs were also analyzed for BA, BMC, and BMD by DEXA. Femurs were put in a plastic water bath with 2 cm of water above the bone and aligned in the anterior-posterior position. The water bath was tested for interference with the scan accuracy. The precision error (as CV%) for excised femur BA, BMC, and BMD was 2.0, 1.0, and 1.1%, respectively. The measurement of single rat bone *ex situ* offers much greater precision (generally <1%) when submerged in aqueous solution than *in situ* measures of the same bone. The *in situ* scans generally fall within a range of 3%-6%, suggesting that the surrounding soft tissues contribute to the precision error (Lochmüller et al. 2001).

Femoral Morphometric Measurements

Femur measurements were obtained with calipers to the nearest 0.01 mm as described by Reichling et al. (2000) and included length, diaphysis thickness, femoral head, neck, and knee joint width. Briefly, femoral length was measured as the proximal-most point on the femur head to the distal-most point of the femur. Diaphysis thickness was measured as the narrowest width across the shaft. The femoral head was measured as the anterior-most point to the posterior-most point on the head. Femoral neck thickness was measured as the narrowest width across the neck and knee joint thickness was measured as the greatest width across the distal femur epiphysis. All measures were re-produced in triplicate by the same examiner.

Biochemical Assays

Serum osteocalcin and Ratlaps were measured by enzyme-linked immunosorbent assays specific for rat osteocalcin (Rat-Mid Osteocalcin, Osteometer BioTech, Herlev Hovengrade, Denmark) and bone-related plasma degradation products of C-terminal peptides of type I collagen in rats (RatLaps, Osteometer Biotech, Herlev Hovedgade, Denmark). Osteocalcin is a marker of bone formation as it is a protein specifically produced by osteoblasts. Ratlaps is a marker of bone resorption, as it measures a breakdown product of collagen released during bone resorption.

Statistical Analysis

Data were analyzed for main effects of lead and zinc, as well as interactions of lead and zinc, by two-way ANOVA using SAS software version 9.1 (SAS institute, Cary, NC). However, in the case when no lead was detectable in the tissues of non-lead treated animals, a one-way

ANOVA was used. Repeated measures analysis was performed for main effects and interactions on weekly body weights, feed efficiency, and growth rates.

Significant differences among treatment group means were determined with Duncan's Multiple Range test. Differences were considered significant at $p < 0.05$, except for interaction effects, which were considered significant at $p < 0.1$ due to the large number of treatments used in the study.

All data are reported as means ± SEM. Data were checked for normality and homogeneity of variance, and transformed when necessary.

IV. RESULTS

The Results chapter reports the results for growth, zinc status, lead status, and micronutrient assessments, followed by the results for liver, kidney, intestine, and bone in separate sections. If there was a significant interaction between lead and zinc, or if the main effects for zinc and lead were not significant, all 8 groups (mean ± SEM) are shown in the Figures. Otherwise, the Figures present data for main effects (mean ± SEM). All data, including the means for the 8 dietary treatment groups, is presented in tabular and graphical form in Appendix A.

Keys to Figures

The following letter codes were used to identify the different treatment groups:

MZ = Marginal Zinc Deficiency

PW = Pair-Weighed to MZ

C = Control

SZ = Supplemental Zinc

The addition of lead is indicated (**+ Pb**) where appropriate; otherwise dietary zinc treatments include both lead treated and non-lead treated animals.

GROWTH ASSESSMENT

To assess the growth of rats during the course of the study, body weights at days 0, 7, 14, and 21 were determined and epididymal fat pad, organ, and femur weights were recorded at termination on day 21. Feed intake was also documented throughout the study to assess feed efficiency.

Weekly Body Weights

Body weights of rats at days 0, 7, 14, and 21 of the dietary treatment were analyzed by repeated measures ANOVA (Figure 6). There were no significant differences in body weight among the treatment groups at the beginning of the study. Lead treatment resulted in reduced body weight regardless of dietary zinc intake and was apparent by Day 7 (Figure 6A). Body weight of lead-treated groups was 4% lower on day 7 and 6% lower on days 14 and 21 compared to non-lead treated groups.

MZ deficiency also resulted in a reduced body weight (6%) in comparison to C rats on day 7, which worsened to a 10% lower weight by days 14 and 21 (Figure 6B). PW rats weighed the same as C rats until the final week of the study when they weighed 11% less than the C group but had a weight equivalent to the MZ group. SZ treatment resulted in a 5% increase in body weight in comparison to C rats on day 14 but this difference was no longer apparent on day 21. The interaction of lead and dietary zinc over time did not reach significance ($p=0.1571$).

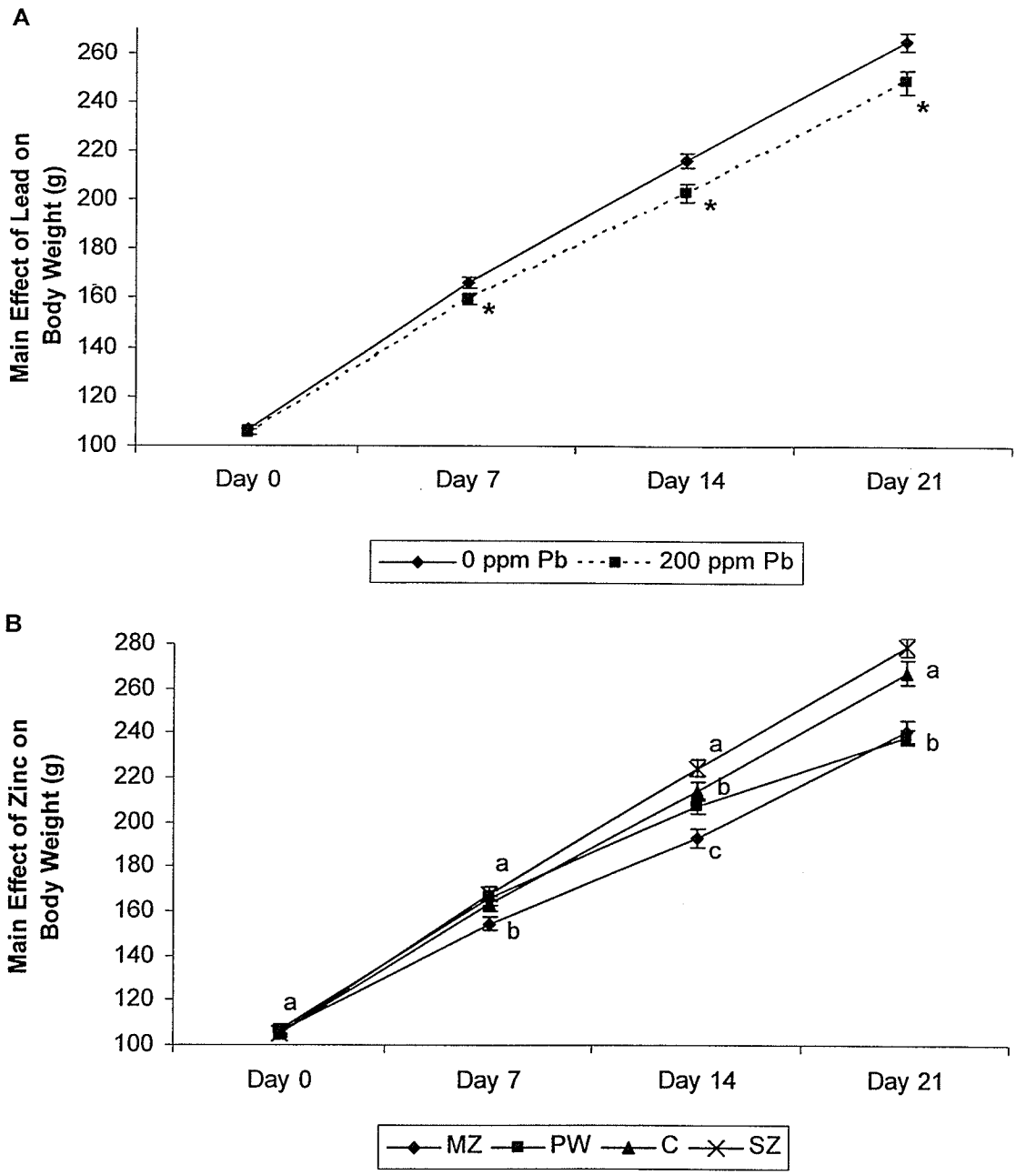


Figure 6. Effect of dietary zinc and lead exposure on body weight at days 0, 7, 14, and 21 of the study. Data points represent group means ± SEM for n=32 (A) and n=16 (B). There were significant main effects of lead (A) and zinc (B) after weeks 1, 2, and 3 ($p<0.0001$), as indicated by an asterisk(*; lead effect) and lower case letters (zinc effect). Data points with an asterisk or different letters are significantly different within the same week as determined by Duncan's multiple range test. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The interaction of lead and dietary zinc over time was not significant ($p=0.1571$).

Feed Intake and Body Weight Assessment

Lead treatment resulted in a 9% lower total feed intake and a 6% lower final body weight compared to non-lead treated groups (Figure 7). MZ and PW groups were not significantly different in terms of total feed intake and final body weight. MZ rats had a lower feed intake (13%) and body weight (10%) than C rats. C and SZ treatments did not differ in terms of either feed intake or body weight.

Total lead dose consumed was not affected by dietary zinc intake when calculated as a total amount or a total amount per kg of body weight (Figure 8).

Total weight gain (Figure 9) followed a similar pattern as feed intake and body weight (Figure 7), with the exception that SZ rats gained 7% more weight than C rats over the course of the study. Weight gain was depressed by 10% with lead treatment, 17% with MZ treatment, and 19% by PW treatment (Figure 9).

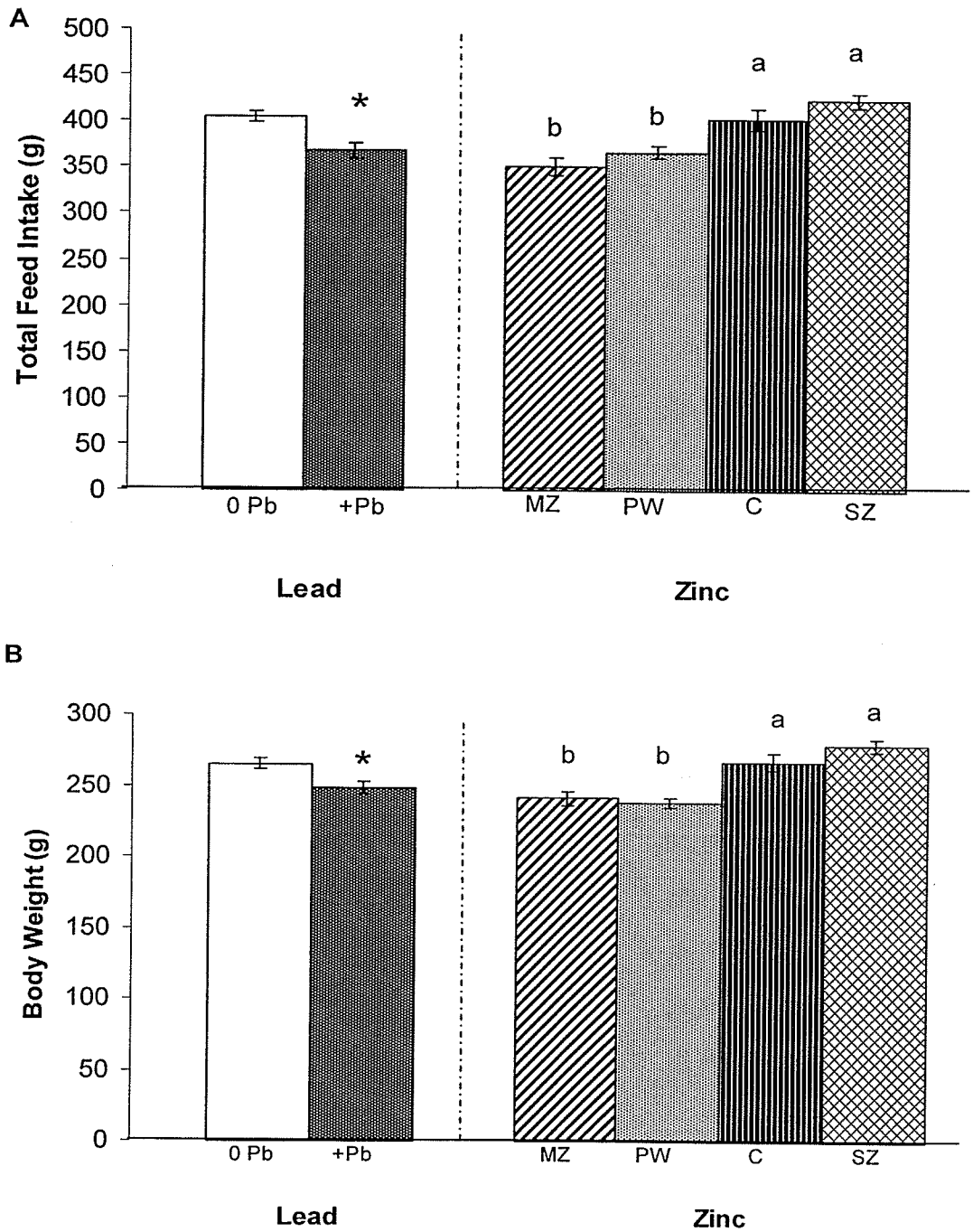


Figure 7. Effect of dietary zinc and lead exposure on total feed intake (A) and final body weight (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There were significant main effects for lead (A, $p<0.0001$; B, $p= 0.0002$), as indicated with an asterisk (*) and significant main effects for zinc (A&B, $p<0.0001$), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (A, $p=0.4877$; B, $p=0.2459$).

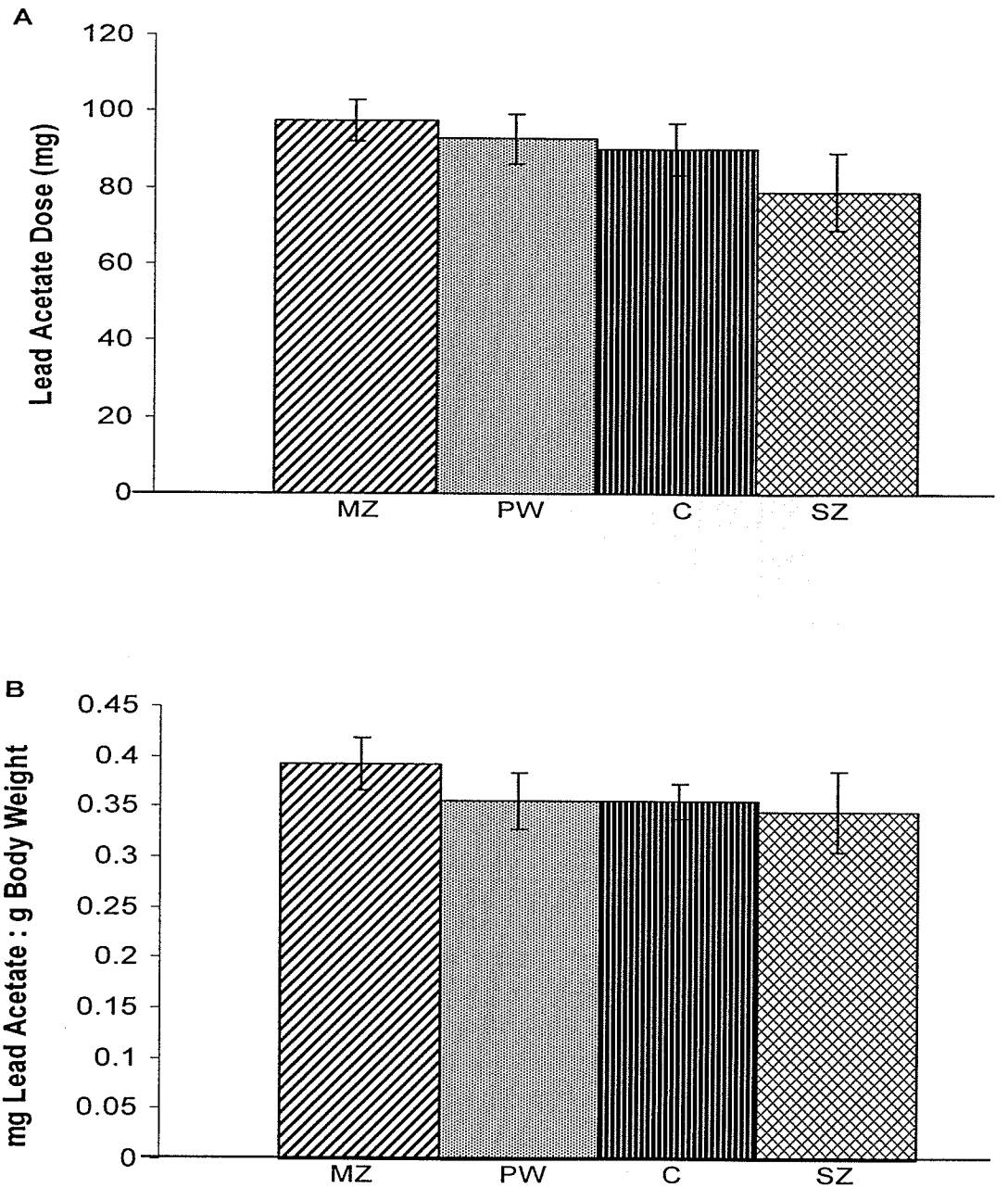


Figure 8. Effect of dietary zinc and lead exposure on total lead dose consumed and total lead dose consumed as a percentage of body weight. Data was analyzed by one-way ANOVA. Columns represent group means ± SEM for n=8. There was no significant effect of zinc on lead dose consumed (A, p=0.3684; B, p=0.6887).

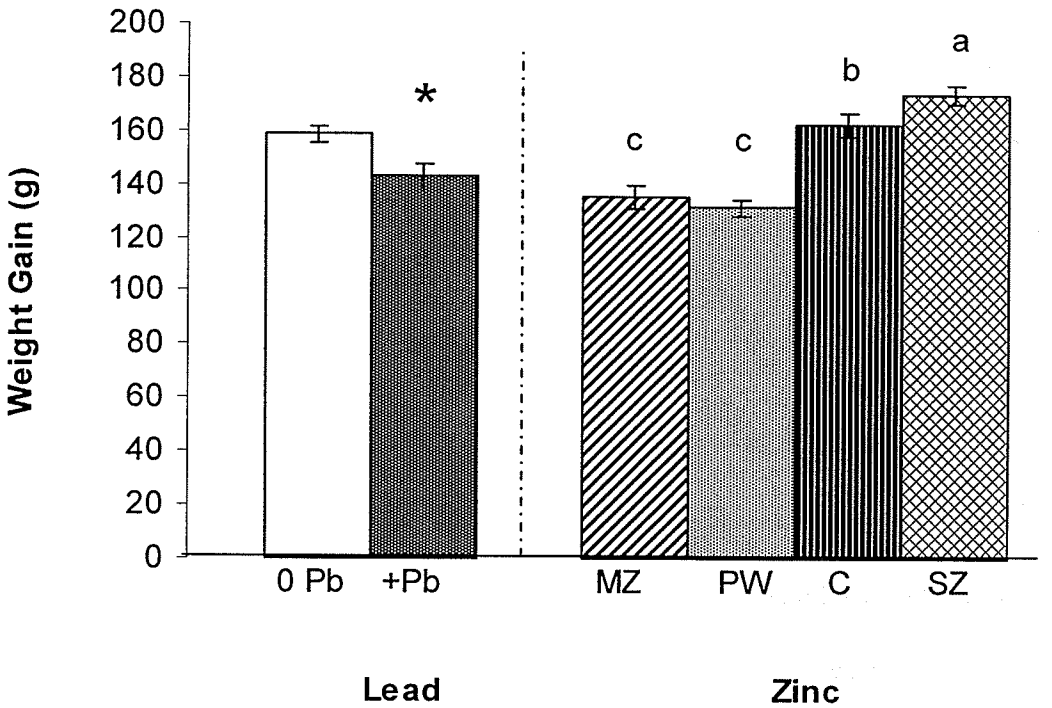


Figure 9. Effect of dietary zinc and lead exposure on total weight gain (g). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There was a significant main effect for lead ($p<0.0001$), as indicated with an asterisk (*) and a significant main effect for zinc ($p<0.0001$), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant ($p=0.2134$).

Feed efficiency was calculated as weekly weight gain divided by weekly feed intake (Figure 10). There was no effect of lead on feed efficiency over time (Figure 10A). However, the feed efficiency of MZ rats was 9% lower than C rats over the first week of the study and 13% less than SZ rats over the second week (Figure 10B). During the third and final week of the study, the MZ groups recovered to the same level of feed efficiency as C and SZ rats while the PW rats dropped to a level 34% lower than C rats. There was no interaction of lead and zinc on feed efficiency over time.

Weekly growth rate was calculated as the mean weight gain per day for weeks 1, 2, and 3 (Figure 11). The change in growth rate was similar to the pattern seen with feed efficiency (Figure 10). Lead did not affect growth rate (Figure 11A). MZ rats had a growth rate of 17% and 24% less than C rats during week 1 and 2, respectively (Figure 11B). PW rats had a growth rate 18% lower than C rats during week 2, which fell to 41% lower during week 3. There was no interaction of lead and zinc on growth rate over time.

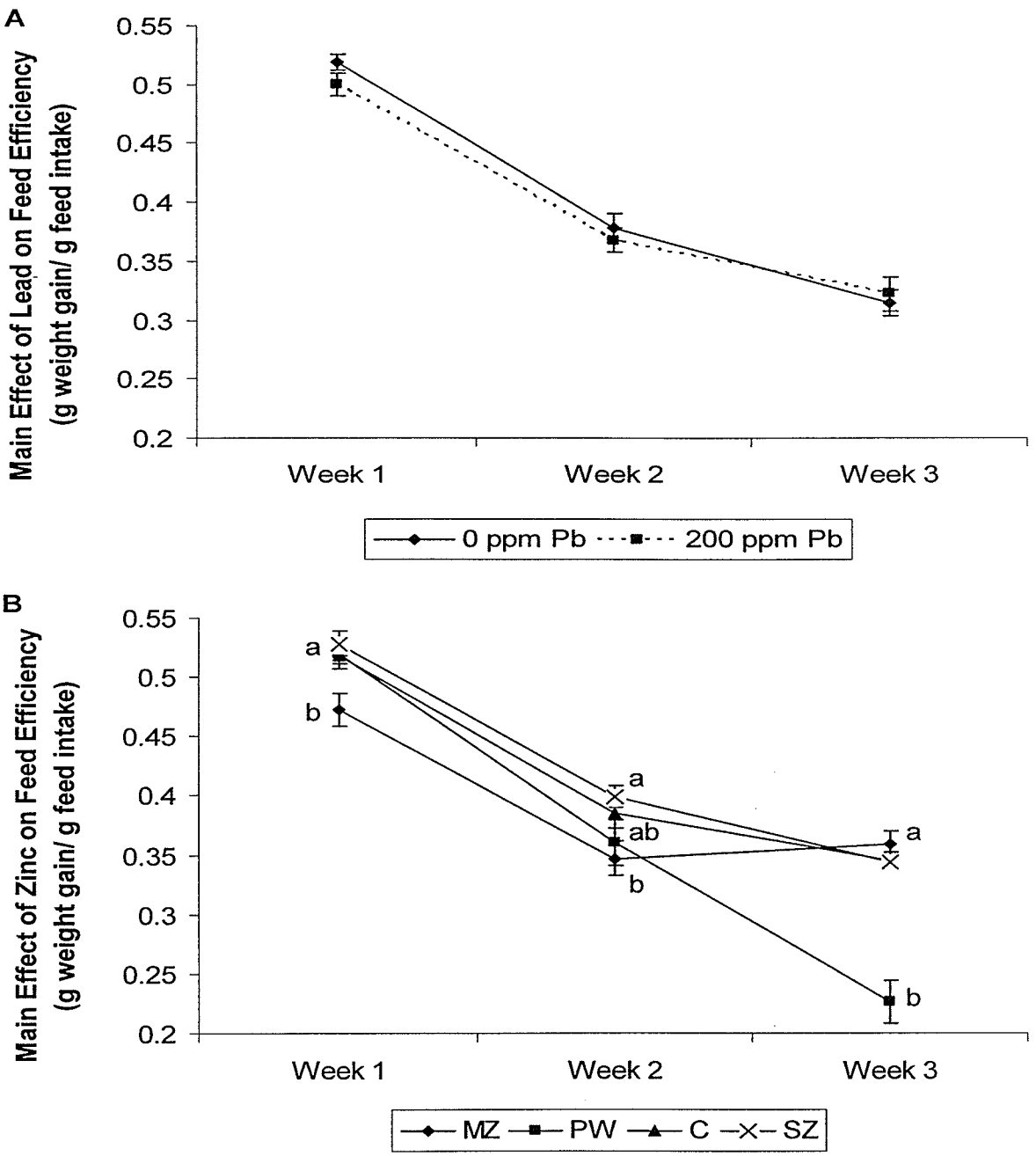


Figure 10. Effect of dietary zinc and lead exposure on feed efficiency, calculated as a weekly average. Data points represent group means ± SEM for n=32 (A) and n=16 (B). There was a significant main effect of zinc (B) over time ($p<0.0001$). Data points with different lower case letters are significantly different within the same week as determined by Duncan's multiple range test. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The main effect of lead and the interaction of lead and dietary zinc over time were not significant ($p=0.3663$ & $p=0.5761$, respectively).

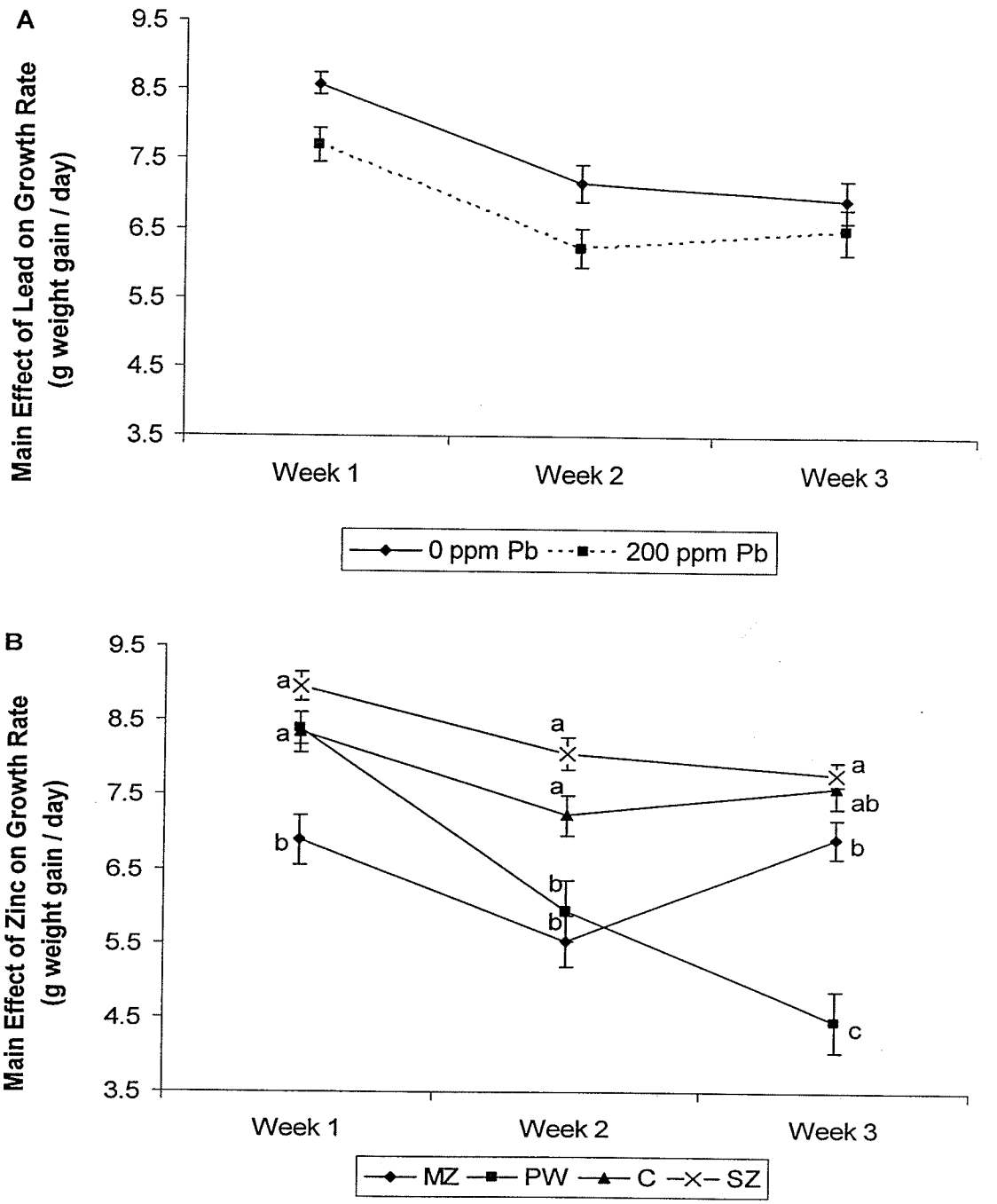


Figure 11. Effect of dietary zinc and lead exposure on growth rate, calculated as a weekly average. Data points represent group means ± SEM for n=32 (A) and n=16 (B). There was a significant main effect of zinc (B) over time ($p<0.0001$). Data points with different lower case letters are significantly different within the same week as determined by Duncan's multiple range test. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The main effect of lead and the interaction of lead and dietary zinc over time were not significant ($p=0.3923$ & $p=0.4411$, respectively).

Epididymal Fat Pad Weight and Epididymal Fat Pad Weight to Body Weight Ratio

Lead treatment resulted in a 12% lower epididymal fat pad weight compared to non-lead treated animals (Figure 12A) but this effect was not seen when expressed as a ratio of body weight (Figure 12B). MZ rats had 25% and 29% less epididymal fat pad weight than C and SZ rats, respectively, while PW rats were not different from either MZ or C rats. However, when expressed as a percentage of body weight, only MZ rats had a lower (17%) fat pad weight compared to C rats.

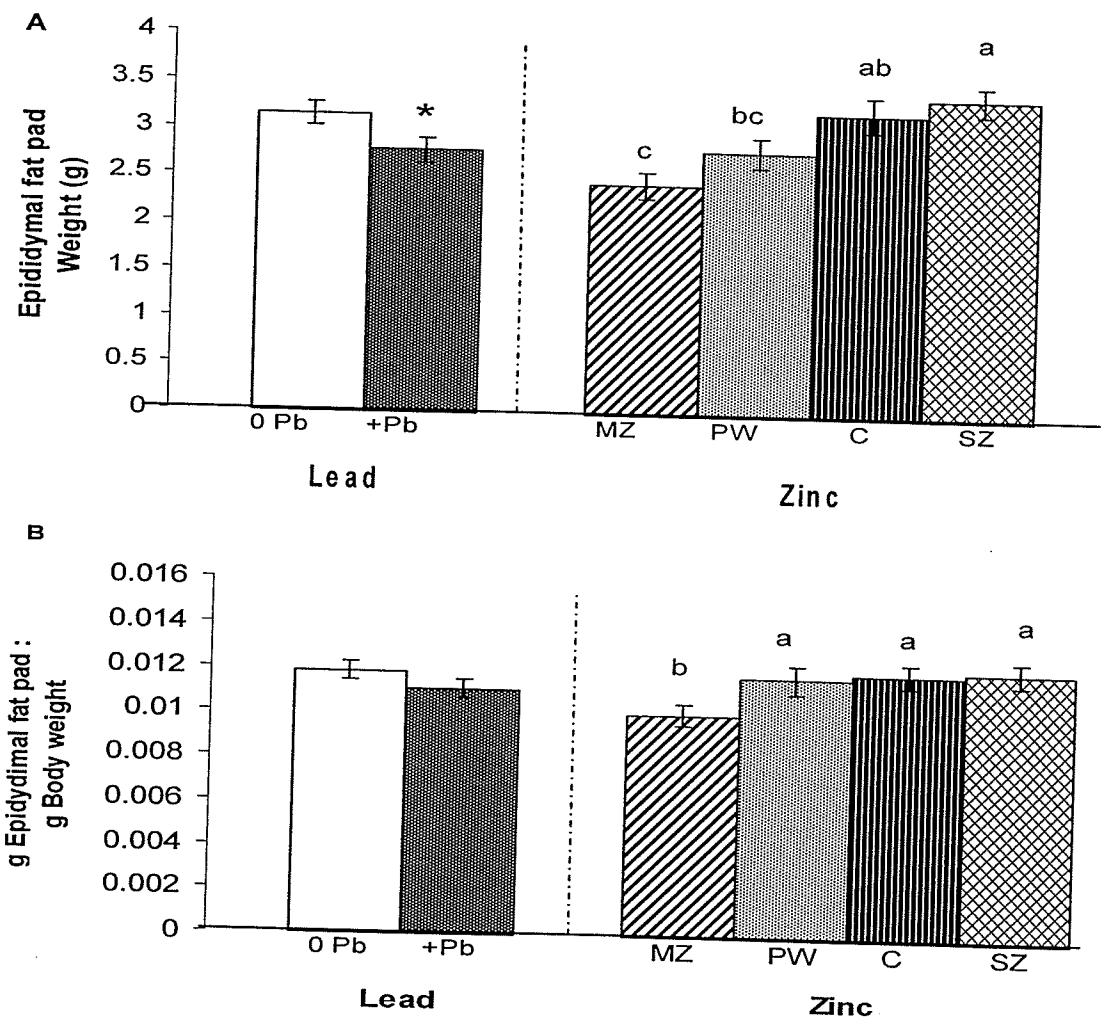


Figure 12. Effect of dietary zinc and lead exposure on epididymal fat pad weight (A) and epididymal fat pad weight to body weight ratio (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was significant (A, p=0.0177), as indicated by an asterisk (*) and the main effect of zinc was significant (A, p< 0.0001; B, p=0.0204), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (A, p=0.9037; B, p=0.8589).

ZINC ASSESSMENT

Short and long term zinc status was assessed by the determination of serum zinc and femur zinc concentrations, respectively. ICP analysis of the experimental diets confirmed that zinc concentration was in close agreement with the values reported for the diet formulation (Table 1). Zinc concentration of the marginal zinc diet was 8.45 ppm, the control diet was 29.17 ppm, and the supplemental zinc diet was 311.51 ppm.

Serum Zinc Concentration

There was an interaction of lead and zinc on serum zinc concentration (Figure 13). However, with the means testing employed, the addition of lead did not significantly change the zinc concentration at any level of dietary zinc. MZ rats had a 57% lower serum zinc concentration than both PW and C rats, while SZ rats had a 27% higher serum zinc concentration than PW and C groups.

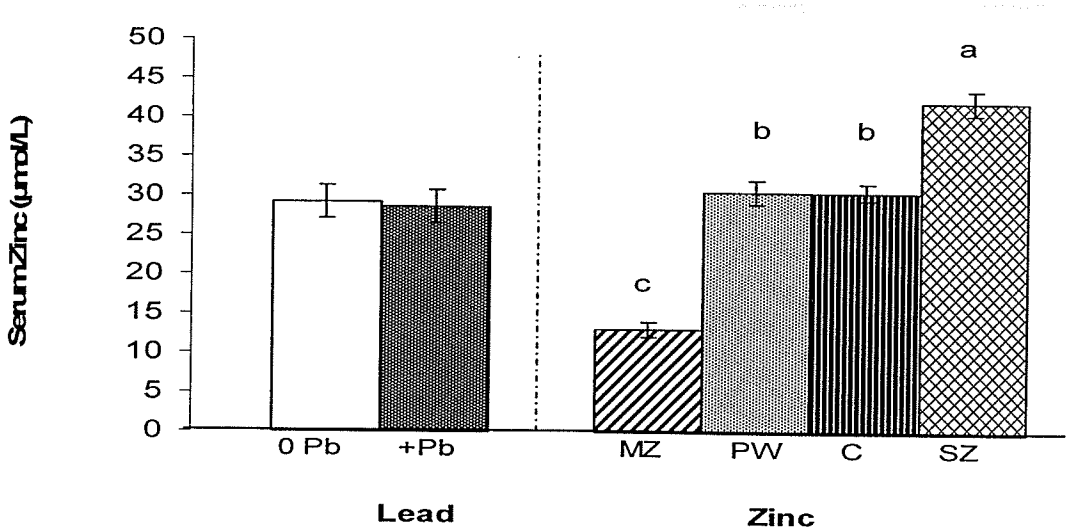


Figure 13. Effect of dietary zinc and lead exposure on serum zinc concentration (µmol/L). Columns represent group means ± SEM for n=31 (lead effect) and n=14-16 (zinc effect). Despite the fact that there was a significant interaction between lead and dietary zinc (p=0.0729), the addition of lead did not affect serum zinc in any treatment group according to Duncan's multiple range test. There was only a main effect of zinc with the MZ groups being lower than both C and PW and the SZ groups having a higher serum zinc concentration than C and PW groups.

Femur Zinc Concentration

There was also an interaction between lead and dietary zinc on femur zinc concentration (Figure 14). Lead treatment lowered femur zinc concentration in PW, C, and SZ rats, but not in MZ rats. Also of note, both SZ treatments had a higher femur zinc concentration than C and PW rats, while MZ rats had a lower concentration than C and PW rats. There was no difference between the PW and C groups or the PW+Pb and C+Pb groups.

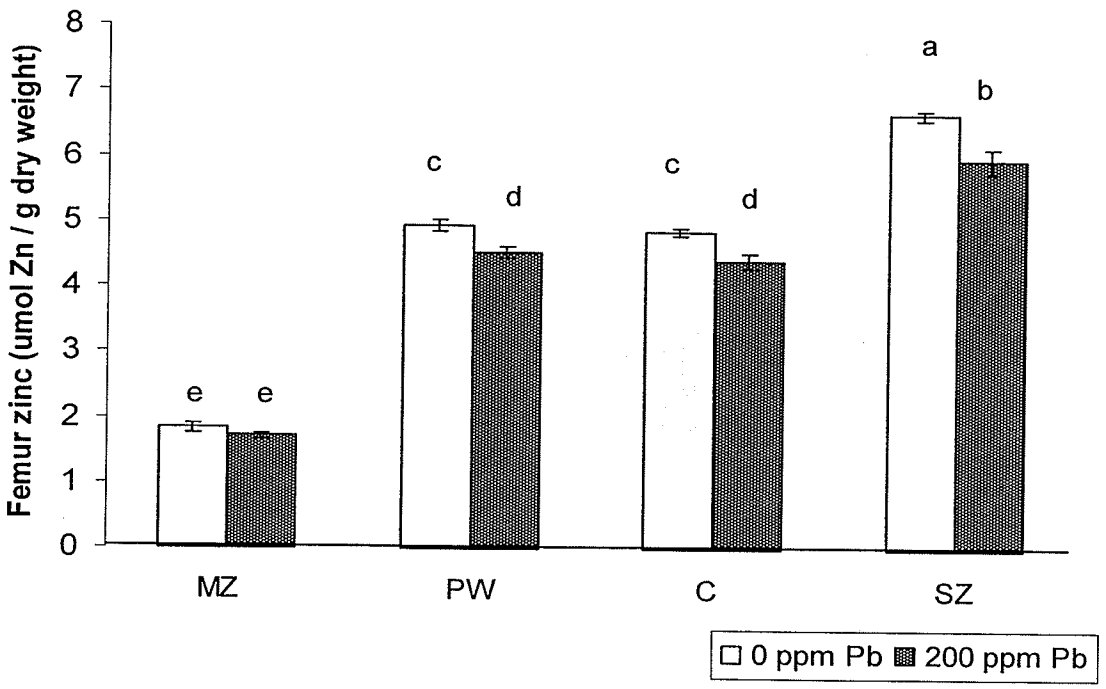


Figure 14. Effect of dietary zinc and lead exposure on femur zinc concentration. Columns represent group means ± SEM for n=8. There was a significant interaction between lead and dietary zinc (p=0.0556), with different letters indicating means are significantly different as determined by Duncan's multiple range test.

LEAD ASSESSMENT

Femur Lead Concentration

Femur lead concentration was 62% higher in MZ rats than C rats, while SZ rats had 65% less lead than C rats (Figure 15). Femur lead was not different between C and PW rats.

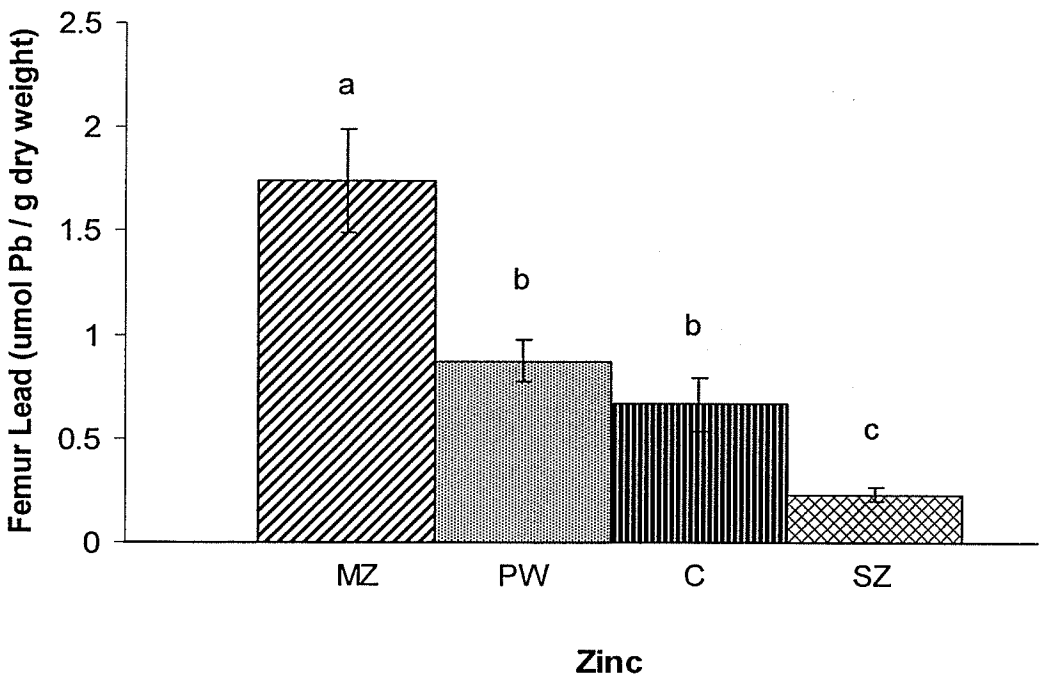


Figure 15. Effect of dietary zinc and lead exposure on femur lead concentration. Data was analyzed by one-way ANOVA. There was a significant effect of zinc on femur lead concentration ($p<0.0001$). Columns represent means ± SEM for n=8; different letters indicate means are significantly different as determined by Duncan's multiple range test. Data was log transformed to correct for normality.

Renal Lead Concentration

Renal lead concentration was 35% higher in the MZ group and 33% lower in the SZ group than the C group (Figure 16). PW rats were not significantly different from either the MZ or C rats.

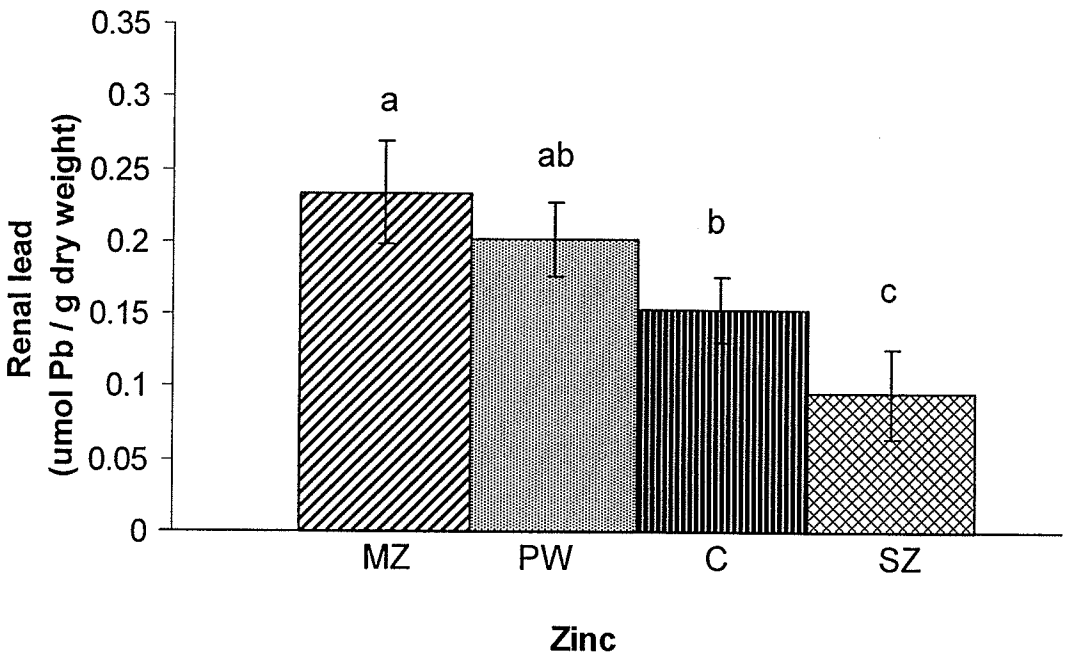


Figure 16. Effect of dietary zinc and lead exposure on renal lead concentration. Data was analyzed by one-way ANOVA. There was a significant effect of zinc on renal lead concentration ($p=0.0002$). Columns represent group means ± SEM for $n=8$; different letters indicate means are significantly different as determined by Duncan's multiple range test.

Femur, Kidney, Liver, and Intestine Lead as a Percentage of Total Tissue Lead

The percentage of tissue lead in each organ was calculated using total tissue lead as the sum of lead in the femur, kidney, liver, and intestine. Tissue lead was expressed as lead content (µg lead, based on wet weight of tissue; Figure 17A) and as lead concentration (µg lead/g dry tissue weight; Figure 17B), Femur lead constituted 22-66% of total tissue lead content (Figure 17A) and 50-81% of total tissue lead concentration (Figure 17B) measured in all treatment groups. Femur lead as a percentage of total tissue lead decreased as dietary zinc increased. Intestinal lead as a percentage of total tissue lead increased with dietary zinc, constituting 6-30% of total tissue lead measured as a concentration, but 19-57% as total lead content. Renal lead accounted for 11-20% of total tissue lead measured (in terms of lead content and lead concentration). Hepatic lead concentration accounted for only 0.9 to 1.5% of the total tissue lead measured as a concentration and 5-10% of lead content.

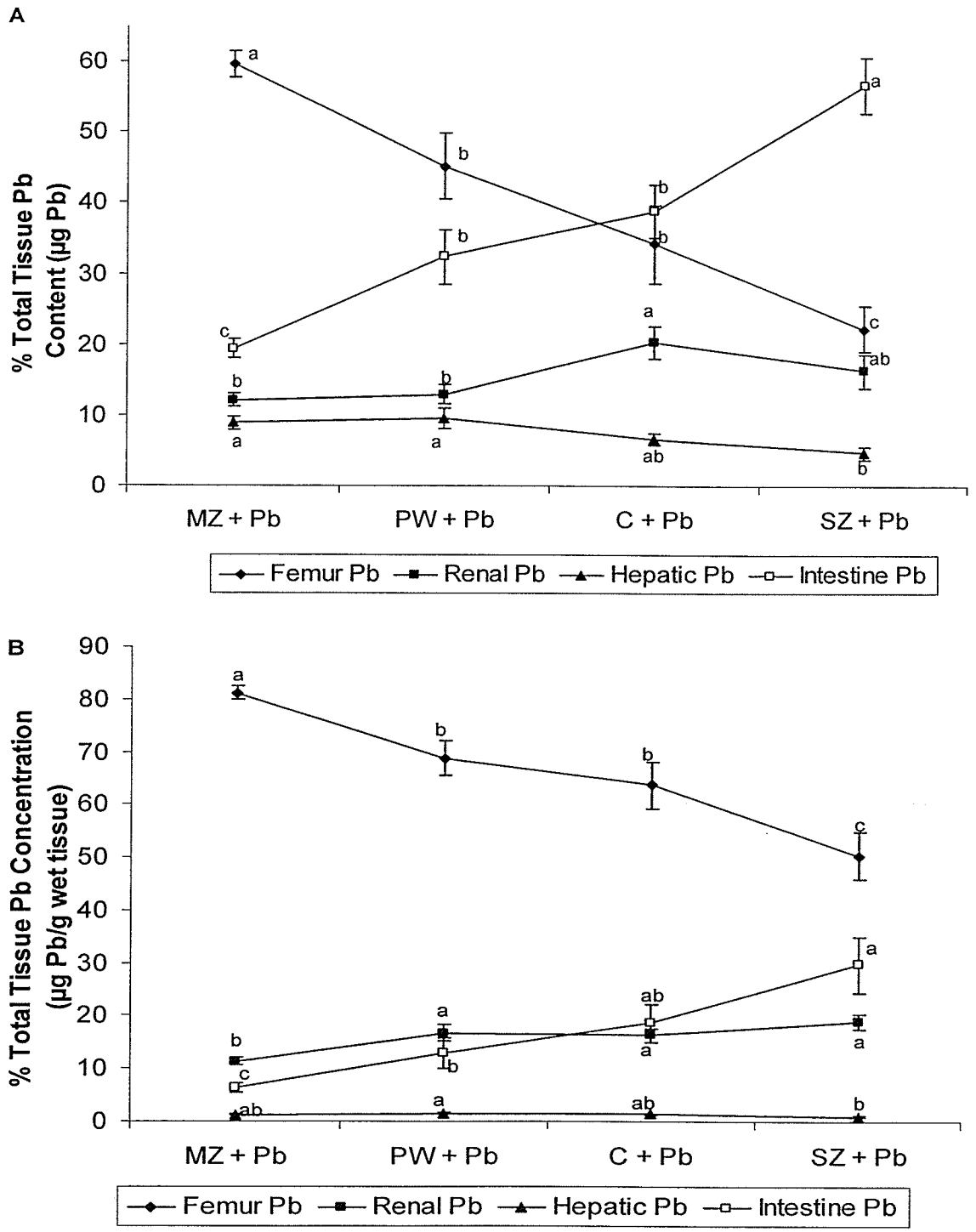


Figure 17. Effect of dietary zinc on tissue lead accumulation, calculated as the percentage of total tissue lead content (µg) and lead concentration (µg/g dry weight) for femur, kidney, liver, and intestine. Data points represent means for n=8. Data points with different lower case letters are significantly different within the same tissue as determined by one-way ANOVA.

Hepatic ALAD Activity

Lead treatment did not have a significant effect on hepatic ALAD activity (Figure 18). There was a significant main effect of dietary zinc such that hepatic ALAD activity was lower in the MZ group than the PW, C, and SZ groups.

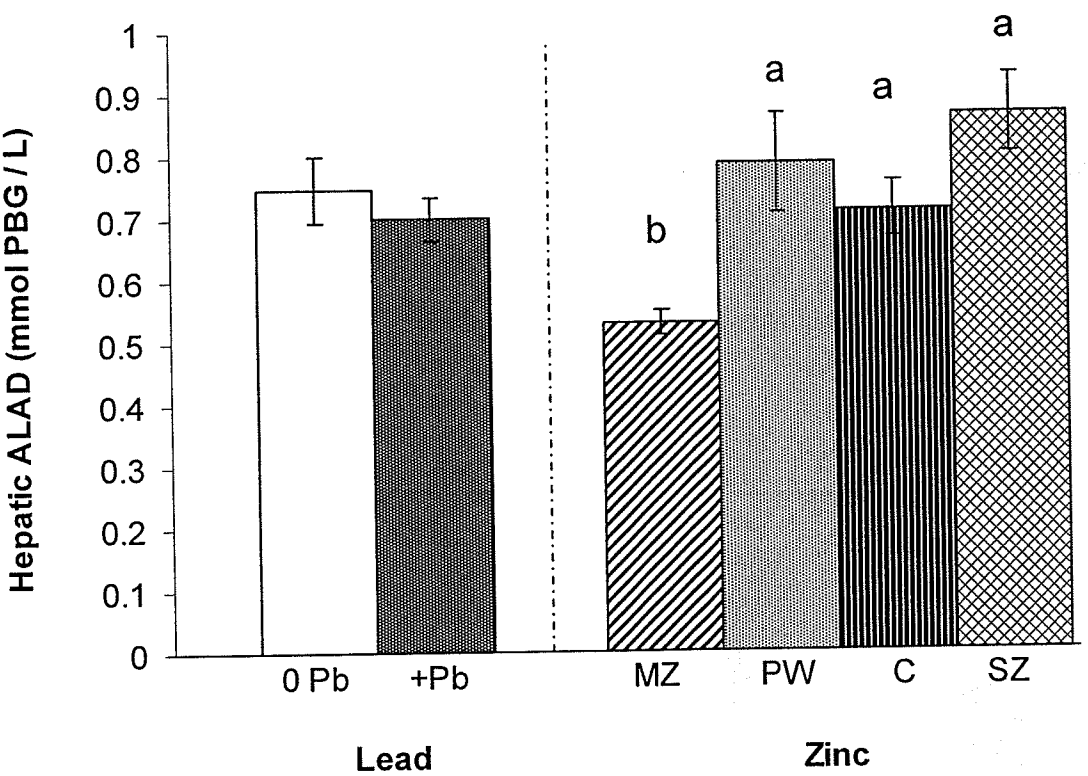


Figure 18. Effect of dietary zinc and lead exposure on hepatic ALAD activity. Columns represent means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There was no significant main effect of lead (p=0.4093). There was a significant main effect of zinc (p=0.0011), with different letters indicating means are significantly different, as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.8402). Data was log transformed to obtain normality.

MICRONUTRIENT STATUS

Hematocrit, hepatic iron concentration, and hepatic copper concentration were measured to assess overall micronutrient status, the presence of anemia, and micronutrient interactions.

Hematocrit was calculated as the percentage of red blood cell volume at termination (Day 21). No significant differences were found among treatment groups (Figure 19).

Hepatic iron concentration was 16% higher in the lead treated rats than the non-lead treated rats, but the main effect of zinc and the interaction of lead and dietary zinc were not significant (Figure 20).

Hepatic copper concentration was 13% lower in the lead treated rats compared to the non-lead treatments (Figure 21). SZ treatment resulted in 32% lower hepatic copper concentrations than the C treatment. Also of note, MZ treatment had a 10% less hepatic copper concentration than the PW treatment, but was not different from C animals.

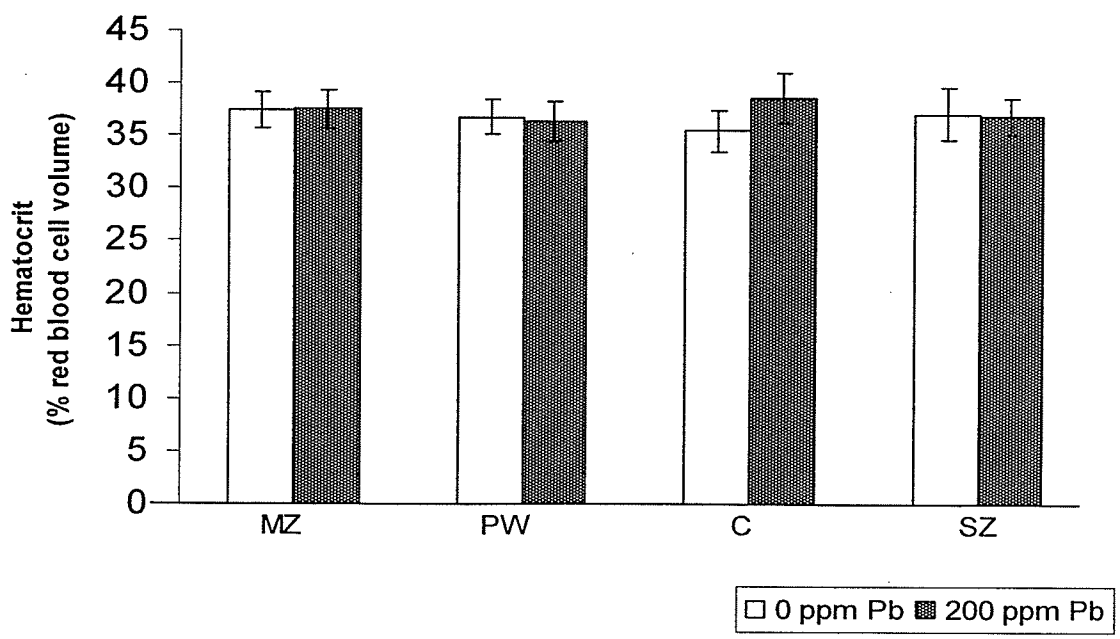


Figure 19. Effect of dietary zinc and lead exposure on hematocrit. Columns represent group means ± SEM for n=8. The main effects of lead, zinc, and their interaction were not significant (p= 0.6288, p=0.9771, p=0.7930, respectively).

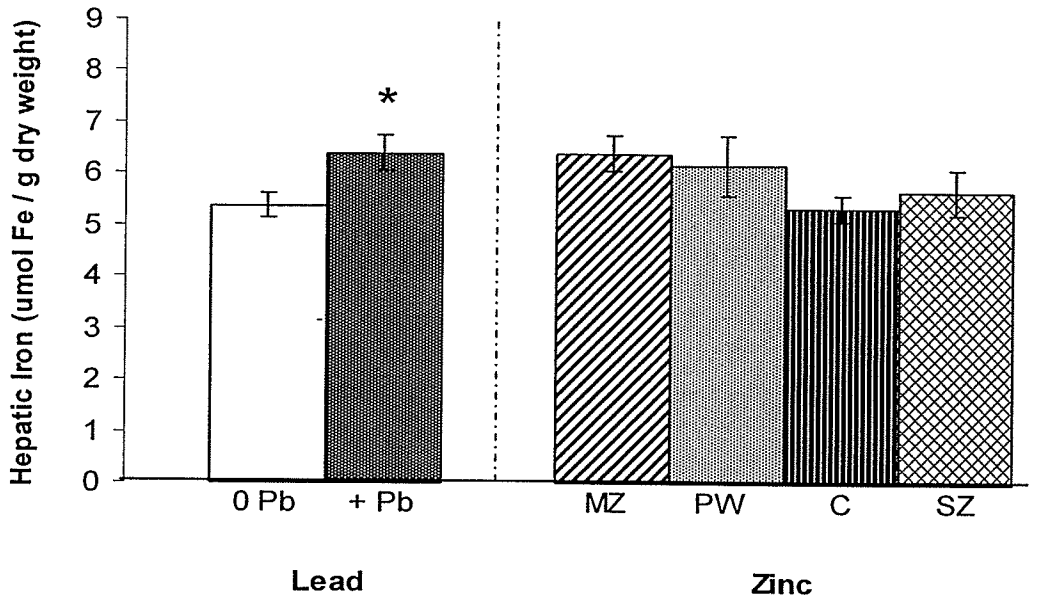


Figure 20. Effect of dietary zinc and lead exposure on hepatic iron concentration. Columns represent means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The lead effect was significant (p=0.0169), as indicated by an asterisk (*). The effect of zinc and the interaction of lead and zinc were not significant (p=0.2500; p=0.7512, respectively). Data was log transformed to obtain normality.

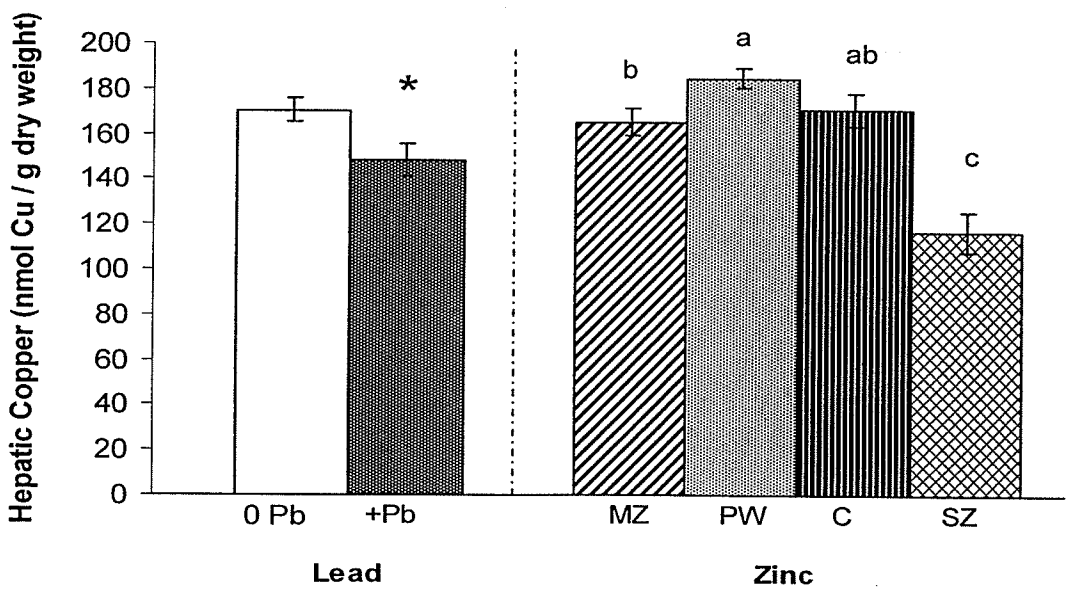


Figure 21. Effect of dietary zinc and lead exposure on hepatic copper concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There were significant main effects for lead (p=0.0011), as indicated by an asterisk (*) and zinc (p<0.0001), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.1122).

Liver

Wet weight and wet weight to body weight ratio

Hepatic weight was 7% lower in lead treated rats than in non-lead treatments (Figure 22A). SZ treatment resulted in an 8%, 19%, and 15% higher hepatic weight than C, PW, and MZ groups, respectively. PW rats had a 12% lower hepatic weight than C rats. Hepatic weight, calculated as a percentage of body weight, was not different among the treatment groups (Figure 22B).

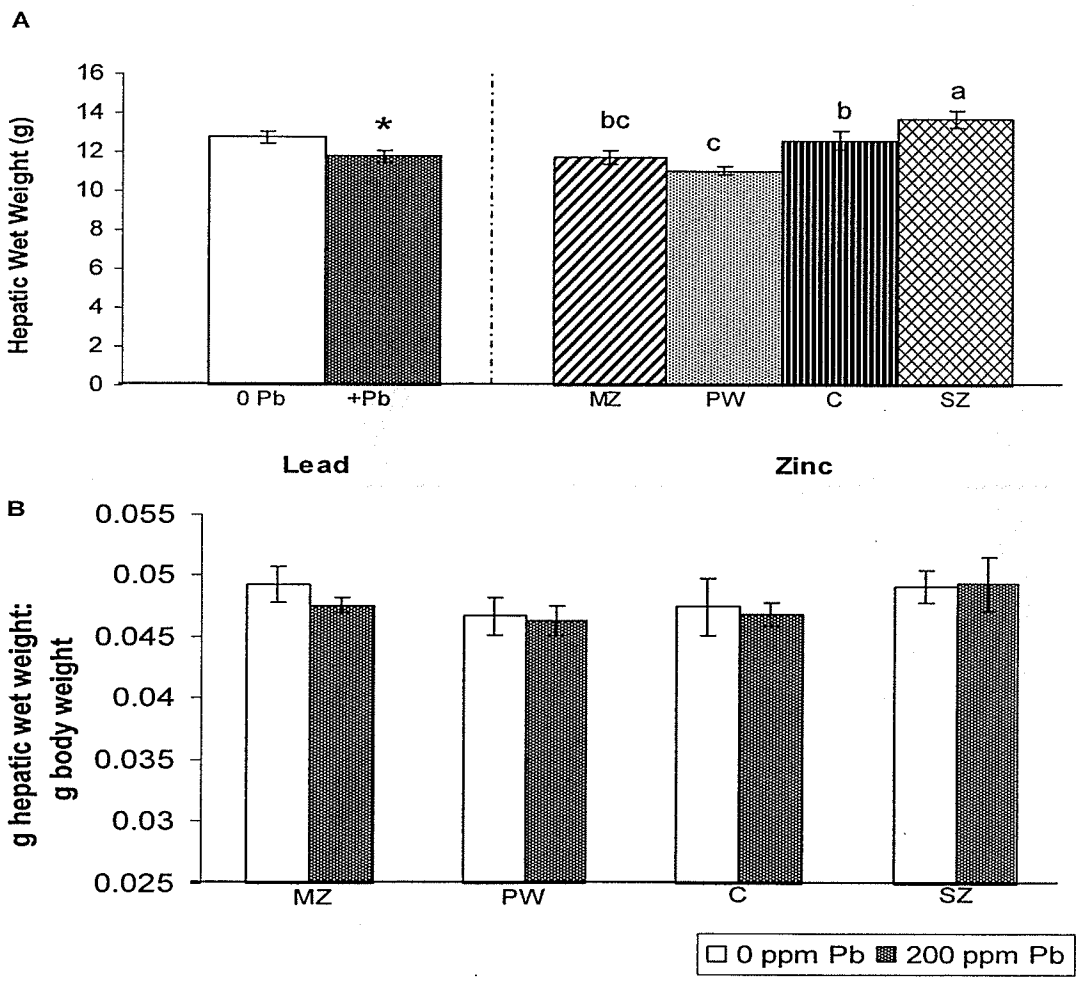


Figure 22. Effect of dietary zinc and lead exposure on hepatic wet weight (A) and hepatic wet weight to body weight ratio (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There was a significant main effect for lead (A, p=0.0125), as indicated by an asterisk (*), and zinc (A, p<0.0001) in hepatic weight, with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (A, p=0.7083; B, p=0.9419). Data (A&B) was log transformed to obtain normality and homogeneity of variance.

Zinc & Lead Concentration

Hepatic zinc concentration was not affected by lead treatment, but was 10% lower in the MZ rats than the PW rats (Figure 23). Neither MZ nor PW rats were different from C or SZ rats. The interaction of lead and dietary zinc on hepatic zinc concentration was not significant.

Hepatic lead concentration was 52% higher in the MZ treatment and 63% lower in the SZ treatment than in the C group (Figure 24). Hepatic lead was 82% lower in the SZ treatment than the MZ treatment. The PW treatment was not significantly different from either the MZ group or the C group.

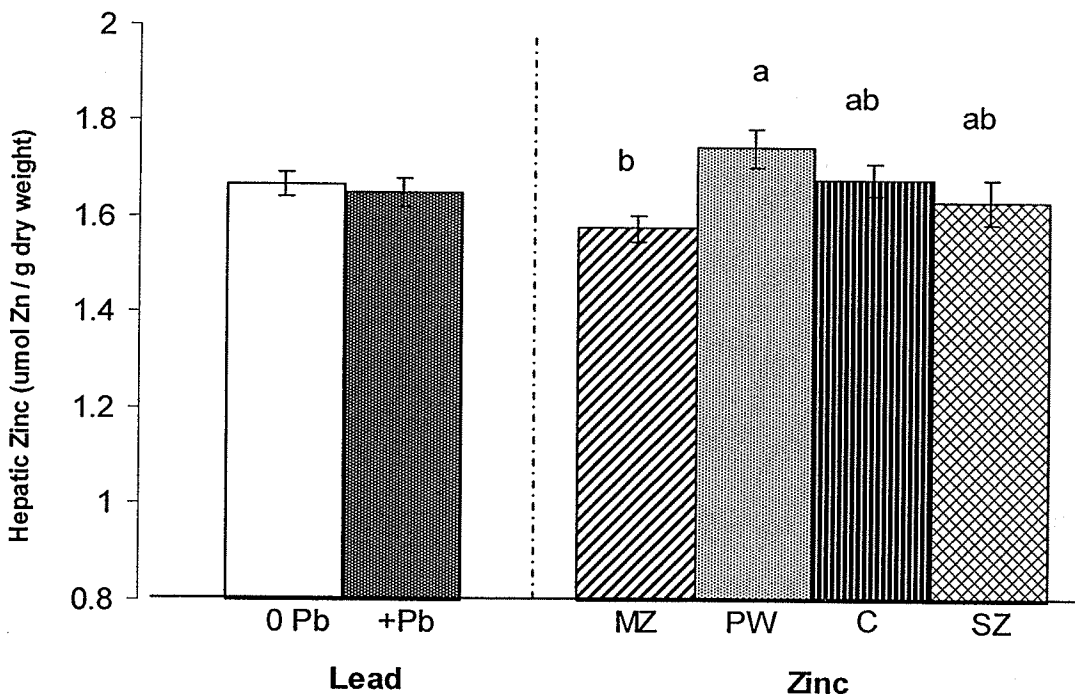


Figure 23. Effect of dietary zinc and lead exposure on hepatic zinc concentration. Columns represent means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There was no significant main effect of lead (p=0.4093), but the main effect of zinc was significant (p=0.0191), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.8402).

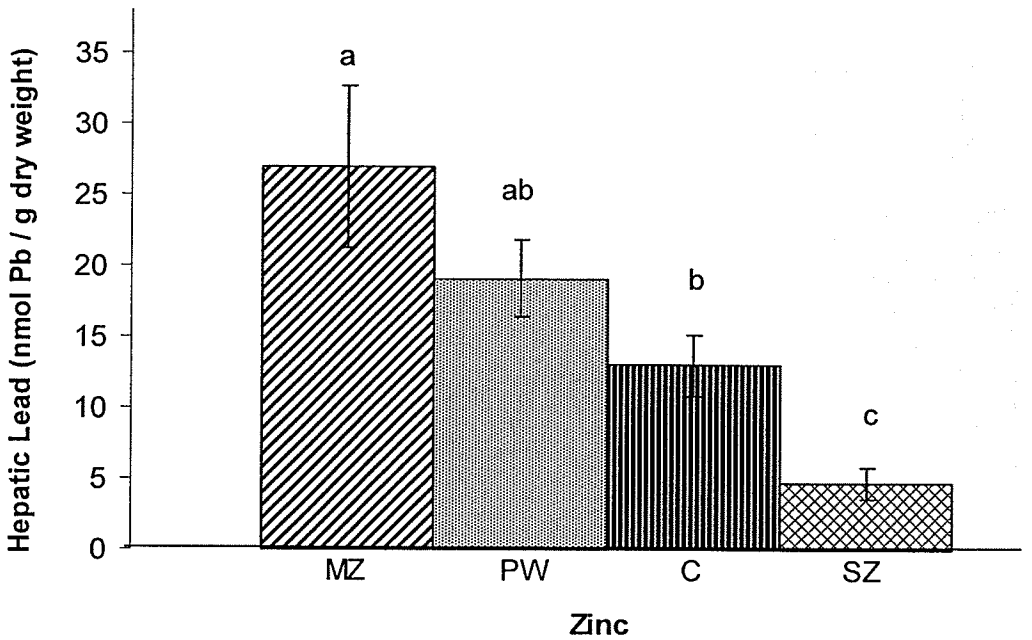


Figure 24. Effect of dietary zinc and lead exposure on hepatic lead concentration. Data was analyzed by one-way ANOVA. Columns represent means ± SEM for n=8. There was a significant effect of zinc (p=0.0006), with different letters indicating means are significantly different as determined by Duncan's multiple range test. Data was log transformed to obtain normality.

Kidney

Wet weight and wet weight to body weight ratio

Renal wet weight was 12% lower in the lead treated rats than in the non-lead treatments (Figure 25A), but not when corrected for body weight (Figure 25B). MZ treatment resulted in 25-29% less renal wet weight than either C or SZ groups. Renal weight of PW rats was not different from the MZ or C groups. In terms of renal weight to body weight ratio, SZ treatment resulted in a 17% lower weight than all other treatments (Figure 25B). The interaction of lead and dietary zinc did not affect renal wet weight or the weight to body weight ratio.

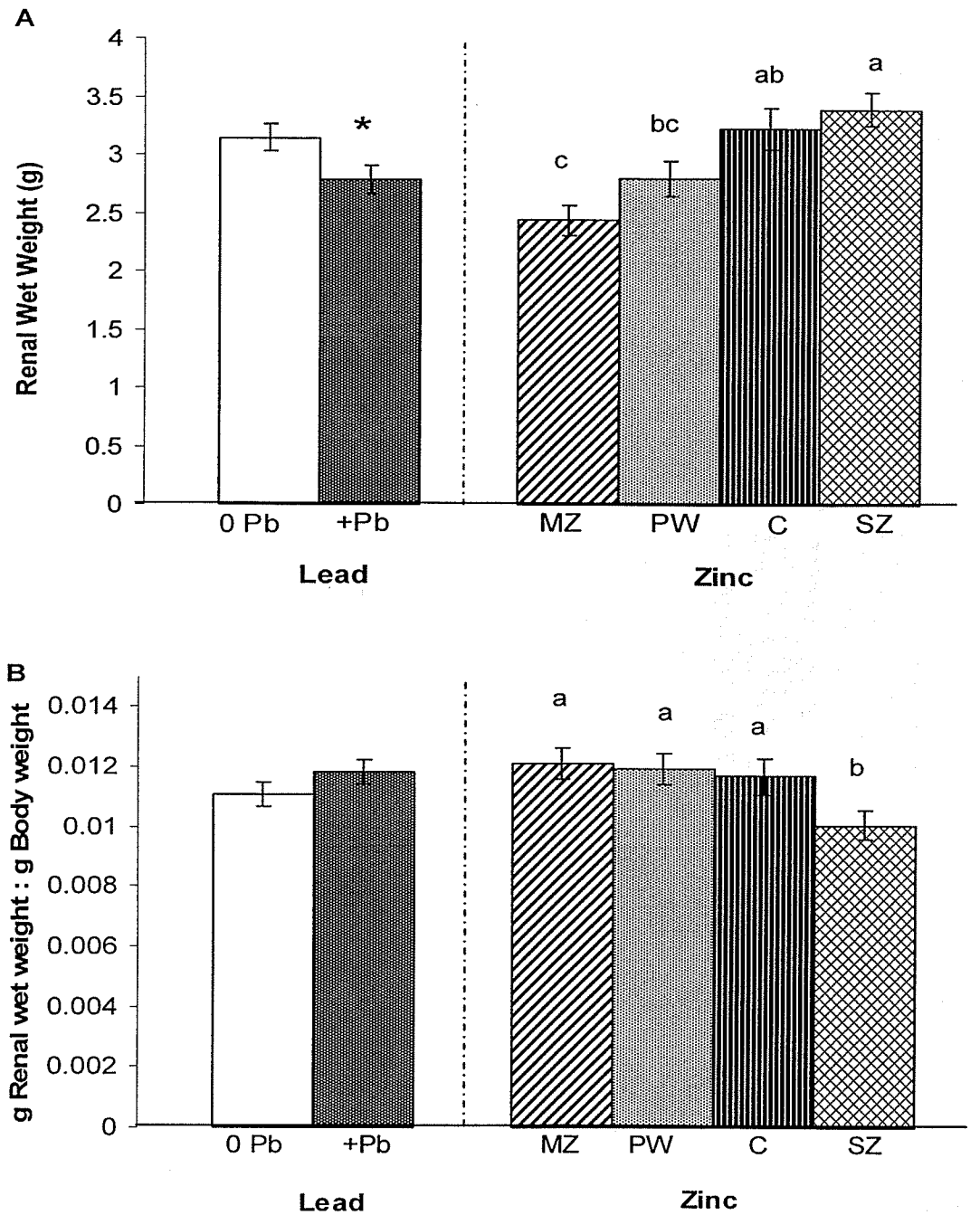


Figure 25. Effect of dietary zinc and lead exposure on renal wet weight (A) and renal wet weight to body weight ratio (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). There was a significant main effect of lead (A, p=0.0177), as indicated by an asterisk (*) and significant main effects of zinc (A, p=0.0001; B, p=0.0204), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interactions of lead and zinc were not significant (A, p=0.9037; B, p=0.8580).

Zinc Concentration

Renal zinc concentration was 5% lower in the lead treated rats than in the non-lead treatments (Figure 26). MZ treatment resulted in 17% - 20% less renal zinc than PW, C, and SZ treatments. SZ rats had 6% higher renal zinc concentrations than C rats. The interaction of lead and dietary zinc on renal zinc concentration was not significant.

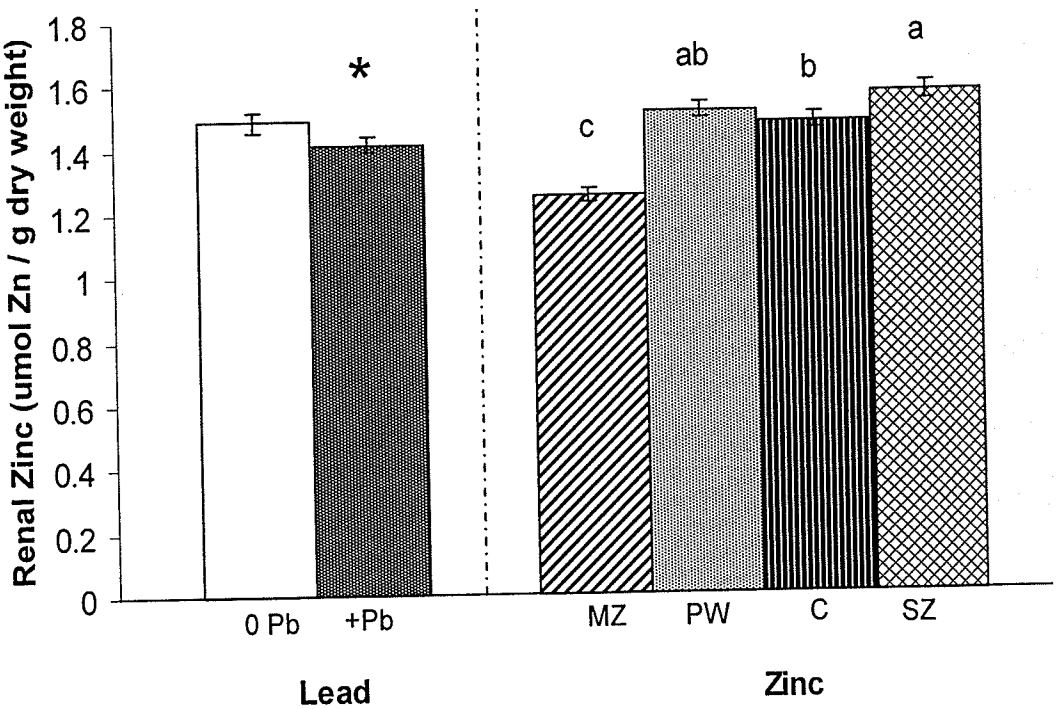


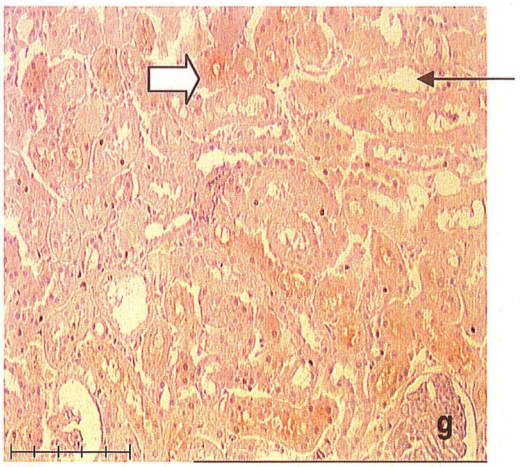
Figure 26. Effect of dietary zinc and lead exposure on renal zinc concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was significant ($p=0.0031$), as indicated by an asterisk (*) and the main effect of zinc was significant ($p<0.0001$), with different letters indicate means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant ($p=0.2053$).

Kidney Metallothionein Immunostaining

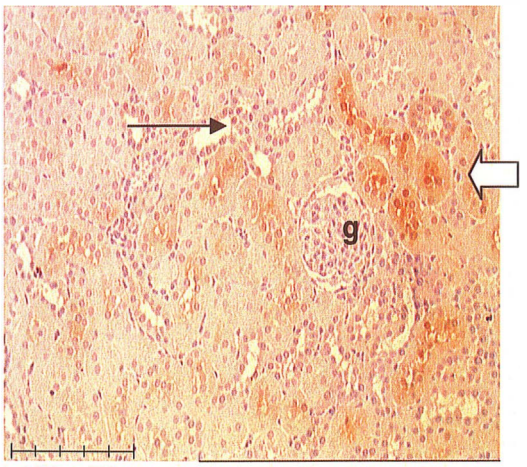
Results of the immunoperoxidase staining for MT in the kidney are presented in Figures 27-29. All negative control sections were absent of metallothionein staining (Figures 27-29), indicating that the staining was specific for MT. There were no apparent differences in staining intensity or distribution between the lead and non-lead treatments. Thus, these results will only be discussed in terms of the response to dietary zinc.

In the kidney, there was strong MT immunostaining in the renal convoluted tubules of the PW and C groups, moderate to strong staining in SZ groups, and weak to moderate staining in MZ groups (Figure 27). The staining was mainly localized within the epithelia of proximal convoluted tubules, rather than distal convoluted tubules, and was present in both the cytoplasm and nuclei of epithelial cells (Figure 28). All groups also had some staining in the lumina of renal tubules and no staining in glomeruli (Figures 27 & 28). The epithelia of large collecting ducts in the base of the renal pelvis also responded to dietary treatment in terms of MT intensity. PW rats had strong metallothionein staining, whereas all other treatments had moderate to strong staining (Figure 28).

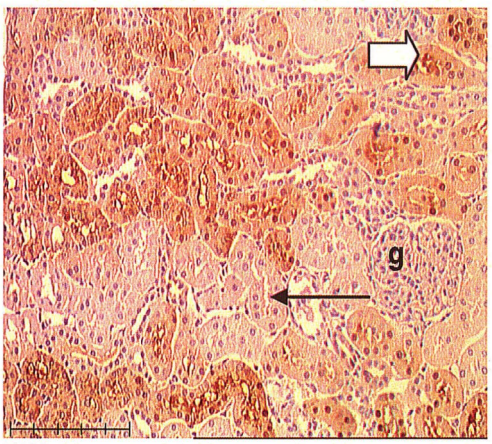
Figure 27. Immunohistological staining for metallothionein in the renal cortex. MT immunostaining was primarily localized within proximal convoluted tubules (white block arrow) and weak or absent from distal convoluted tubules (arrow). No MT immunostaining was present in glomeruli (g). Note the strong MT immunostaining in PW, PW+Pb, C, and C+Pb, whereas SZ & SZ+Pb treatments had moderate to strong staining and MZ & MZ+Pb treatments had moderate to weak staining. Scale bars at the bottom left corner equal 160 µm.



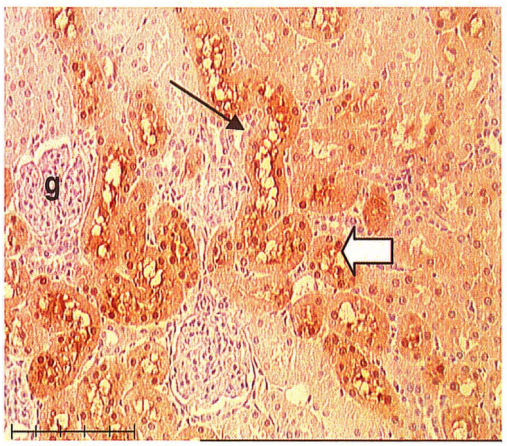
MZ



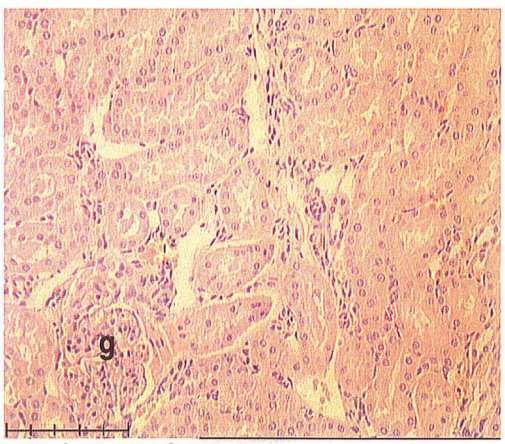
MZ + Pb



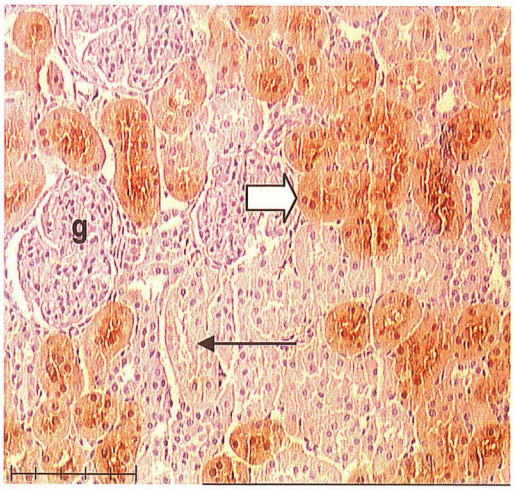
PW



PW + Pb



NEGATIVE CONTROL



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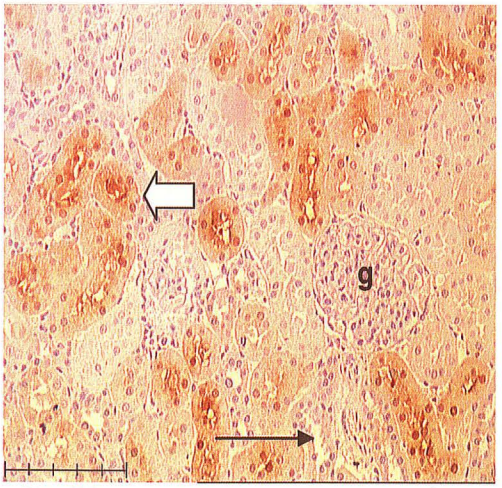
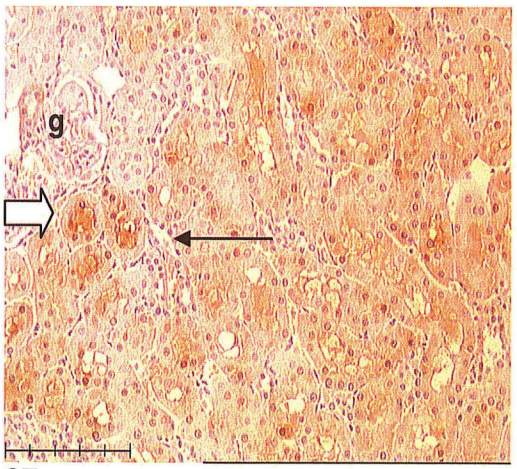
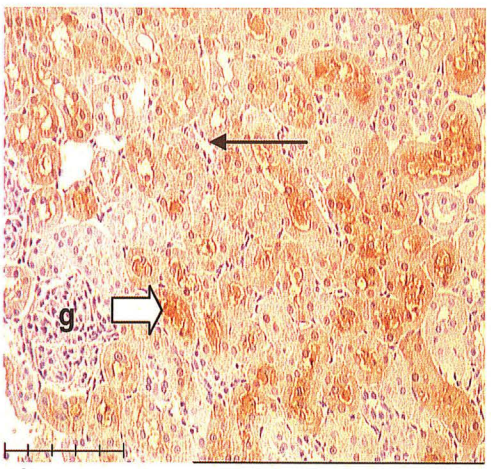


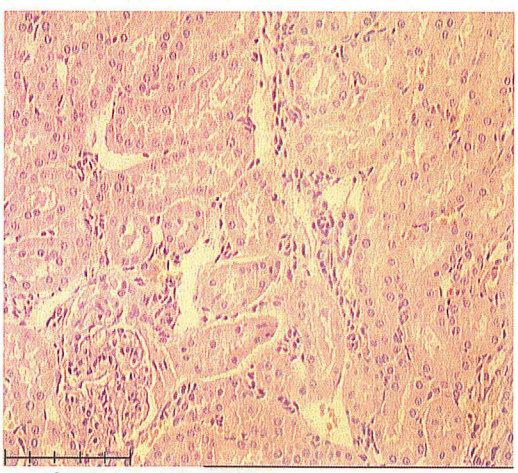
C + Pb



SZ



SZ + Pb



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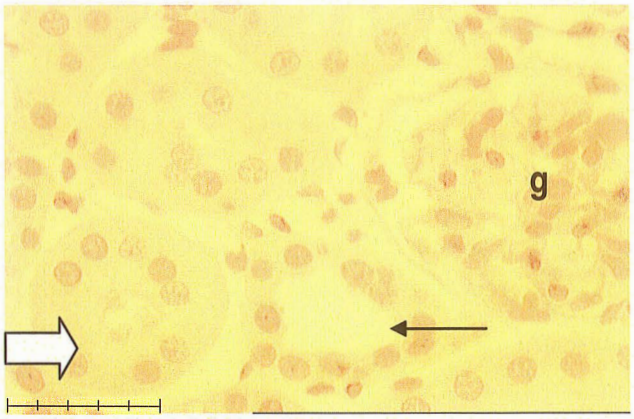
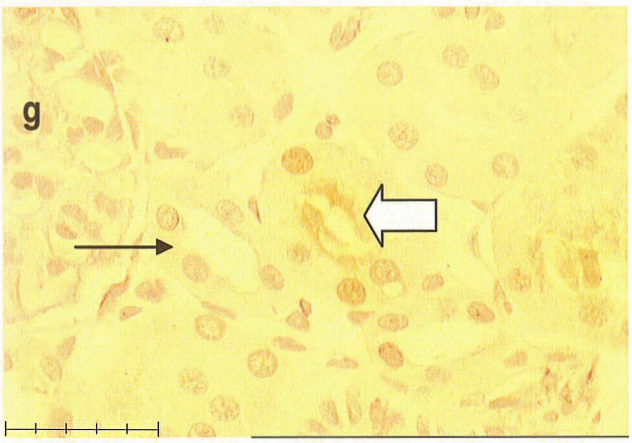
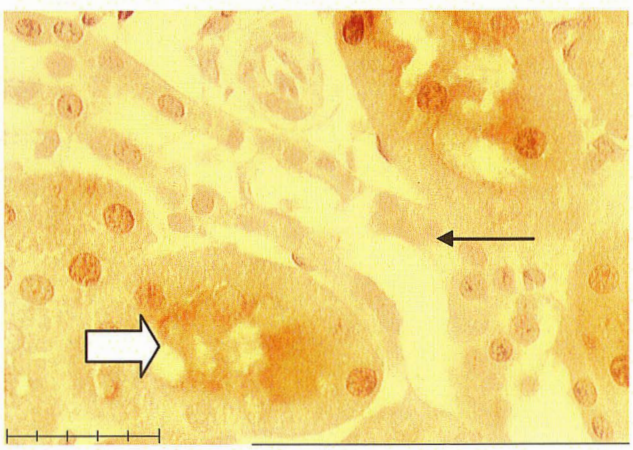


A NEGATIVE CONTROL



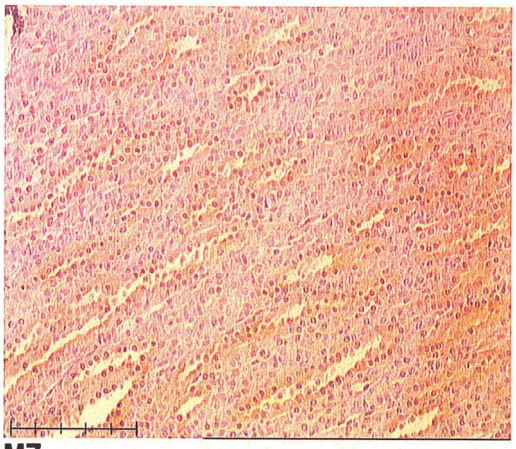
B MODERATE IMMUNOSTAINING



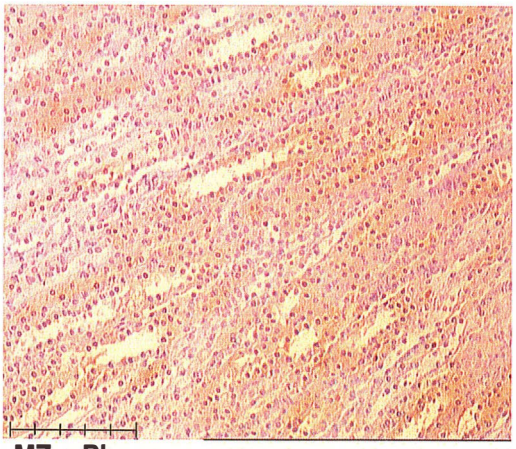
C STRONG IMMUNOSTAINING

Figure 28. Immunohistological staining for metallothionein in the renal convoluted tubules. MT staining was localized within proximal convoluted tubules (white block arrow), with weak or absent staining in distal convoluted tubules (arrow). Proximal convoluted tubules are distinguishable by their distinct brush border, basally placed nuclei, and occluded lumen; whereas distal convoluted tubules lack a brush border, have apically located nuclei, and wide, open lumens. Immunostaining was absent from glomeruli (g). Scale bars in bottom left corners equal 40 µm.

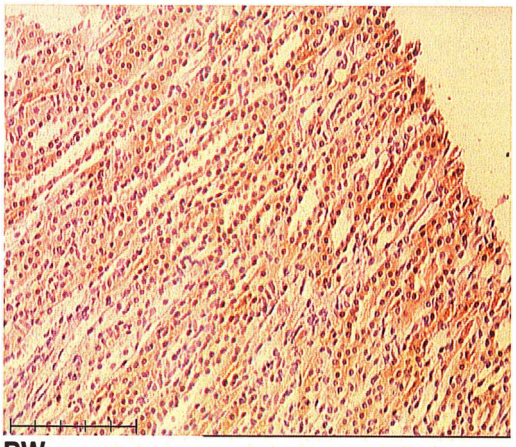
Figure 29. Immunohistological staining for metallothionein in the renal pelvis. Note the strong MT immunostaining in the collecting ducts of the renal pelvis in the PW and PW+Pb treatments. All other treatments (MZ, MZ+Pb, C, C+Pb, SZ, SZ+Pb) had moderate to strong staining. Scale bars at the bottom left corner equal 160 μm.



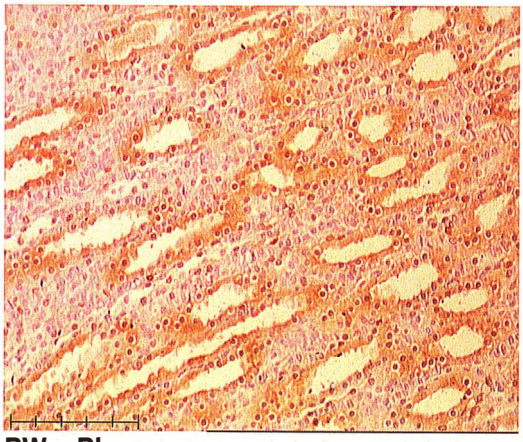
MZ



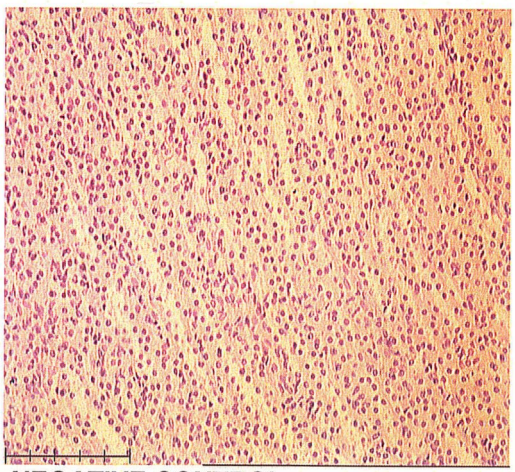
MZ + Pb



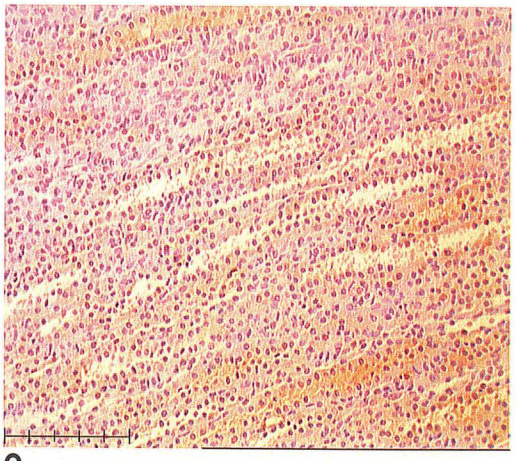
PW



PW + Pb



NEGATIVE CONTROL



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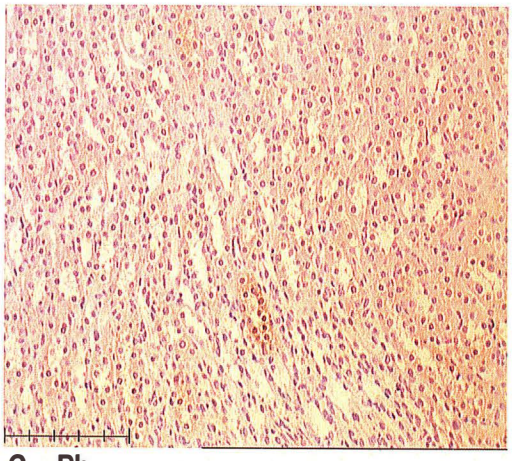
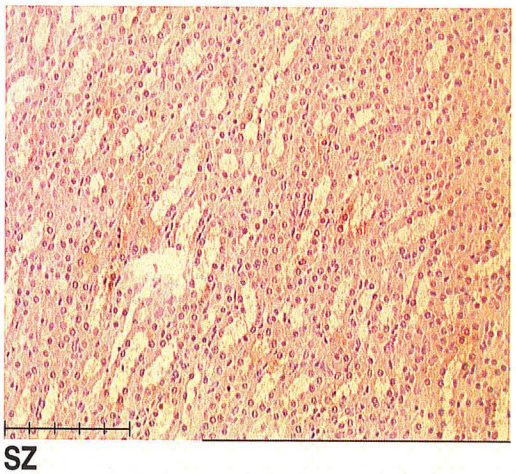
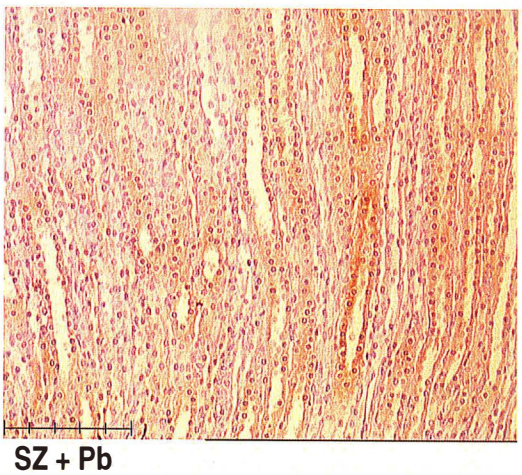


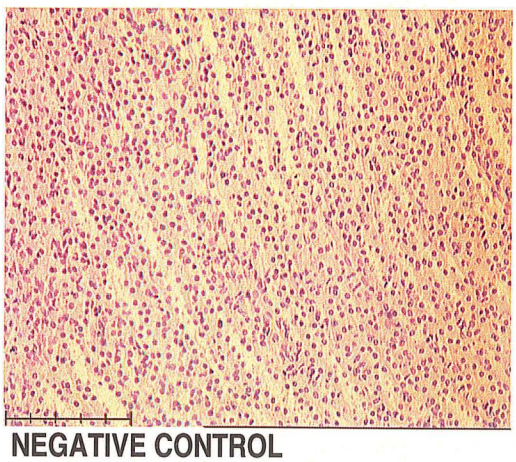
C + Pb



SZ



SZ + Pb



NEGATIVE CONTROL

Intestine

Wet weight and Wet weight to body weight ratio

Intestinal weight and intestinal weight to body weight ratio were not affected by lead treatment (Figure 30). Intestinal weight was 12% lower in the MZ group than the C group (Figure 30A), but not when expressed as a percentage of body weight (Figure 30B). However, the intestinal weight of the PW treatment was not significantly different from either the MZ or C groups. The interaction of lead and dietary zinc on intestinal wet weight and weight to body weight ratio were not significant.

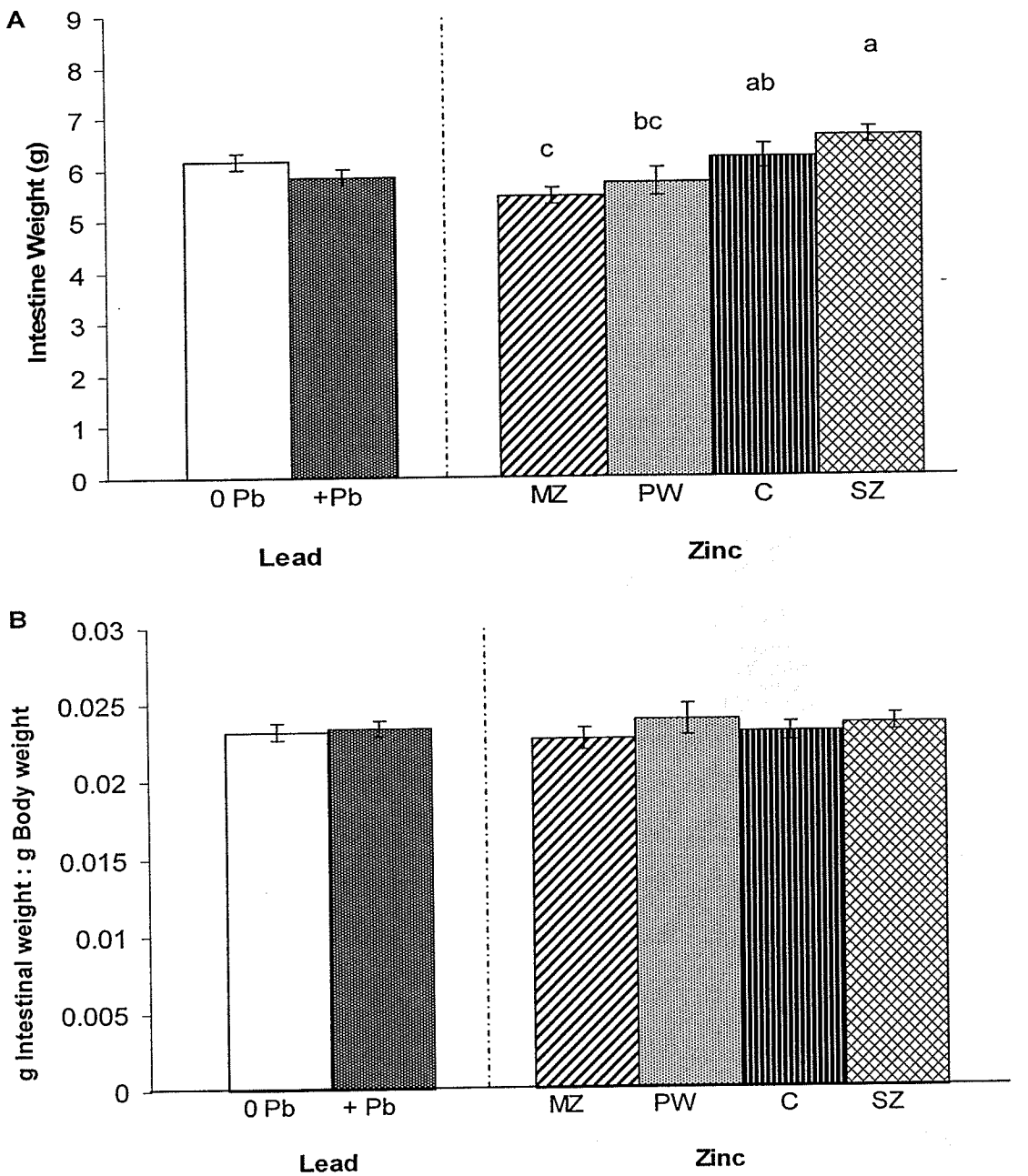


Figure 30. Effect of dietary zinc and lead exposure on intestinal wet weight (A) and intestinal wet weight to body weight ratio (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was not significant (A, p=0.1225; B, p=0.7937). The main effect of zinc was significant (p=0.0014) for intestinal weight (A), but not as a ratio of body weight (B), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (A, p=0.5612; B, p=0.6975).

Intestine Length

Lead treatment did not have a significant effect on intestinal length (Figure 31). MZ rats had 4%-5% less intestinal length than the C and SZ groups, but the intestinal length of the PW groups was not significantly different from either the MZ or C and SZ groups. The interaction of lead and dietary zinc on intestinal length was not significant.

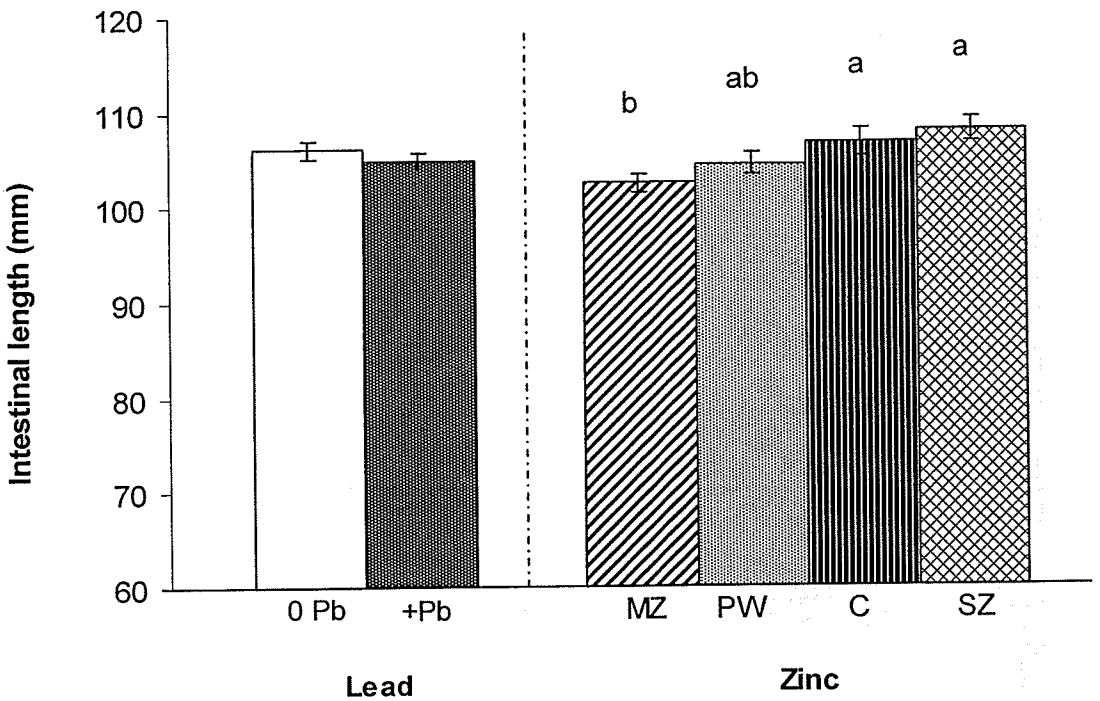


Figure 31. Effect of dietary zinc and lead exposure on intestinal length. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was not significant (p=0.3214). There was a significant main effect of zinc on intestinal length (p=0.0125), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.2950).

Duodenum Zinc & Lead Concentration

Duodenum zinc concentration was not affected by lead treatment, but was 30% lower in the MZ and 38% higher in SZ groups than C rats (Figure 32). There was no difference between C and PW rats in terms of duodenum zinc concentration. The interaction of lead and dietary zinc was not significant.

There were no significant differences among treatment groups for duodenum lead concentration (Figure 33).

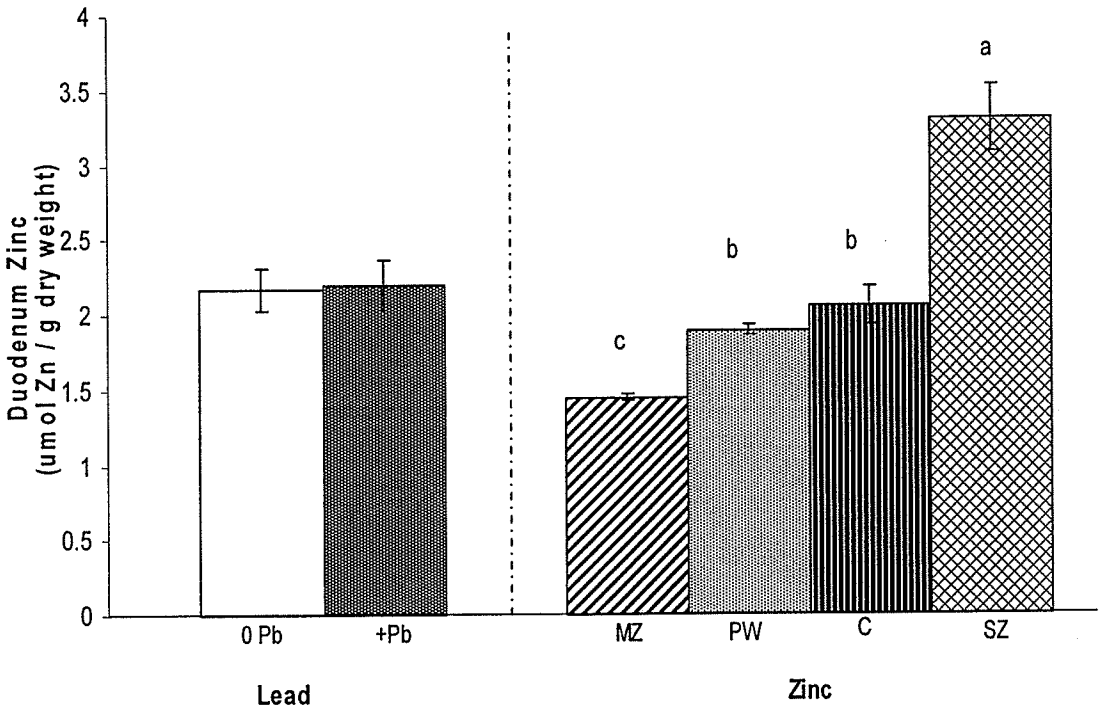


Figure 32. Effect of dietary zinc and lead exposure on duodenum zinc concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=15-16 (zinc effect). The lead effect was not significant (p=0.7596). There was a significant main effect of zinc (p<0.0001), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.7705).

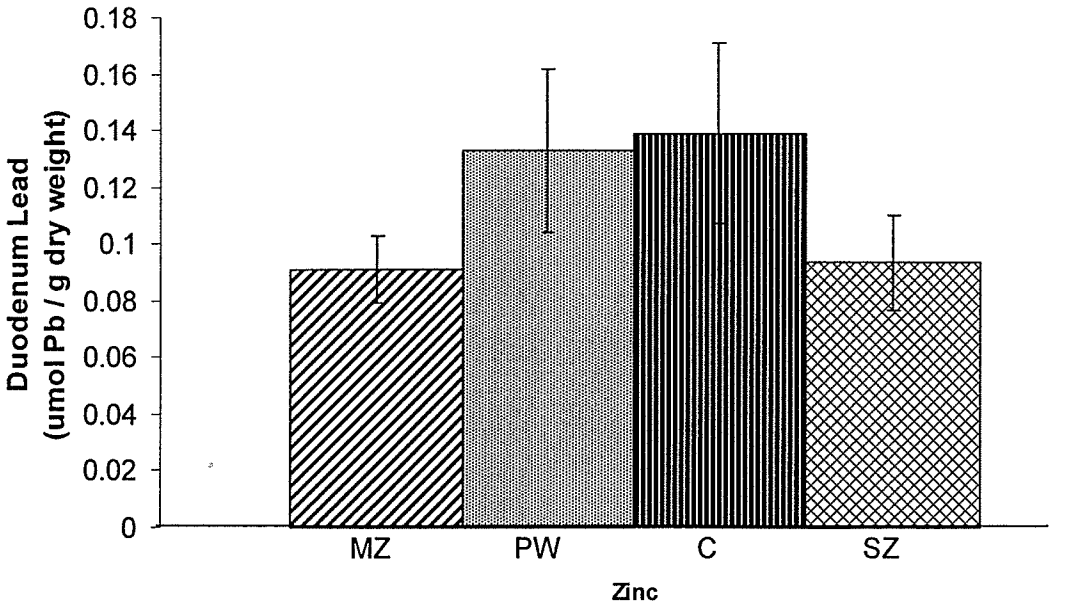


Figure 33. Effect of dietary zinc and lead exposure on duodenum lead concentration. Data was analyzed by one-way ANOVA. Columns represent means ± SEM for n=8. Means were not significantly different (p=0.5496) as determined by one-way ANOVA.

Jejunum Zinc & Lead Concentration

Jejunum zinc concentration was not affected by lead treatment, but was 36% lower in MZ rats and 36% higher in SZ rats compared to both PW and C rats (Figure 34). There was no significant interaction of lead and dietary zinc for jejunum zinc concentration.

Jejunum lead concentration was not significantly different among treatment groups (Figure 35).

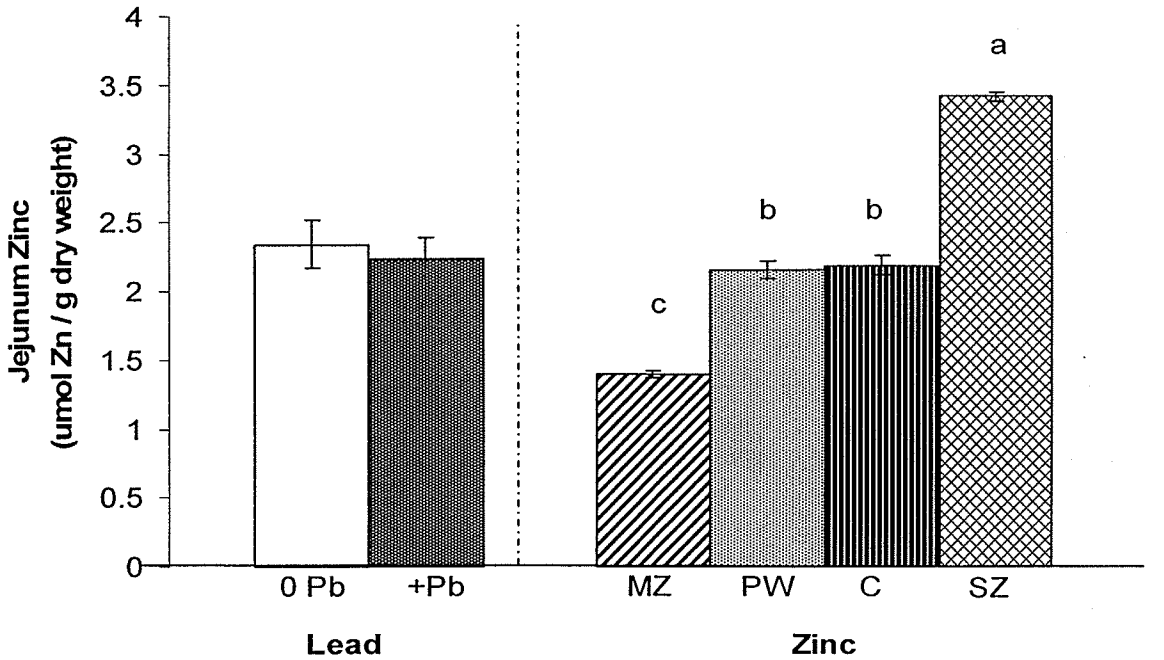


Figure 34. Effect of dietary zinc and lead exposure on jejunum zinc concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was not significant (p=0.4987). There was a significant main effect of zinc (p<0.0001), with different letters indicating that means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.8959).

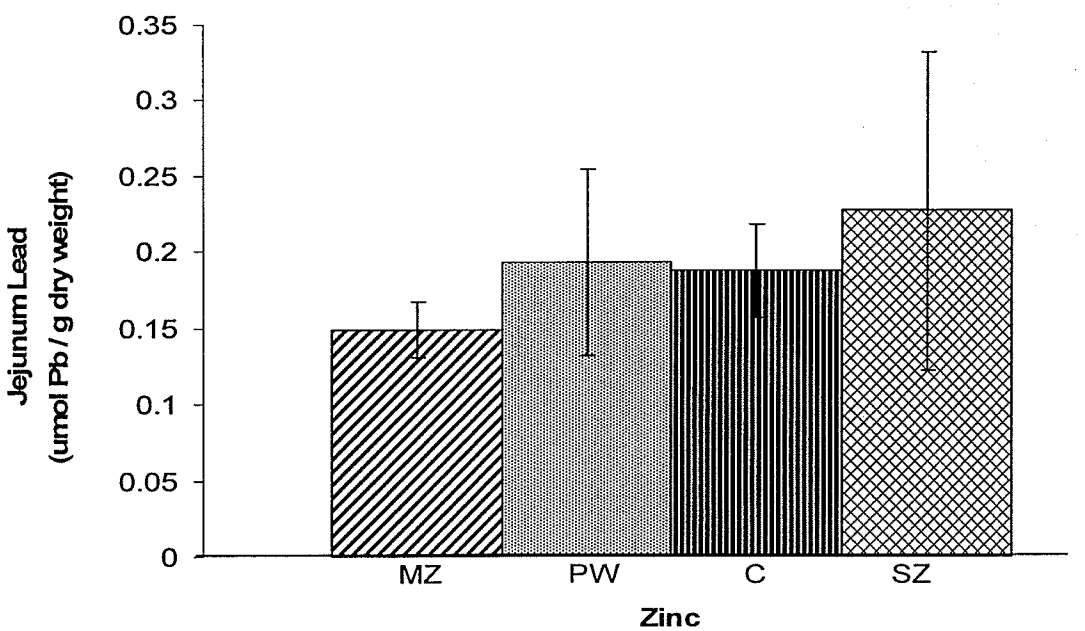


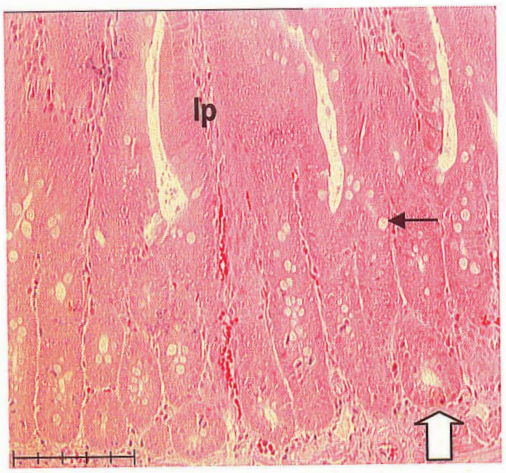
Figure 35. Effect of dietary zinc and lead exposure on jejunum lead concentration. Data was analyzed by one-way ANOVA. Columns represent means ± SEM for n=8. Means were not significantly different (p=0.8594) as determined by one-way ANOVA.

Intestinal Metallothionein Immunostaining

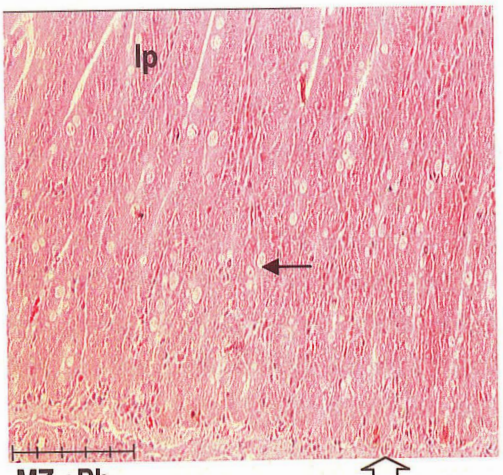
Results of the immunoperoxidase staining for MT in the intestine are presented in Figures 36-40. All negative control sections were absent of metallothionein staining (Figures 36-40), indicating that the staining was specific for MT. There were no apparent differences in staining intensity or distribution between the lead and non-lead treatments for intestinal MT. Thus, these results will only be discussed in terms of the response to dietary zinc. In addition, the results from the jejunum are mainly reflective of the results from the duodenum, with few exceptions, thus they will be discussed together, except where indicated.

There was strong nuclear and cytoplasmic MT staining in Paneth cells of intestinal crypts in all treatments except the MZ groups (Figures 36-38). MZ treatment resulted in weak staining, which was limited to very few cells in each intestinal section (Figure 36, 37 & 40). MT staining of the nuclei and cytoplasm of villi surface epithelial cells, with the exception of goblet cells, was weak to nil in all treatment groups, except in duodenal sections of the SZ groups, which stained strongly (Figures 36, 37, & 39). In general, MT staining was more intense in the duodenum than the jejunum and was mainly present in the proliferative region of the villi, rather than the apical tip (Figures 36, 37, & 40). There was no MT staining detectable in the lamina propria, submucosa, muscularis, or vasculature in intestinal sections of any treatment group.

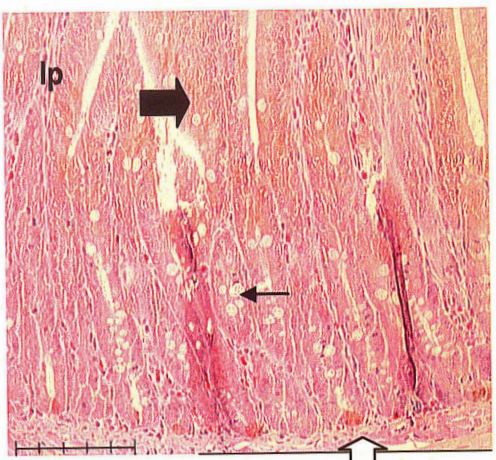
Figure 36. Immunohistological staining for metallothionein in rat duodenum. There was strong nuclear and cytoplasmic metallothionein staining in Paneth cells (white block arrow) in all treatments except MZ and MZ+Pb. Metallothionein staining in villi epithelial cells (black block arrow) was strong in SZ and SZ+Pb groups, weak to nil in C, C+Pb, PW, and PW+Pb groups and nil in MZ and MZ+Pb groups. No metallothionein staining was present in goblet cells (arrow) and lamina propria (lp) in all treatments. Scale bars shown at the bottom left corner equal 160 μm.



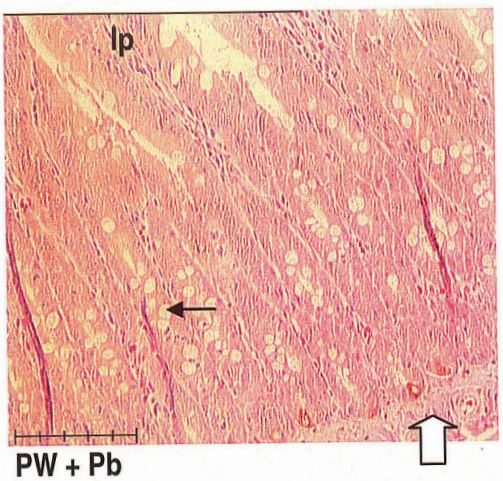
MZ



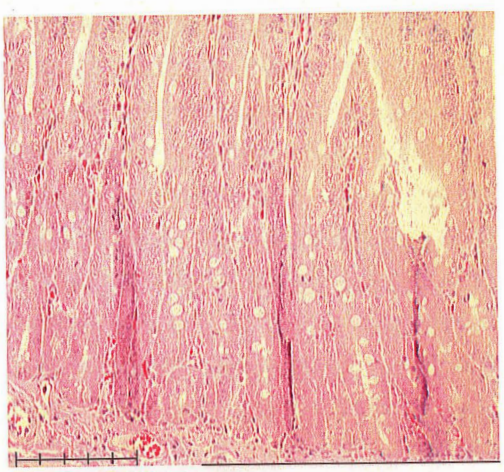
MZ +Pb



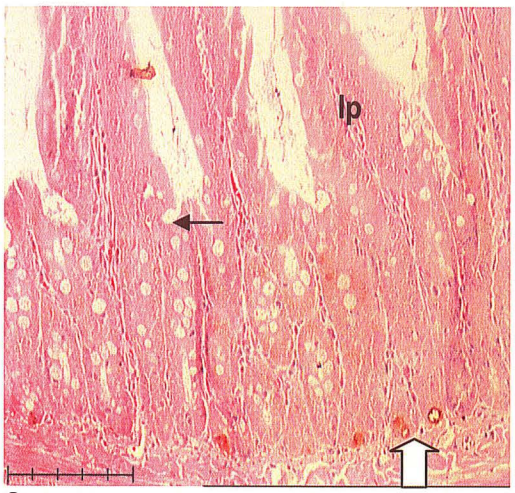
PW



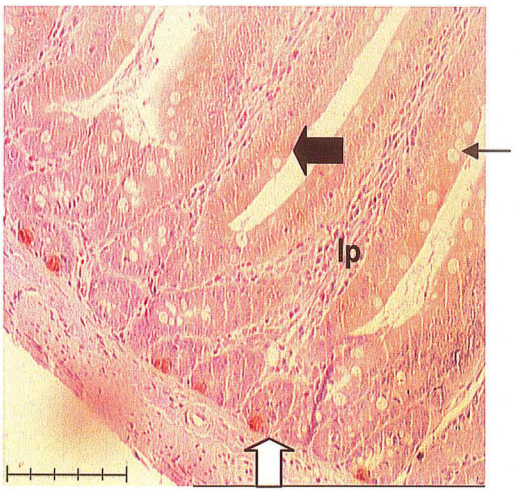
PW + Pb



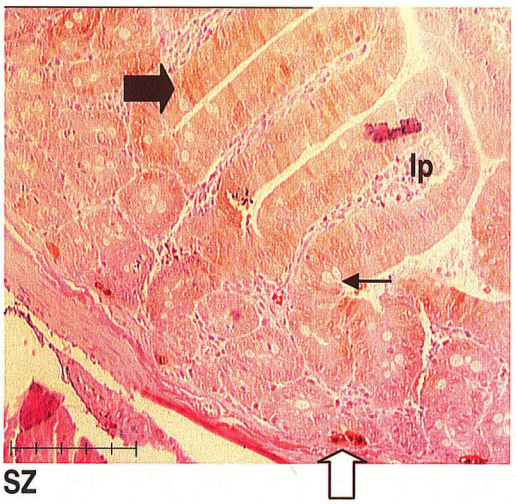
NEGATIVE CONTROL



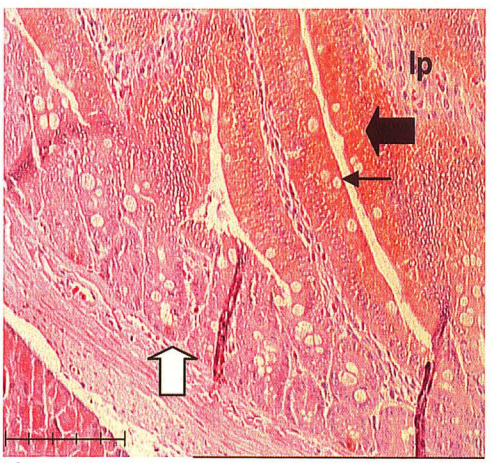
C



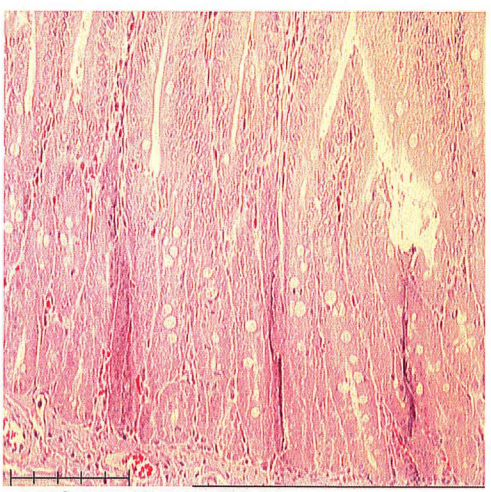
C + Pb



SZ

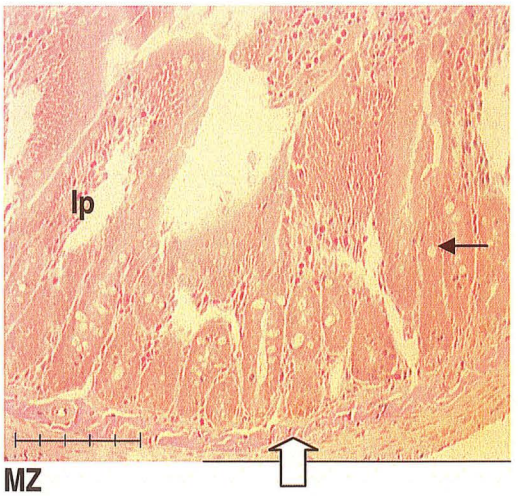


SZ + Pb

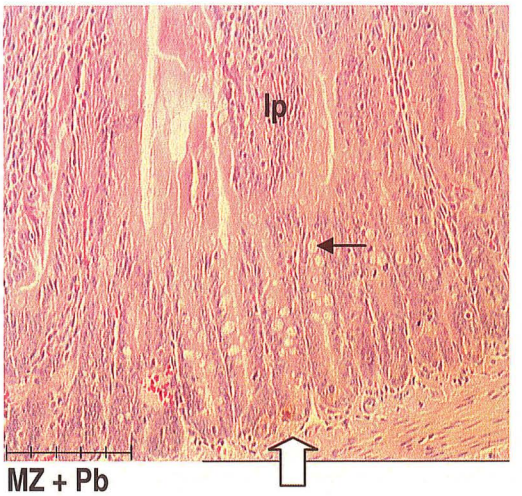


NEGATIVE CONTROL

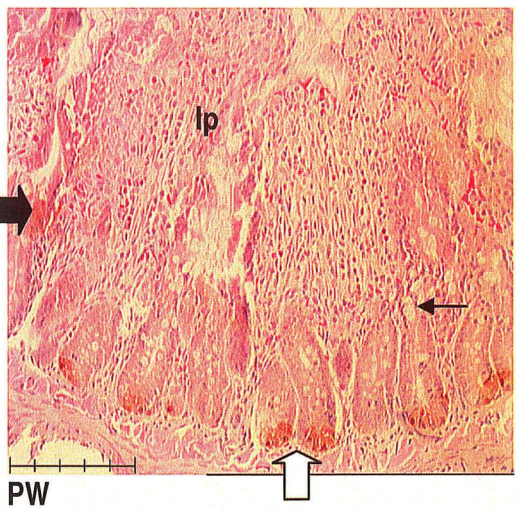
Figure 37. Immunohistological staining for metallothionein in rat jejunum. There was strong nuclear and cytoplasmic metallothionein staining in Paneth cells (white block arrow) in all treatments except MZ and MZ+Pb. Metallothionein staining in villi epithelial cells (black block arrow) was weak to nil in SZ, SZ+Pb, C, C+Pb, PW, and PW+Pb groups and nil in MZ and MZ+Pb groups. No metallothionein staining was present in goblet cells (arrow) and lamina propria (lp) in all treatments. Scale bars shown at the bottom left corner equal 160 μm.



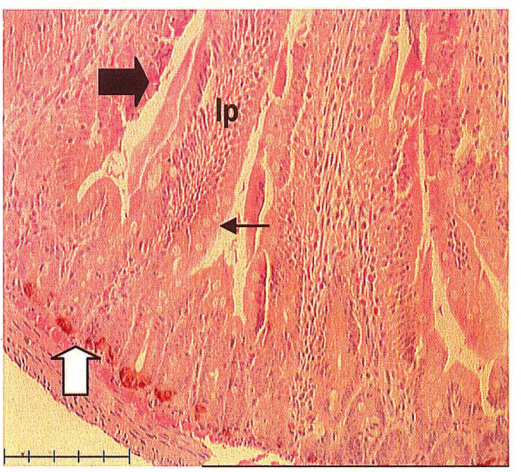
MZ



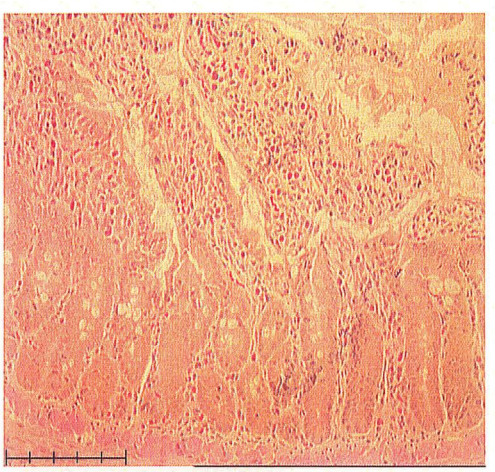
MZ + Pb



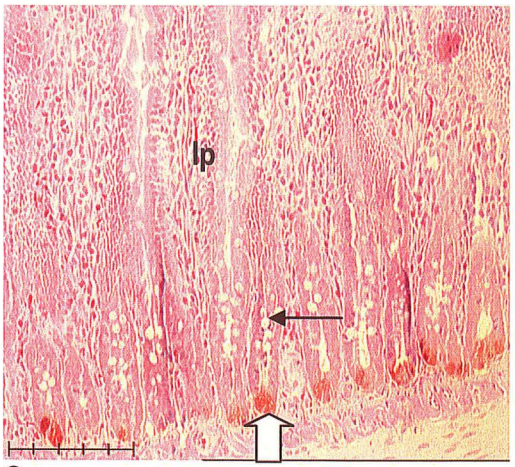
PW



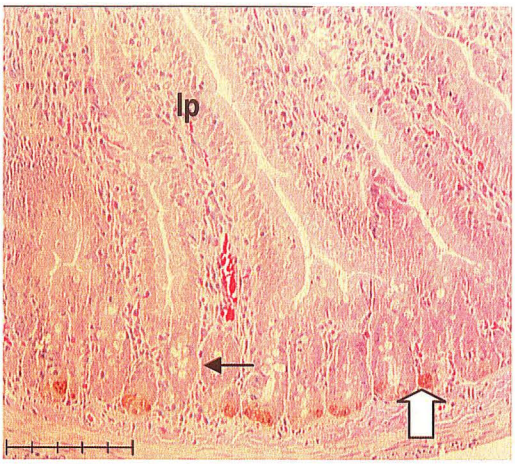
PW + Pb



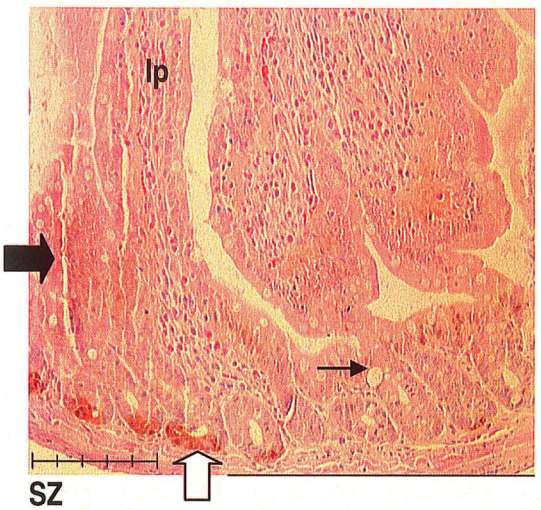
NEGATIVE CONTROL



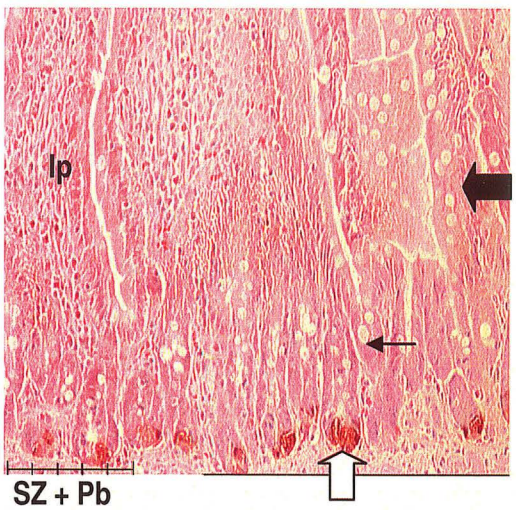
C



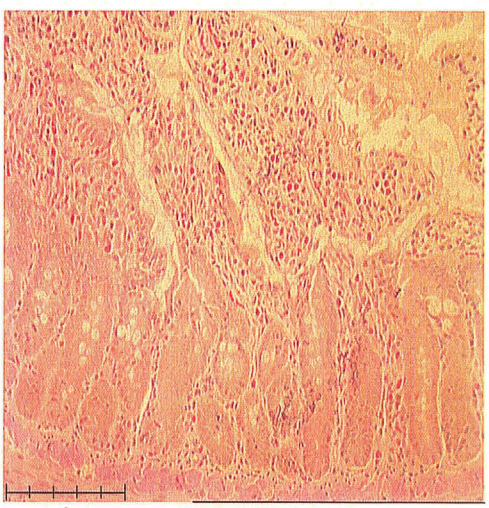
C + Pb



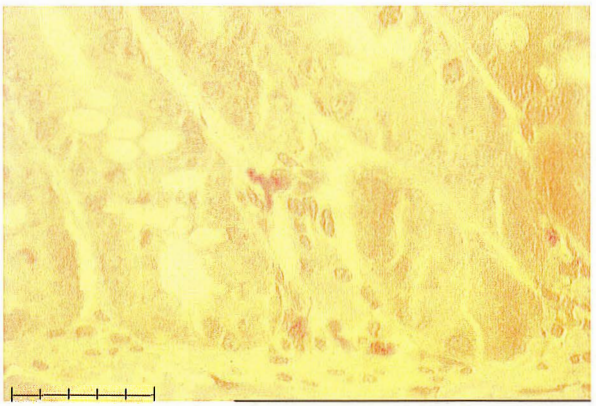
SZ



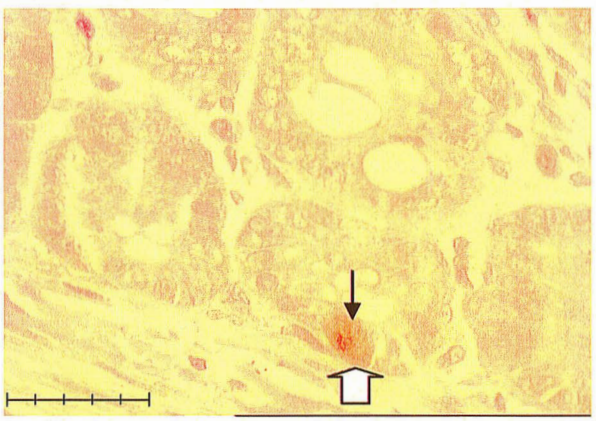
SZ + Pb



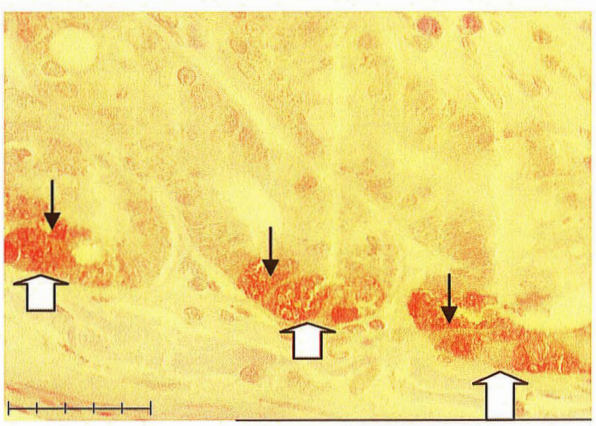
NEGATIVE CONTROL



A Negative Control

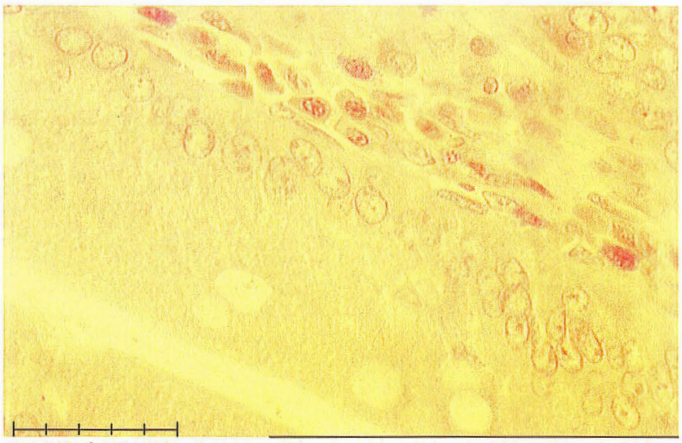


B Moderate Immunostaining



C Strong Immunostaining

Figure 38. Immunohistological staining for metallothionein in Paneth cells of rat intestine. Metallothionein staining was localized within the nuclei and cytoplasm of Paneth cells (white block arrow) in the base of intestinal crypts. The secretory granules (arrow) of the Paneth cells are clearly evident at 400X magnification with strong MT immunostaining (C). Scale bars at the bottom left corner equal 40 µm.



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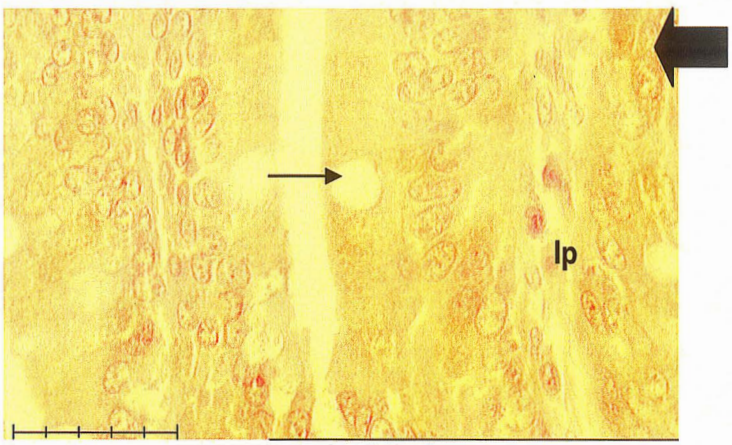
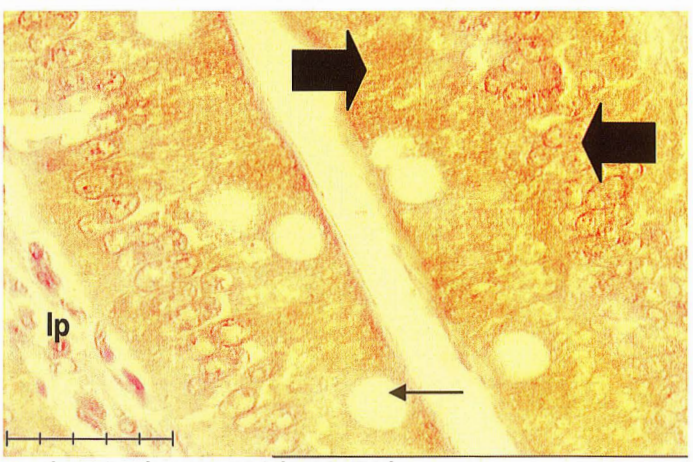
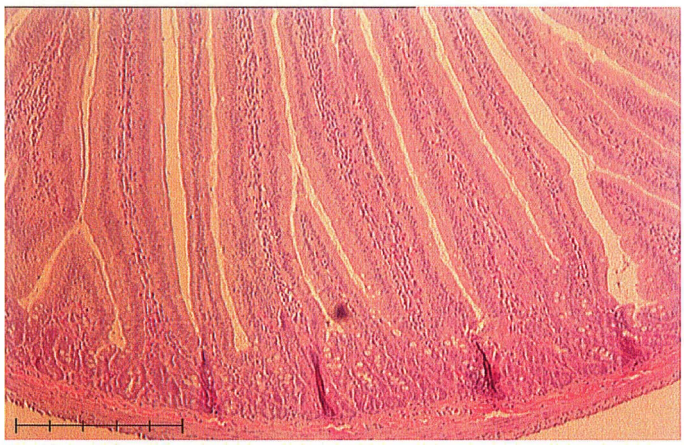


B MODERATE IMMUNOSTAINING

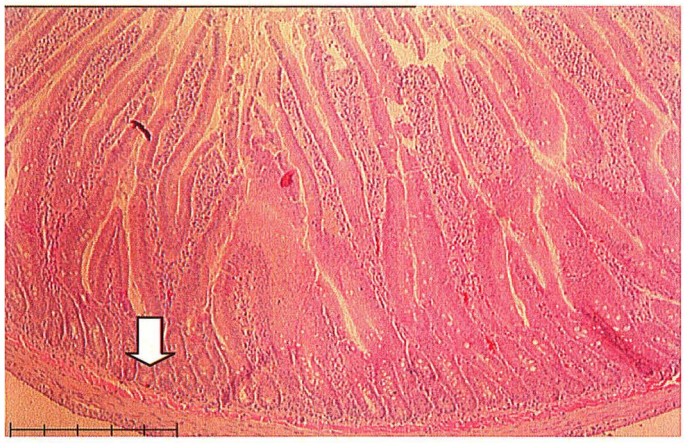


C STRONG IMMUNOSTAINING

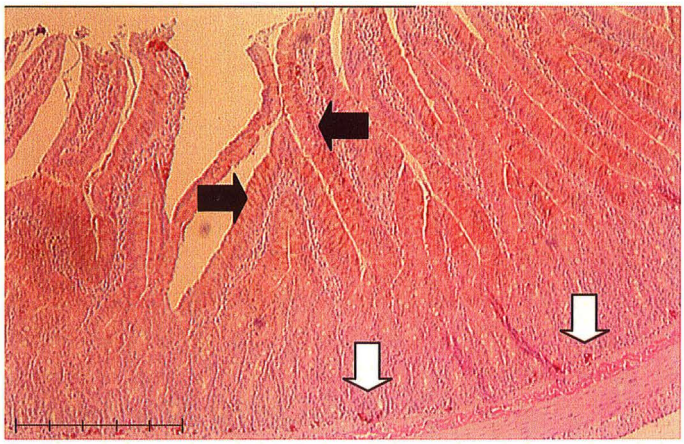
Figure 39. Immunological staining for metallothionein in rat intestinal villi epithelia. Metallothionein was localized within the nuclei and cytoplasm of surface epithelial cells (black block arrow), but absent from Goblet cells (arrow) and lamina propria (lp). Scale bars in bottom left corners equal 40 µm.



A Negative Control



B Weak Immunostaining



C Strong Immunostaining

Figure 40. Immunohistological staining for metallothionein in rat intestinal section. Metallothionein immunostaining in Paneth cells (white block arrow) and villi epithelial cells (black block arrow) at 40X magnification. Scale bars shown at the bottom left corner equal 160 µm.

Duodenum RT-PCR Analysis

Duodenum MT mRNA levels were not affected by lead treatment but there was a clear effect of dietary zinc (Figure 41). MT mRNA levels were lower in MZ rats than all other treatments. MZ treatment was 56% lower than PW, 67% lower than C and 82% lower than SZ.

Duodenum CRIP mRNA levels were not altered by lead exposure or dietary zinc (Figure 42).

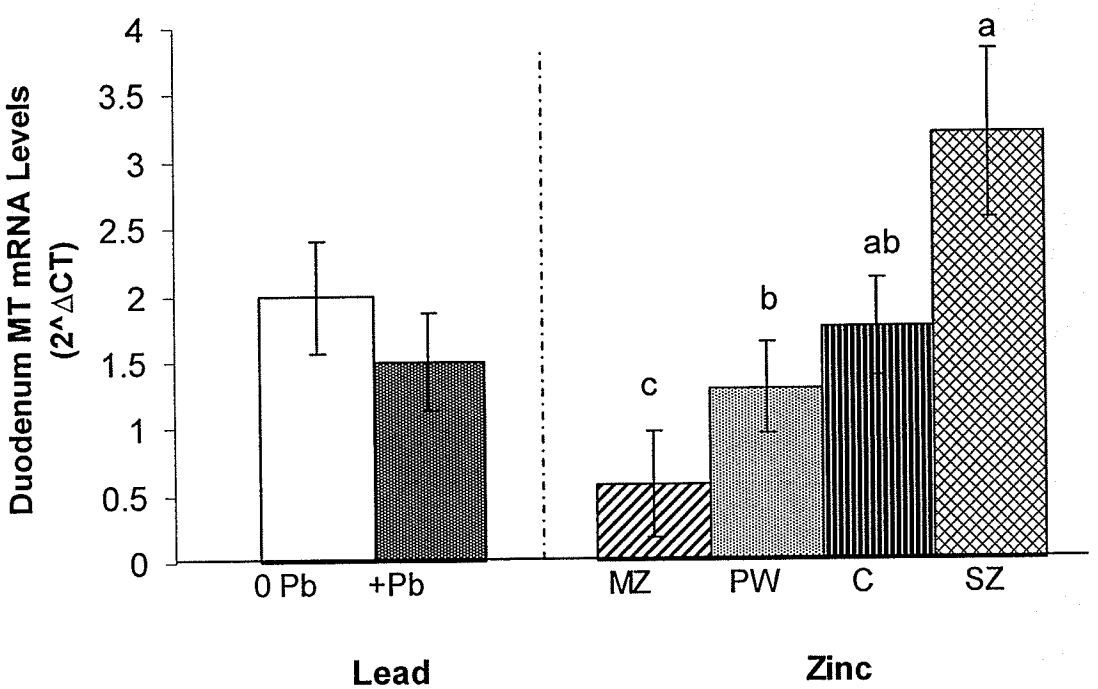


Figure 41. Effect of dietary zinc and lead exposure on duodenum MT mRNA levels. Data are expressed as the difference between control and treatment MT mRNA expression, calculated from the CT difference using the formula $2^{\Delta CT}$. Columns represent means ± SEM for n=16-17 (lead effect) and n=8-9 (zinc effect). The main effect of lead was not significant (p=0.4240). The main effect of zinc was significant (p=0.0040), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.2499).

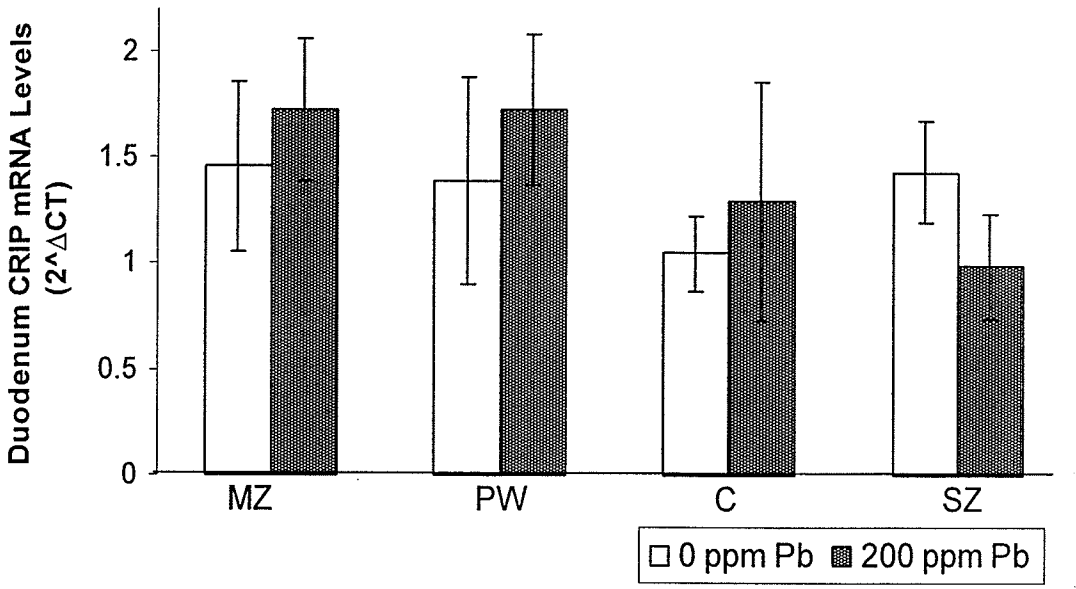


Figure 42. Effect of dietary zinc and lead exposure on duodenum CRIP mRNA levels. Data are expressed as the difference between control and treatment CRIP mRNA expression, calculated from the CT difference using the formula $2^{\Delta CT}$. Columns represent means ± SEM for n=4-5. There were no significant effects of lead, zinc, or their interaction (p=0.7012, p=0.5398, and p=0.6701, respectively).

Jejunum RT-PCR Analysis

Lead exposure did not have a significant effect on jejunum MT mRNA levels (Figure 43). There was, however, a main effect of dietary zinc, with MZ rats having levels 89% lower than C, 93% lower than PW, and 96% lower than SZ rats. Of note, SZ rats had MT mRNA levels 62% higher than C rats.

There were no significant main effects or interactions of lead and dietary zinc on CRIP mRNA levels in the jejunum (Figure 44).

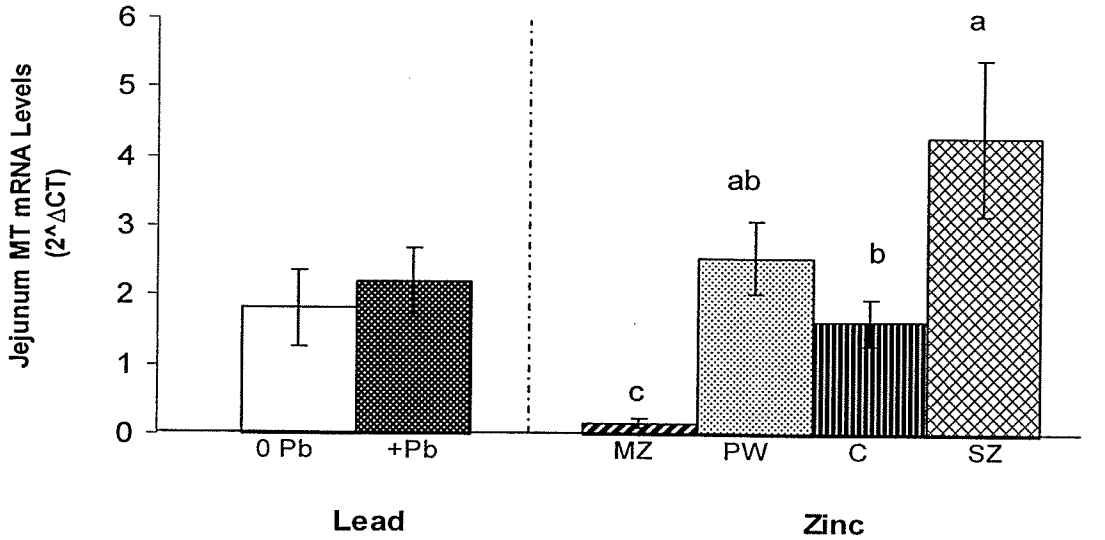


Figure 43. Effect of dietary zinc and lead exposure on jejunum MT mRNA levels. Data are expressed as the difference between control and treatment MT mRNA expression, calculated from the CT difference using the formula $2^{\Delta CT}$. Columns represent means ± SEM for n=18-23 (lead effect) and n=9-12 (zinc effect). The main effect of lead was not significant (p=0.6507). The main effect of zinc was significant (p=0.0005), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.9301).

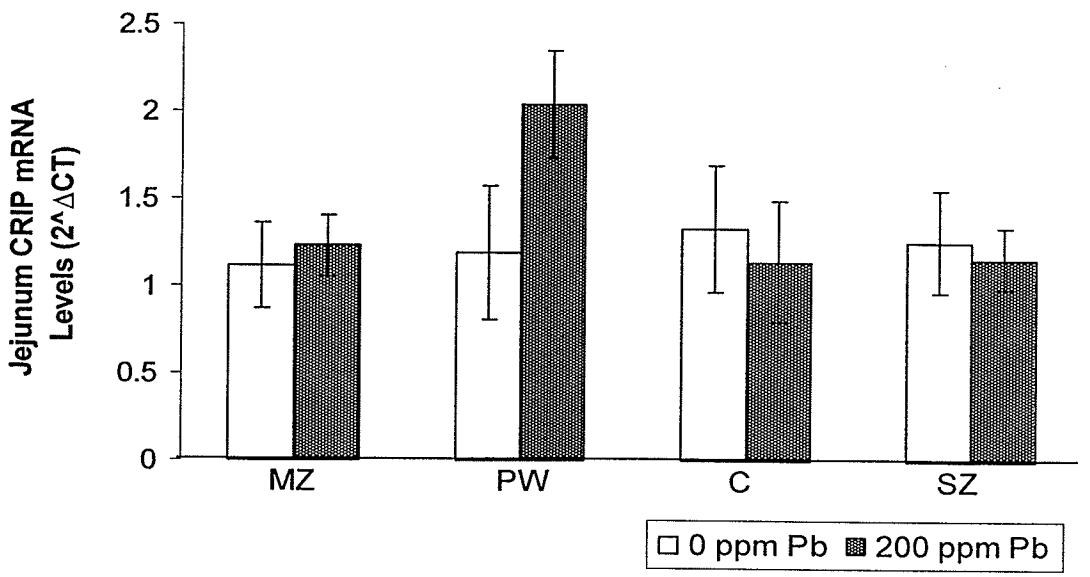


Figure 44. Effect of dietary zinc and lead exposure on jejunum CRIP mRNA levels. Data are expressed as the difference between control and treatment CRIP mRNA expression, calculated from the CT difference using the formula $2^{\Delta CT}$. Columns represent means ± SEM for n=3-6. There were no significant effects of lead, zinc, or their interaction (p=0.4796, p=0.5854, and p=0.4693, respectively).

Femur

Dry Weight and Dry Weight to Body Weight Ratio

Lead treated rats had 6% lower femoral weight than non-lead treatments (Figure 45A), but not when calculated as a percentage of body weight (Figure 45B). MZ and PW groups had 5% lower femoral weights than both the C and SZ groups (Figure 45A). However, when femoral weight was calculated as a percentage of body weight, MZ rats had 14-16% lower femoral weight than all other treatments (Figure 45B). There was no interaction of lead and zinc in terms of dry femoral weight or weight to body weight ratio.

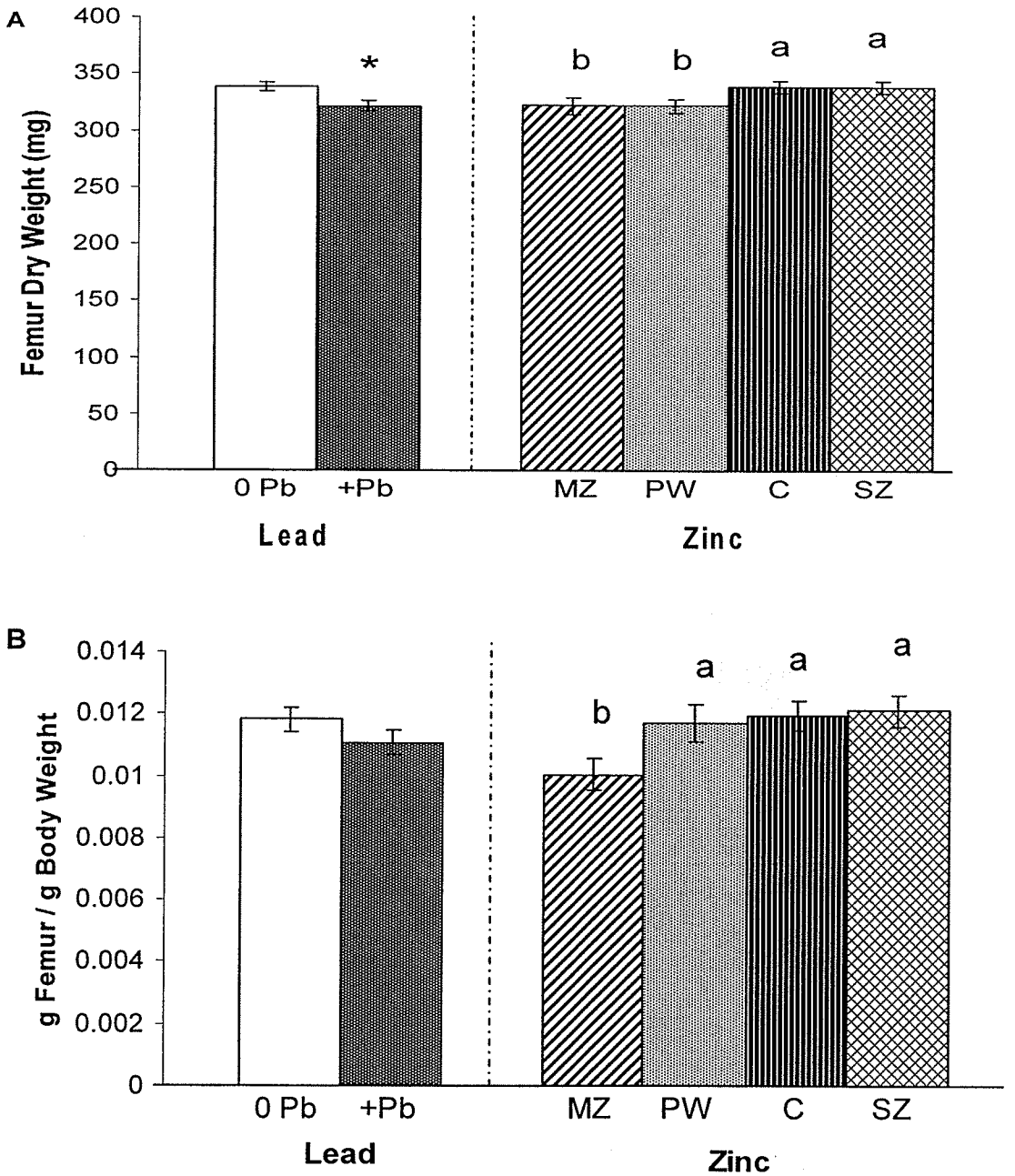


Figure 45. Effect of dietary zinc and lead exposure on femur dry weight (A) and femur dry weight to body weight ratio (B). Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was significant (p=0.0394) for femur weight (A), as indicated by an asterisk (*). The main effect of zinc was also significant (A, p=0.0038; B, p=0.0204), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interactions of lead and zinc were not significant (A, p=0.1549; B, p=0.8580).

Femur Calcium & Phosphorus Concentration

Lead exposure did not affect femoral calcium concentration (Figure 46). MZ treatment resulted in 4% less femoral calcium than PW, C and SZ rats. The interaction of lead and zinc did not affect femoral calcium.

Lead exposure did not affect femoral phosphorus concentration (Figure 47). MZ treatment resulted in 4% less femoral phosphorus than PW, C and SZ rats. The interaction of lead and zinc did not affect femoral phosphorus.

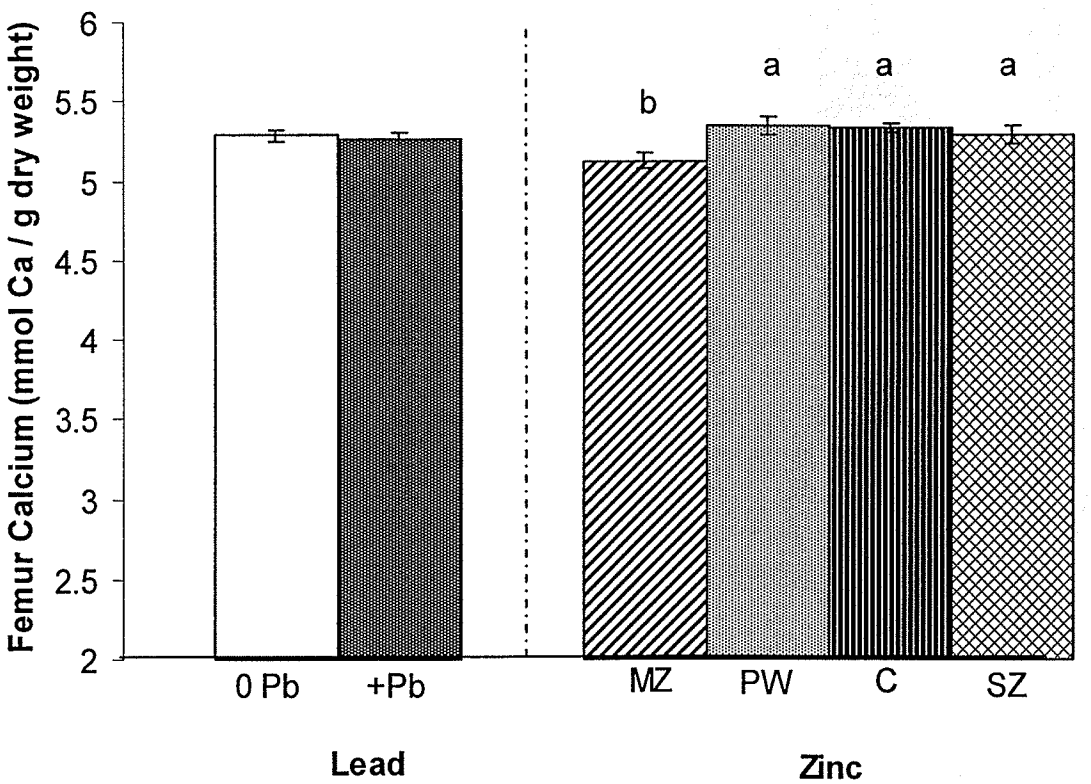


Figure 46. Effect of dietary zinc and lead exposure on femoral calcium concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was not significant (p=0.6765). The main effect of zinc was significant (p=0.0088), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.5997).

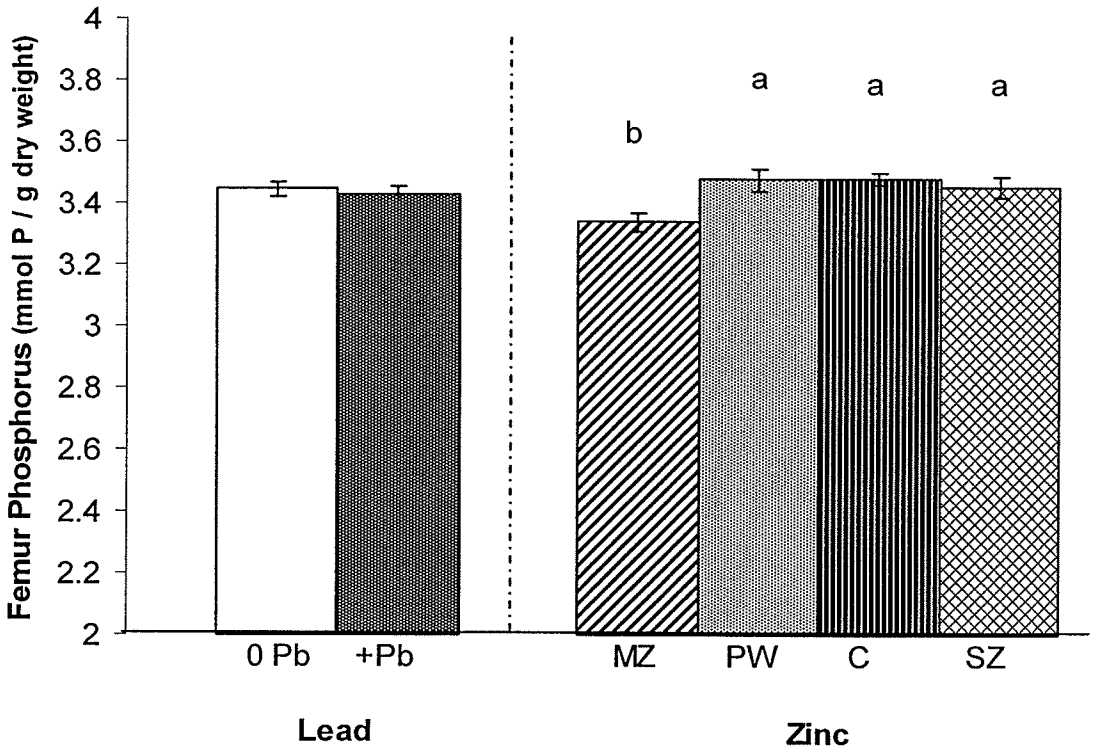


Figure 47. Effect of dietary zinc and lead exposure on femoral phosphorus concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was not significant (p=0.6123). The main effect of zinc was significant (p=0.0052), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.4981).

Morphometric Analysis

Femur length (Figure 48) was 2% lower in the lead treated rats compared to the non-lead groups. MZ and PW treatments resulted in 2% lower femoral length than the SZ treatment, but not C. There was no interaction of lead and dietary zinc on femur length.

There was a significant interaction between lead and dietary zinc on femoral knee width (Figure 49). The addition of lead resulted in 4% less femoral knee width in the MZ treatment but did not have an effect in the PW, C, or SZ treatments.

Femoral head width was 2% lower in the lead treatments than in the non-lead treated groups (Figure 50). Dietary zinc and the interaction of zinc with lead did not have significant effects on the femoral head. Femoral neck width and diaphysis width were not significantly different among treatment groups (Figure 51 & 52).

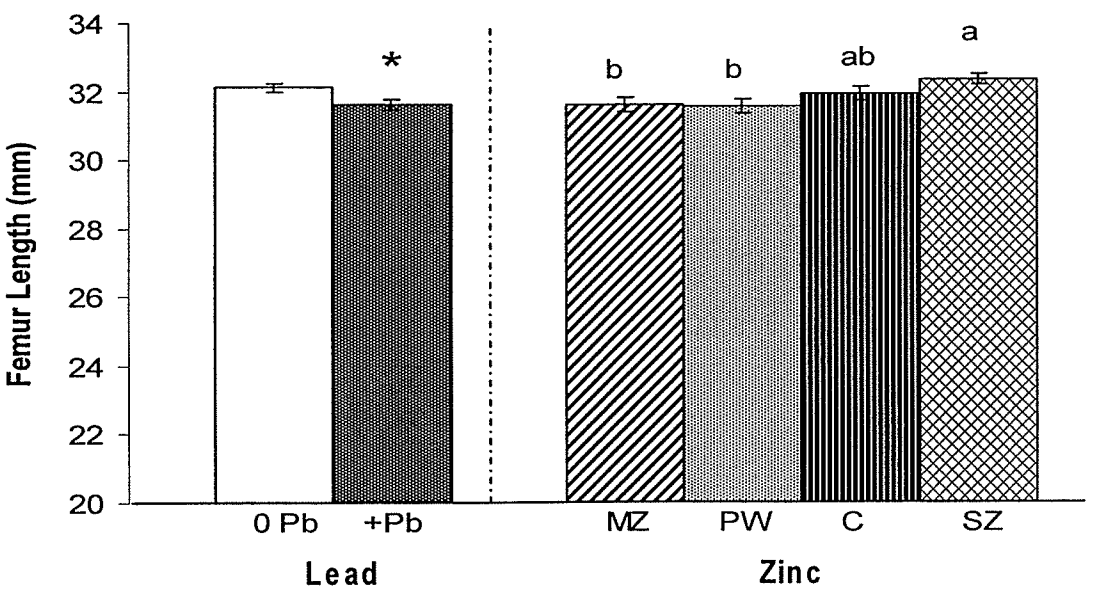


Figure 48. Effect of dietary zinc and lead exposure on femoral length. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was significant (p=0.0064), as indicated by an asterisk(*). The main effect of zinc was also significant (p=0.0145), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and dietary zinc was not significant (p=0.4375).

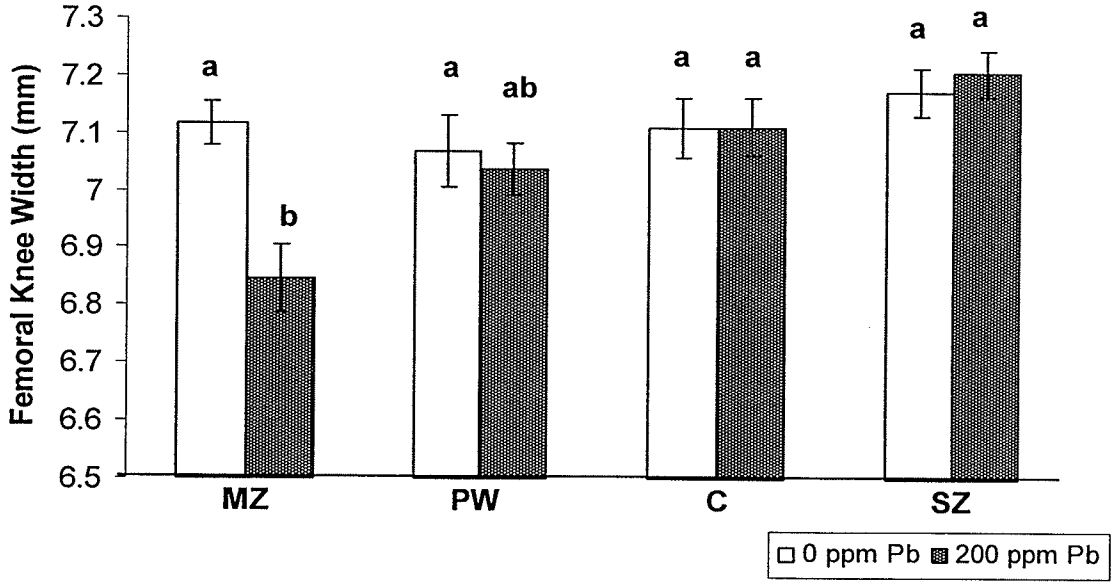


Figure 49. Effect of dietary zinc and lead exposure on femoral knee width. Columns represent group means ± SEM for n=8. The interaction of lead and zinc was significant (p=0.0519), with different letters indicating means are significantly different as determined by Duncan's multiple range test.

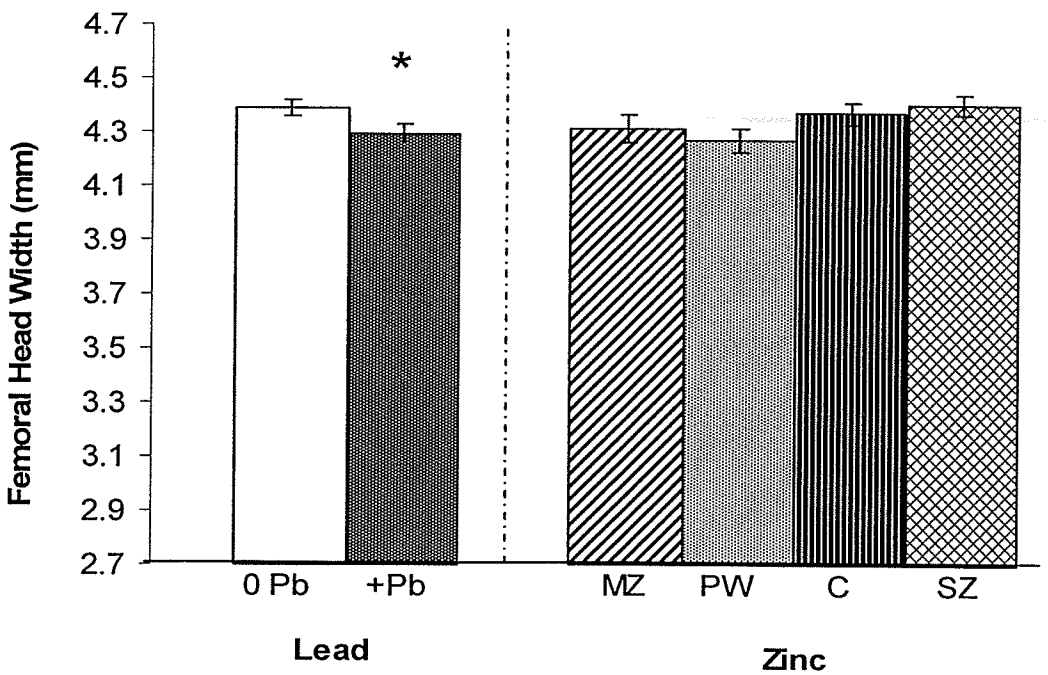


Figure 50. Effect of dietary zinc and lead exposure on femoral head width. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The lead effect was significant (p=0.0382), as indicated by an asterisk(*). The main effect of zinc and the interaction of lead and zinc were not significant (p=0.1372 and p=0.3691, respectively).

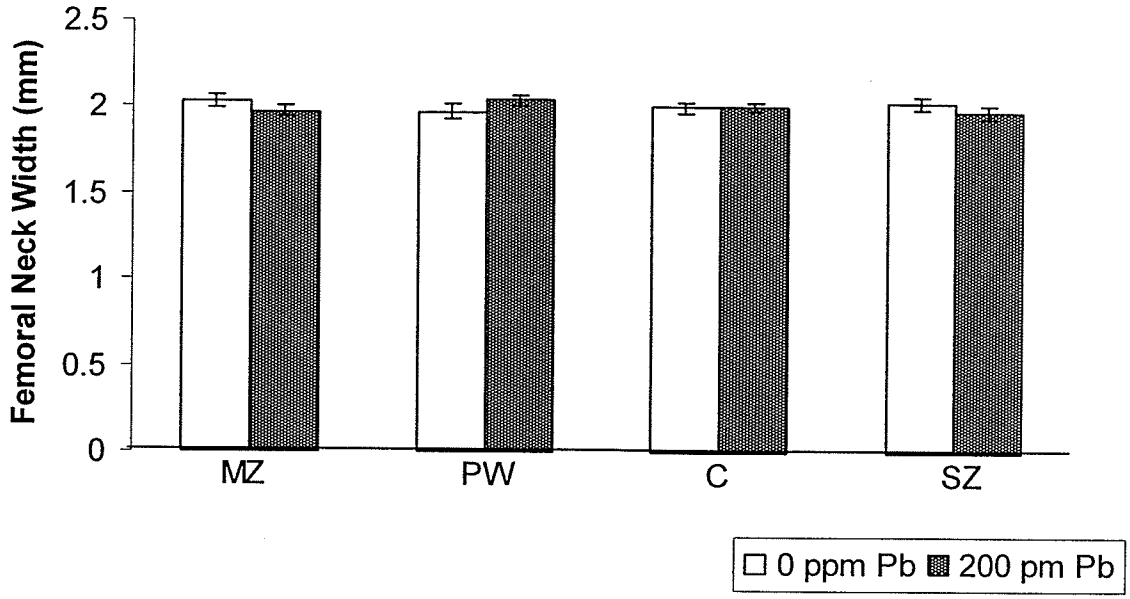


Figure 51. Effect of dietary zinc and lead exposure on femoral neck width. Columns represent group means ± SEM for n=8. The main effects of lead and zinc and the interaction of lead and zinc were not significant (p=0.6246, p=0.9999, and p=0.1364, respectively).

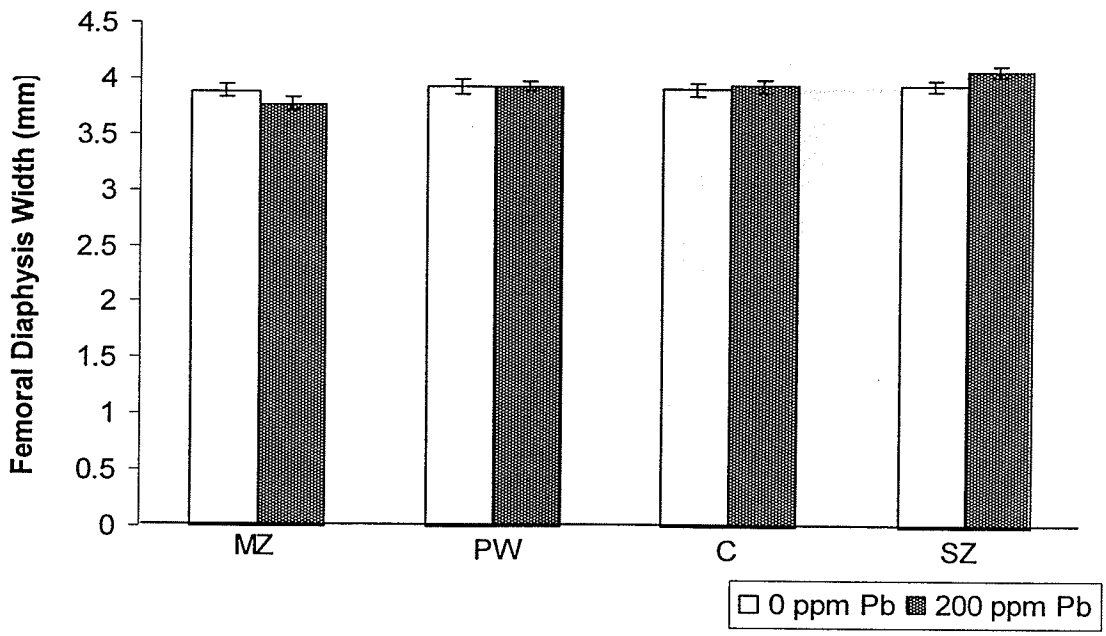


Figure 52. Effect of dietary zinc and lead exposure on femoral diaphysis width. Columns represent group means ± SEM for n=8. The main effects of lead and zinc and the interaction of lead and zinc were not significant (p=0.7953, p=0.0908, and p=0.3744, respectively).

Excised Femur DEXA Scans

Femoral area was not affected by lead exposure (Figure 53). MZ treatment resulted in 6-8% less femoral area than C and SZ treatments. MZ and PW groups were not significantly different. The interaction between lead and dietary zinc was not significant.

Femoral BMC was 6.5% lower in lead treated than non-lead treated animals (Figure 54). MZ and PW treatments had 8-9% less femoral BMC than C rats. SZ treatment was not different from MZ, PW, or C groups.

Femoral BMD was 3.5% lower in lead treated rats compared to non-leaded treated rats (Figure 55). PW and SZ treatments had a 6% lower femoral BMD than C rats. MZ rats were not different from PW, C, or SZ treatments.

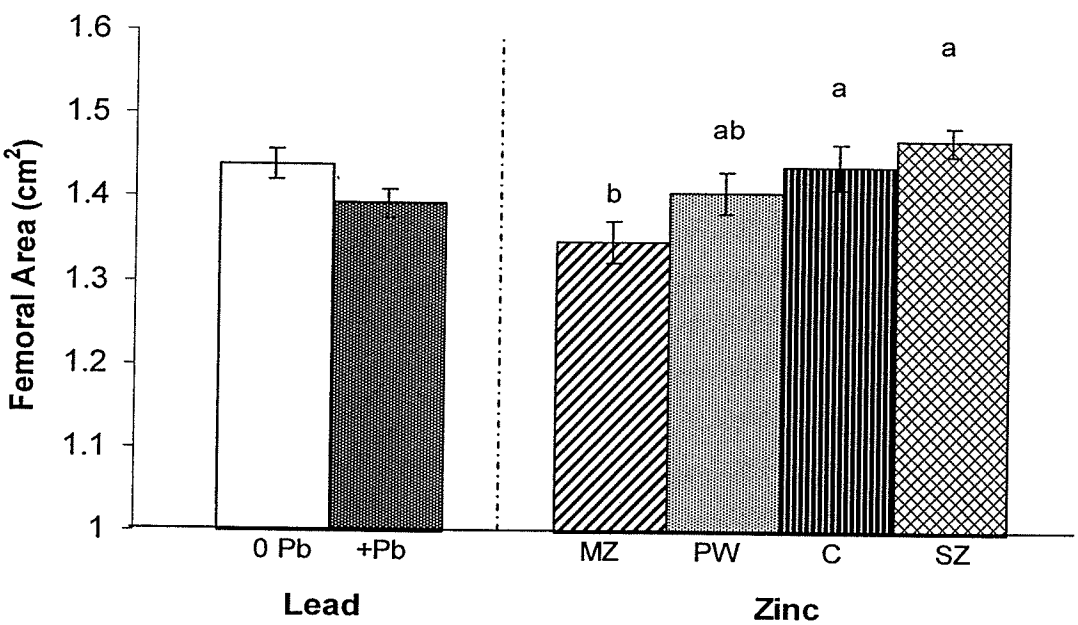


Figure 53. Effect of dietary zinc and lead exposure on femoral area. Columns represent group means ± SEM for n=26-27 (lead effect) and n=11-15 (zinc effect). The lead effect was not significant (p=0.0675). The main effect of zinc was significant (p=0.0058), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.7484).

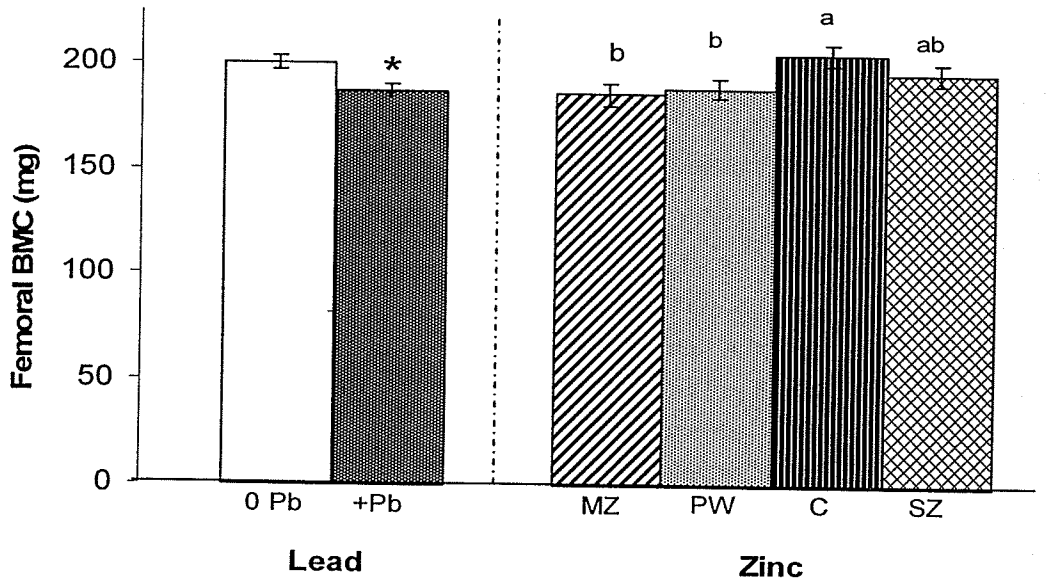


Figure 54. Effect of dietary zinc and lead exposure on femoral bone mineral content. Columns represent group means ± SEM for n=26-27 (lead effect) and n=11-15 (zinc effect). The lead effect was significant (p=0.0085), as indicated by an asterisk (*). The zinc effect was also significant (p=0.0484), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.8139).

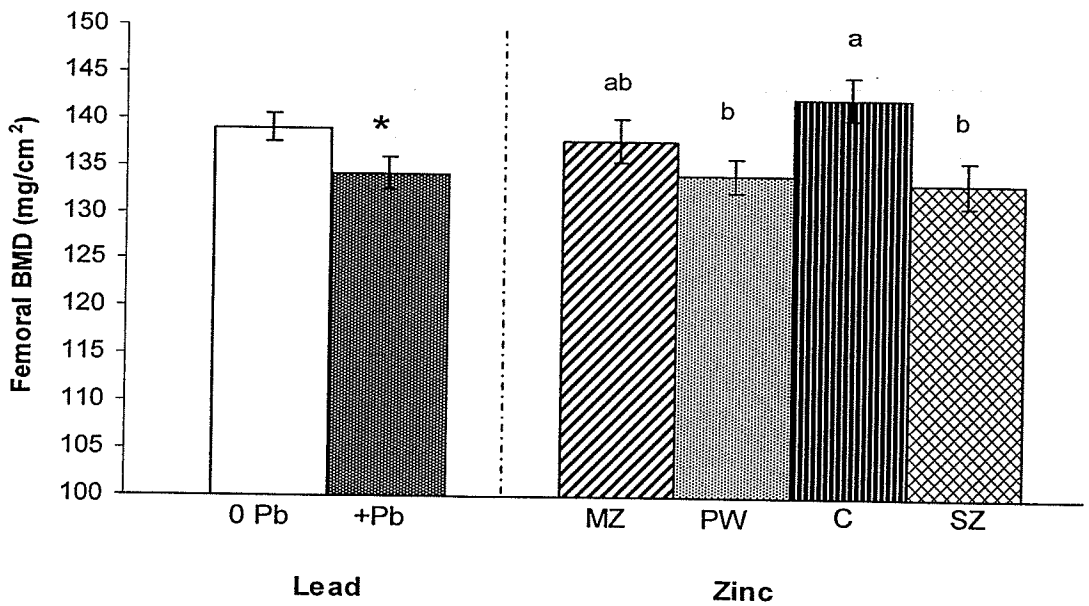


Figure 55. Effect of dietary zinc and lead exposure on femoral bone mineral density. Columns represent group means ± SEM for n=26-27 (lead effect) and n=11-15 (zinc effect). The lead effect was significant (p=0.0209), as indicated by an asterisk (*). The main effect of zinc was also significant (p=0.0191), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.8304).

In Situ Whole Body DEXA Scans

The *in situ* analysis of the whole body is presented in Figure 56. *In situ* whole body area and BMC were 5% and 6% lower, respectively, in lead treated groups than non-lead treatments. MZ rats had 7-9% less area and BMC than C and SZ rats, but MZ and PW rats did not differ in terms of area or BMC. BMD was not different among the various treatments.

Similar results were found with several *in situ* scans of selected bones. Lead treatment resulted in lower humeral, tibial, femur, and combined femoral and tibial bone area than non-lead treatments (Appendix, Tables 49-53). Lead treatment was also found to have a lower spinal BMC than non-lead treatments. Spinal bone area was lower in MZ and PW groups than C and SZ groups, as was found for the whole body. BMD was not different in the spine, humerus, tibia, femur, and combined tibia and femur *in situ* scans.

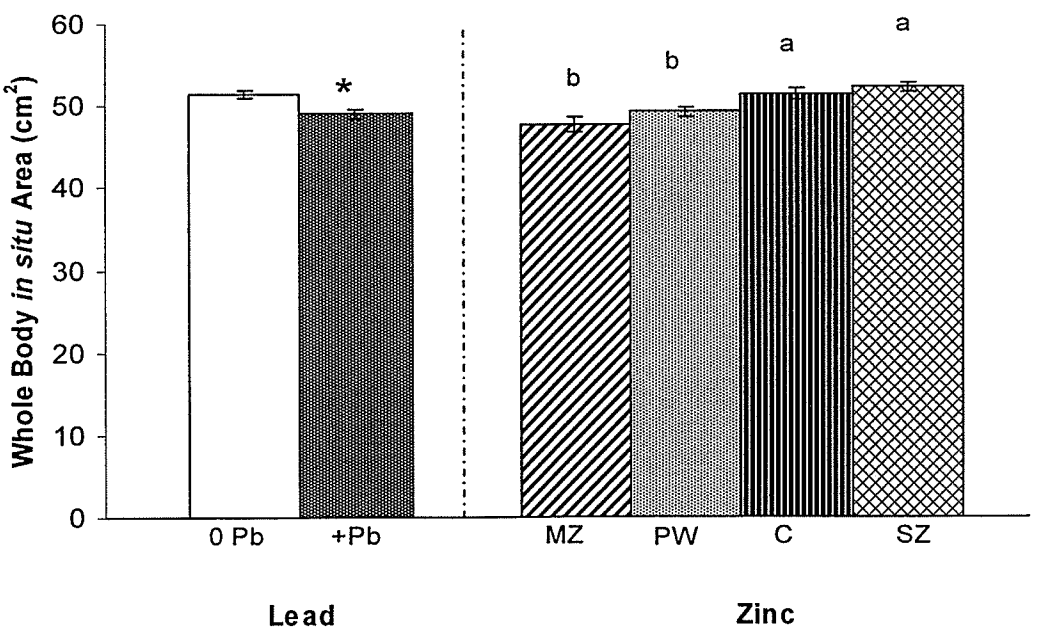


Figure 56. Effect of dietary zinc and lead exposure on whole body area. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effect of lead was significant (p=0.0004) as indicated by an asterisk (*). The main effect of zinc also was significant (p<0.0001), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.1183).

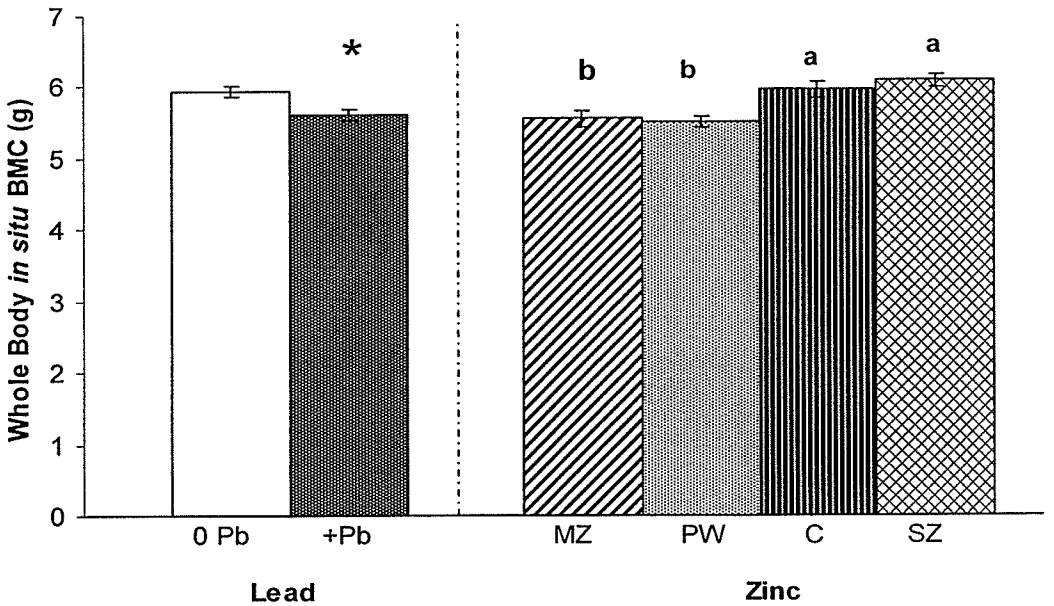


Figure 57. Effect of dietary zinc and lead exposure on whole body BMC. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The lead effect was significant (p=0.0007), as indicated by an asterisk (*). The zinc effect also was significant (p<0.0001), with different letters indicating means are significantly different as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.1604).

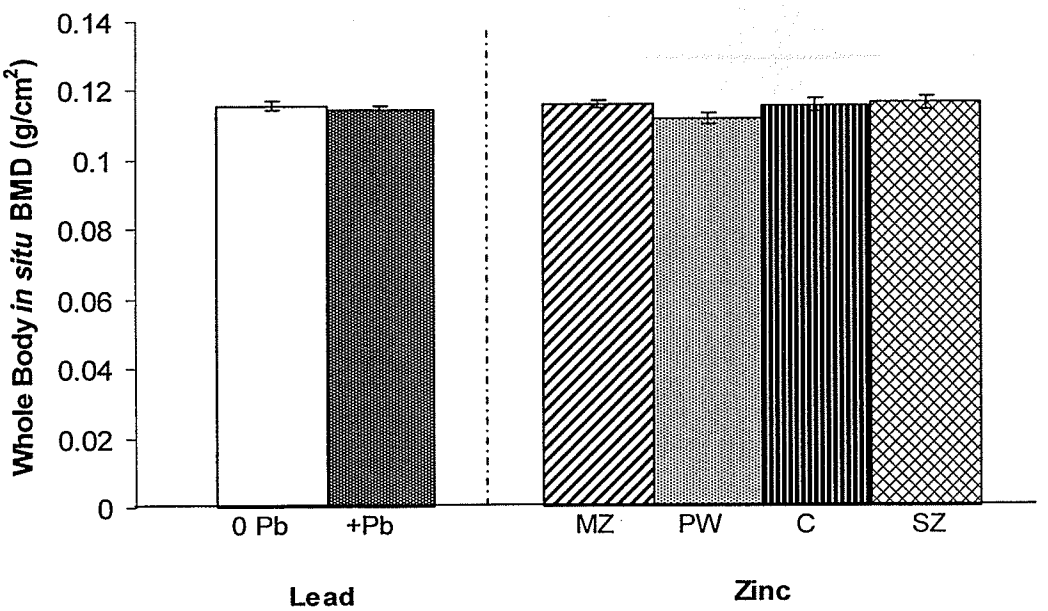


Figure 58. Effect of dietary zinc and lead exposure on whole body BMD. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The main effects of lead, zinc, and their interaction were not significant (p=0.3705, p=0.1248, and p=0.4400, respectively).

Skeletal Formation & Remodeling

Serum Osteocalcin & Ratlaps Concentration

Serum osteocalcin concentration is biochemical marker of bone formation. Dietary zinc, but not lead exposure affected serum osteocalcin concentration (Figure 57). MZ rats had 12-16% lower serum osteocalcin than C and SZ. PW rats were not different from MZ or C rats.

Serum Ratlaps is a biochemical marker of bone resorption. Lead treatment did not affect serum Ratlaps (Figure 58). There was a main effect of dietary treatment on serum Ratlaps. The PW groups had 23-24% less serum Ratlaps than C and SZ groups, but MZ rats were not different from PW, C, or SZ groups.

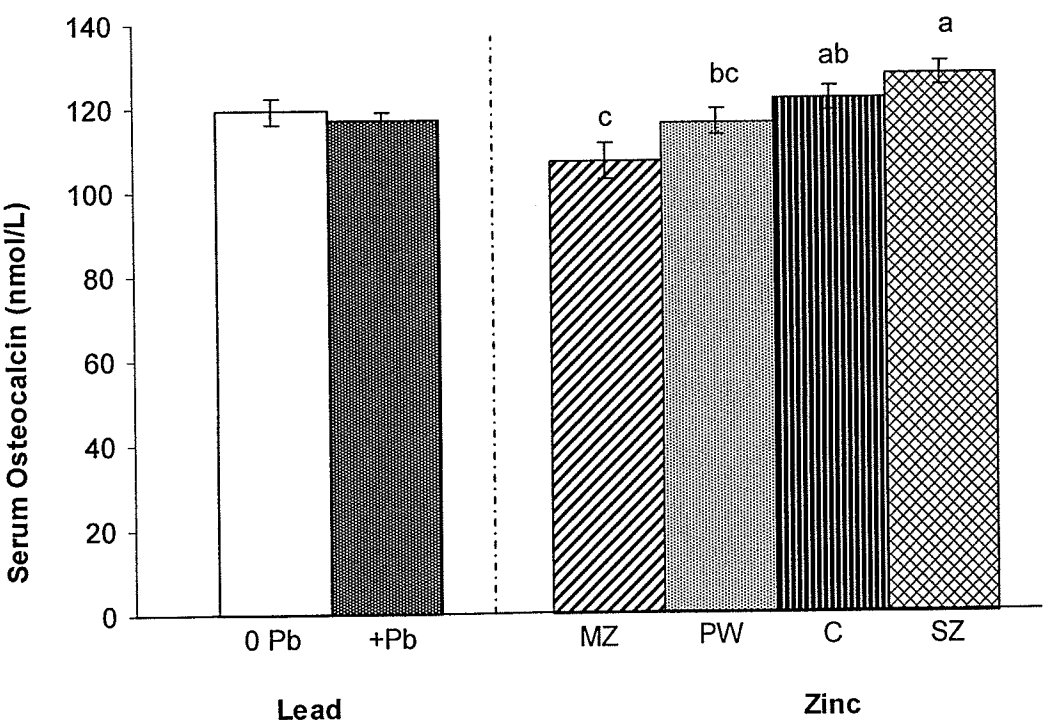


Figure 59. Effect of dietary zinc and lead exposure on serum osteocalcin concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The lead effect was not significant (p=0.4913). The main effect of zinc was significant (p=0.0008), with different letters indicating means are significantly different, as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.9312).

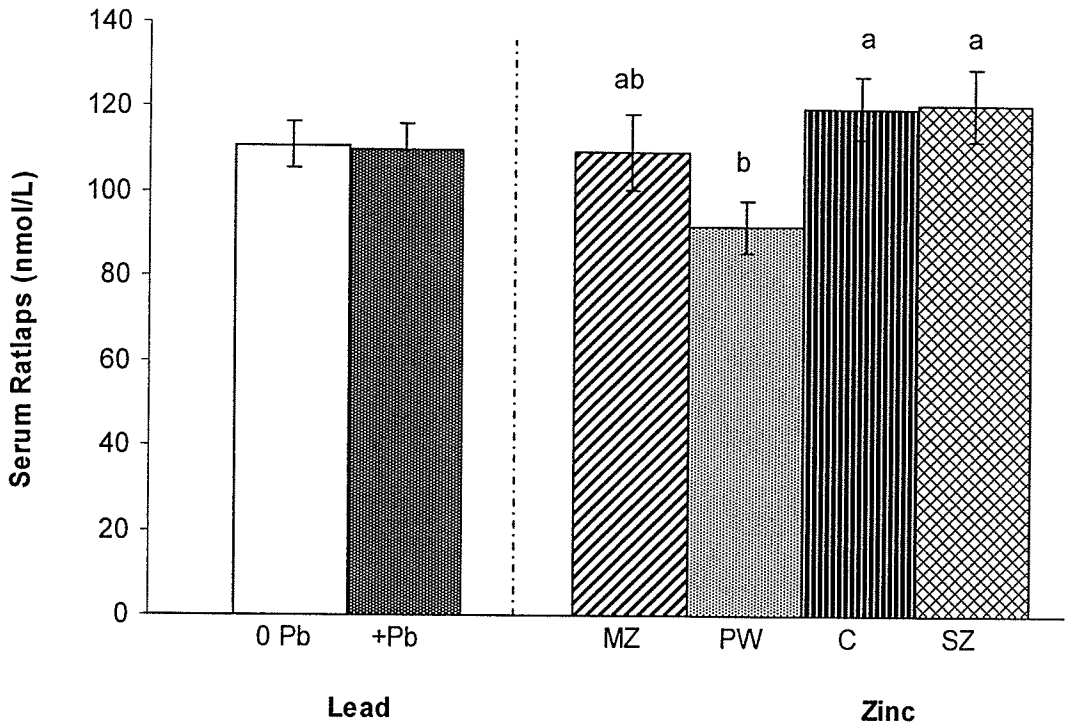


Figure 60. Effect of dietary zinc and lead exposure on serum Ratlaps concentration. Columns represent group means ± SEM for n=32 (lead effect) and n=16 (zinc effect). The lead effect was not significant (p=0.9017). The main effect of zinc was significant (p=0.0394), with different letters indicating means are significantly different, as determined by Duncan's multiple range test. The interaction of lead and zinc was not significant (p=0.2433).

V. DISCUSSION

Growth

Throughout the dietary treatment phase of the study, the adequately nourished C groups and the SZ groups experienced rapid growth and development as demonstrated by greater body, kidney, intestine, femur, and epididymal fat pad weights than the MZ groups (Figures 6, 25, 30, 45, 12) and greater body, liver, and femur weights than the PW groups (Figures 6, 22, and 45). Of note, liver weight and body weight gain (Figures 22 & 9) were the only parameters in which the SZ groups had a higher weight than the C groups. So although body weight was not higher in SZ animals than C, total body weight gain over 3 weeks was greater with SZ treatment, suggesting that zinc supplementation stimulated growth.

In comparison, the MZ and PW groups displayed growth retardation. A reduction in body weight in the MZ treatments was first evident on Day 10. Therefore, dietary restriction of the PW groups commenced on Day 11 of the study. Final body, organ, femur, and epididymal fat pad weights were not significantly different between the MZ and PW groups (Figures 6, 22, 25, 30, 45, & 12). However, femoral and epididymal fat pad weight as a percentage of body weight, was significantly lower in the MZ groups than PW, C, and SZ (Figures 45 & 12). Body, kidney, intestine, femur, femur to body weight ratio, epididymal fat pad weight, and epididymal fat pad to body weight ratios of MZ animals were 10%, 25%, 12%, 6%, 25%, 17%, and 17%, respectively, of the C animals.

Reduced epididymal fat pad weight is characteristic of energy malnutrition. MZ rats appeared to have a greater loss of adiposity than PW rats. Though there was no difference in epididymal fat pad weight between these groups, MZ rats had a lower fat pad weight to body

weight ratio (Figure 12), indicating a more severe response. In contrast, weanling rats pair-fed for both 16 days and 3 weeks showed the same reduction in epididymal fat pad weight and epididymal fat pad to body weight ratio as severely zinc deficient rats (Szczurek, 2000; Prescod, 1998). However, in this study the PW rats were only diet restricted for the final 12 days of the study, rather than the full 3 weeks of treatment. Additionally, the growth inhibition resulting from a marginally zinc deficient diet was much less pronounced than a severely zinc deficient diet.

Although organ and femur weights were not different between MZ and PW groups, femur weight as a percentage of body weight was lower in MZ animals (Figure 45). This suggests that skeletal growth was more depressed by marginal zinc deficiency than dietary restriction. In addition, kidney and intestinal weights were not different between PW and C groups (Figures 25 & 30), as they were between MZ and C groups, also indicating a more severe response with MZ treatment. In contrast, liver weight was lower in PW rats than C, but there was no difference between C and MZ rats. This finding suggests that dietary restriction had a more profound effect on liver growth than marginal zinc deficiency.

In comparison to a severe zinc deficiency, the growth suppression seen in the MZ-fed rats in this study was minimal. Weanling, SD, male rats fed <1 ppm zinc for 3 weeks achieved a final body weight of only 147 ± 2 g compared to the *ad* libitum-fed control group (30 ppm zinc) that weighed 289 ± 6 g, a reduction of 49% (Hosea et al. 2003). In contrast, the same age, sex, and species of rats fed 8 and 30 ppm zinc in this study weighed 255 ± 5 g and 272 ± 8 g, respectively, or a reduction of only 6% (Figure 6). While severely zinc-restricted rats (<1 ppm zinc) have decreased feed intake within days and stop growing after the first week (Hosea et al. 2004), the MZ rats in this study (8 ppm zinc) had a reduced growth rate during week 2 but were able to recover

during the third week to a level not significantly different from C and SZ rats (Figure 11), suggesting a metabolic adaptation to the low zinc diet or reduced zinc requirements for growth with age. Additionally, while the feed efficiency of the PW rats was considerably lower than all other treatments (32-39%) by the end of the study (Figure 10), MZ-fed rats were able to maintain their feed efficiency at a rate equivalent to the C and SZ rats. In contrast, severely zinc deficient rats reportedly have a lower feed efficiency than their pair-fed controls (White, 1988). However, Prescod (1998) also a reported a 13% lower feed efficiency in pair-fed rats than control rats, with no difference between control and severely zinc deficient animals.

These findings are in accordance with previous studies such as by Williams & Mills (1970) who fed 30 male weanling rats 3, 6, 9, or 12 ppm zinc diets for 35 days. At 3 ppm zinc, growth was sharply depressed by day 7 but rats continued to grow extremely slowly over the next 4 weeks (~10 g total weight gain). Within the range of 6-12 ppm zinc, a close relationship was found between the rate of weight gain and the dietary zinc concentration. However, the 12 ppm zinc diet resulted in a weight gain similar to the control (83 ppm zinc), with only the 6 and 9 ppm zinc diets showing a decrease in weight by day 14. The weight gain with the 9 ppm zinc diet was greater than the 6 ppm diet at 21, 28, and 35 days. Additionally, two recent studies have reported no difference in feed intake or weight gain in female SD rats fed a 10 ppm zinc diet for approximately 3 months (Chowanadisai et al. 2004; Kelleher & Lönnerdal, 2003). Thus, a diet of 9 ppm zinc appears to be sufficiently low in zinc to produce a growth-limiting zinc deficient state in growing male rats.

El-Gazzar et al (1978) fed weanling, male SD rats a 5 or 50 ppm zinc diet for 140 days and found a slower growth rate with the low zinc diet in the initial stages of the study. Interestingly, by the end of the study the low zinc fed rats caught up to the control rats in body weight, suggesting

that the requirement for zinc is higher in the earlier stages of life (El-Gazzar et al. 1978).

In contrast, the study by Cerklewski & Forbes (1976) which was based on a similar design as the present study, found no significant difference in weight gain among weanling, male SD rats fed 8, 30, or 200 ppm zinc with and without 200 ppm lead for 3 weeks (mean weight gain 115 ± 1 g). In comparison, 3 week weight gain in the present study ranged from 121 ± 3 g to 178 ± 4 g and was significantly affected by both lead and zinc (Figure 9). Although this study used a higher level of zinc supplementation (300 ppm vs. 200 ppm), the C groups (30 ppm zinc) also had a higher weight gain than the MZ and PW groups. This discrepancy may be attributed to the stricter attention paid to avoid environmental zinc contamination of the MZ groups in the present study, especially with respect to diet preparation and feeding practices. In addition, the Cerklewski & Forbes (1976) study did not confirm a marginal zinc status in the 8 ppm zinc-fed animals by serum or bone zinc analysis.

Lead exposure also reduced body weight (Figure 6). As a main effect, lead treatment resulted in lower body weight (6%), liver (7%), kidney (11%), femur (6%), and epididymal fat pad weight (10%), compared to non-lead treated groups. However, organ, femur, and epididymal fat pad weight, as a percentage of body weight, were not different between the lead and non-lead treatments (Figures 22, 25, 45, & 12), suggesting that the loss in tissue weight was directly proportional to the loss in whole body weight.

Lead has been found to alter the feed intake pattern of weanling rats, independently of nutrient intake (Minnema & Hammond, 1994). In a 10 day study, weanling rats fed a nutritionally complete diet and drinking water containing 250 ppm lead acetate were found to have decreased body weight, tail length, and cumulative feed intake compared to control animals. In the initial 6

days, lead exposed rats reduced the size and duration of each meal consumed during the dark phase. After 6 days, meal size and duration returned to control values but the number of nocturnal meals was decreased so that the total daily feed intake remained depressed (Minnema & Hammond, 1994).

While there were marked effects of zinc deficiency, dietary restriction, and lead exposure on growth, there were no interactions between lead and dietary zinc on any ponderal growth outcome measured in the present study. This may be due to several factors including: growth suppression resulting from different mechanisms, the attainment of maximal growth suppression at these particular levels of lead and zinc, or insufficient power in the experimental design to detect an interaction. Of note, the lead dose and dose per gram of body weight were not different between treatment groups (Figure 8). These findings agree with the results of a similar study by Bushnell & Levin (1982) in which growing, male SD rats were fed 2 or 20 ppm zinc and 0 or 100 ppm lead for 3 weeks. While zinc deficiency and lead exposure both suppressed growth, the interaction was not statistically significant (Bushnell & Levin, 1982).

In summary, marginal zinc deficiency, dietary restriction, and lead exposure all moderately depressed the growth of weanling rats in this study. The effects of zinc deficiency and lead exposure on growth were not additive. MZ treatment was more detrimental than diet restriction alone in terms of loss in adiposity and femoral weight. Zinc supplementation had a stimulatory effect on weight gain over the course of the study.

Zinc Status

Zinc status was assessed through serum and femur zinc concentration. Serum zinc can

not be used alone as an indicator of whole body zinc status, as it is amenable to stressful conditions such as starvation and is only reflective of extreme intakes of dietary zinc (Vallee & Falchuk, 1993; Blanchard and Cousins, 1996). Consequently serum zinc may not be an accurate and reliable reflection of true zinc status. Fortunately, bone contains a metabolically active zinc pool in rats and is a sensitive and useful marker of dietary zinc intake (Blanchard and Cousins, 1996). The marked reductions in serum and femur zinc in the MZ rats of the present study (Figures 13 & 14) confirmed that the experimental diets produced at least a marginal to moderate zinc deficiency. Serum and femur zinc were 57% and 62% lower, respectively, in MZ rats than C rats. In addition, dietary restriction did not affect either indicator of zinc status. These results are in accordance with the Sczcurek (2000) study, in which severely zinc deficient rats had 77% and 71% lower serum and femur concentrations, respectively, than *ad libitum* fed control animals. However, the degree of change in serum zinc is somewhat surprising, as a 3 week study in weanling, SD rats fed 7 ppm zinc only resulted in a 17% lower serum zinc concentration (Hall et al. 2005). The Hall et al (2005) study also reported a 60% lower femoral zinc concentration, which does agree with the present results. The release of zinc from bone during periods of inadequate dietary intake is thought to be a homeostatic response to provide zinc for use to other tissues or a decline in zinc uptake by bone, in response to low plasma zinc (King et al. 2000).

In addition to the response to zinc restriction, there was an effect of zinc supplementation on zinc status. SZ rats had 27% and 26% higher serum and femur zinc concentrations, respectively, than C rats (Figures 13 & 14), suggesting increased zinc absorption and tissue retention. These results are not surprising in light of the amount of zinc fed. The 300 ppm zinc diet is 10 times the zinc requirement for growing rats (Reeves et al. 1993). Reeves (1995) also

reported a significant increase in serum zinc concentrations of rats chronically fed 350 ppm zinc diets. Serum zinc remained elevated for 28 days and then declined to a level not different than the control animals (Reeves 1995). Femur zinc has been shown to reflect zinc supplementation in rats, as well. Growing, SD rats receiving 0, 160, 320, and 640 ppm zinc diets over a 3 month period showed a corresponding increase in femoral zinc concentration, although it appeared to plateau at the 320 ppm dose (Llobet et al. 1988).

There was also an interaction between lead and dietary zinc in terms of femoral zinc concentration (Figure 14). Lead exposure resulted in lower femoral zinc concentrations by 8-11% in PW, C, and SZ rats. This trend was also seen in the MZ rats, although the effect did not reach significance. This effect may be due to a re-distribution of zinc in response to lead intoxication. Zinc is thought to be protective against lead absorption at the gastrointestinal level (Hempe and Cousins, 1992). Therefore, zinc may be released from the metabolically active zinc pool and re-directed to the intestine, or more critical sites, as a protective mechanism to impede lead absorption and toxicity. However, lead treatment did not result in an increase in duodenal or jejunal zinc concentrations so this effect may also be related to zinc excretion and/or a re-distribution of zinc to alternate sites. The lack of an effect in the MZ rats may be due to the already limited supply of zinc in these animals, which would hinder their ability to re-distribute zinc among body tissues.

Lead Status

Lead status was assessed by femoral and renal lead concentration, as well as hepatic ALAD activity. Serum lead concentration was undeterminable due to the lack of access to

equipment sensitive enough for detection. Lead was undetectable in the femurs and kidneys of all non-lead exposed treatment groups, confirming the absence of any significant lead contamination in these animals.

Renal lead concentration was assessed as the kidney is the primary site of accumulation following lead exposure. The half-life of lead in the blood and soft tissues is approximately 28-36 days, after which it is primarily deposited in bone (WHO, 1995). Renal lead concentration decreased as dietary zinc increased (Figure 16), with the SZ rats having 57% less lead present than the MZ rats. Renal lead concentration of the PW group was intermediate to the MZ and C rats, but not significantly different from either. This is likely related to the dietary restriction of the PW rats, as lead is absorbed more readily across the gastrointestinal tract when food is not present (WHO, 1995).

Femoral lead concentration was assessed as bone is the long term storage site for lead in the body and is therefore reflective of whole body lead burden (Peraza et al. 1998). Femoral lead concentration was markedly affected by dietary zinc treatment (Figure 15). The MZ animals had more than 7 times the lead concentration of SZ animals, while C rats had almost 3 times as much lead as SZ rats. Thus, having adequate and excess zinc was highly protective against lead accumulation in the skeleton. This is an important finding, as skeletal lead can be mobilized during periods of increased calcium demand and bone remodelling, such as growth and pregnancy. Once the lead is mobilized to the blood, it is able to act on sensitive targets within the body such as the nervous and haematopoietic systems (Peraza et al. 1998). Also of note, there was no significant difference between PW and C rats in terms of femoral lead concentration, suggesting that the dietary restriction employed in the present study did not increase whole body lead burden.

Hepatic lead concentration (Figure 24) followed the same pattern as seen with renal lead concentration (Figure 16). However, the liver contained much less total lead content, as well as content per gram of tissue, than the kidney. Overall, the decrease in tissue lead concentrations seen in the femur, kidney, and liver with the adequate and supplemental zinc treatments suggests either a decrease in lead absorption at the gastrointestinal level or an increase in lead excretion. Although urinary lead excretion was not measured in the present study, Cerklewski and Forbes (1976) reported no effect of 8, 30, and 200 ppm zinc diets on lead excretion over a 3 week period in growing, male, SD rats.

To further examine the relationship between dietary zinc and tissue lead retention, tissue lead content and concentration were calculated, as a percentage of the sum of tissue lead measured in the femur, kidney, liver, and intestine (Figure 17). The effects of dietary zinc were similar in terms of lead content and concentration, although the magnitude of the effect on the intestine was much greater when expressed as total lead content. As dietary zinc increased, the percentage of total tissue lead found in the femur markedly dropped. At the same time, the percentage of total tissue lead found in the intestine markedly rose. This suggests a protective effect of zinc against body lead accumulation and an increase in intestinal lead accumulation, which may be due to retention of lead in the enterocyte as a means of elimination from the body. In comparison to the intestine and femur, the percentage of change in total tissue lead in the kidney and liver were not greatly affected by dietary zinc.

Hepatic ALAD activity was also assessed as an indicator of lead intoxication. ALAD is a cytosolic enzyme that catalyzes the second step of heme biosynthesis, and is reflective of intracellular lead toxicity with a high degree of sensitivity and specificity (Goering and Fowler,

1985). The Cerklewski & Forbes (1976) study reported significant inhibition of renal ALAD in growing rats exposed to 200 ppm lead for 3 weeks. In addition, hepatic ALAD is reportedly much more sensitive to inhibition by lead than renal ALAD (Goering and Fowler, 1985). However, in the present study hepatic ALAD was not significantly inhibited by lead (Figure 18). This finding may be due to a higher than expected inter-individual variation among the animals in each treatment group and/or insufficient power in the experimental design.

Despite the lack of inhibition of hepatic ALAD, effects of lead intoxication were seen in terms of growth inhibition (Figures 6, 7 & 9) and confirmed by tissue lead concentrations (Figures 15, 16, & 24). Also of note, hepatic ALAD was reduced in MZ-fed rats in comparison to all other treatments (Figure 18). This finding is supportive of the poor zinc status of these rats, as ALAD is a zinc-dependent enzyme.

Micronutrient Assessment

Hematocrit Analysis

Hematocrit was measured at termination as an indicator of anemia, since a lead-induced depression of hematopoiesis or an interaction with iron and/or copper absorption or metabolism in the SZ treatments could result in anemia. Acute lead toxicity has been shown to produce a microcytic, hypochromic anemia by interfering with iron and copper metabolism and may also decrease hematocrit and hemoglobin levels (Mugahi et al. 2003). However, there were no differences in hematocrit among any of the dietary treatments (Figure 19), suggesting that the lead and high zinc treatments were not severe enough to produce anemia in a 3 week period.

Dietary Zinc & Copper Status

Zinc, as opposed to lead and other metals, is considered to be relatively non-toxic as it does not accumulate within a permanent body reserve (Calesnick & Dinan, 1988). Although zinc concentrations at or in excess of 240 ppm can induce symptoms of copper deficiency (L'Abbé & Fischer, 1984a;1984b), death, anemia, anorexia, and reduced growth rates are only found at concentrations of 5000 ppm or more (Hambidge,1985).

Pharmacological doses of zinc (from 30:1 to 100:1, with respect to copper intake) are thought to inhibit intestinal copper absorption via zinc-induced intestinal MT, which preferentially binds copper (Linder & Hazegh-Azam, 1996). Zinc supplementation at 240 ppm for 2 weeks has been shown to produce biochemical signs of copper deficiency in weanling rats, including reduced activity of the copper-requiring enzymes superoxide dismutase, ceruloplasmin, and cytochrome c oxidase in the liver, serum, and heart, respectively (L'Abbe & Fischer, 1984b). In addition, hepatic copper concentration was reduced by 27% compared to the control, although not to the same degree (50% reduction) as seen with a copper deficient diet (0.6 ppm copper). These findings suggest both a reduction in copper reserves as well as functional activity at dietary levels of 8 times the recommended intake in rats (30 ppm zinc). However, other studies in this area have produced equivocal results. Reeves et al (1995) fed 8 week old SD rats a 350 ppm zinc diet for 5 weeks and found no evidence of declining copper status, in terms of serum, hepatic, or renal copper concentrations. Various factors such as the relative copper and zinc concentrations of the diet, the overall diet composition, the age of the animals in each study, and the duration of the studies likely contributed to the disparity in results.

In the present study, SZ treatment (300 ppm zinc) resulted in 32%, 37%, and 30% lower

hepatic copper concentrations than C, PW, and MZ groups, respectively (Figure 21). These results are in accordance with the L'Abbe & Fischer study (1984b); less severe than a copper deficient diet but more severe than 240 ppm zinc for 2 weeks. Saari (2002) reported that weanling, male SD rats fed a 0 and 1.5 ppm copper diet had a 91% and 23% reduction in liver copper concentration, whereas diets of 3 ppm and 4.5 ppm copper were not significantly different from the control (6 ppm copper) in terms of hepatic copper. Thus, the hepatic copper concentrations that the SZ diet produced indicates at least a moderate degree of copper deficiency. However, copper deficiency also results in microcytic, hypochromic anemia and hepatic accumulation of iron (Lönnerdal, 1996), which were not found with the SZ groups in this study (Figure 20). The sequestration of iron within the liver is the result of either dietary or genetic ceruloplasmin deficiency (Linder & Hazegh-Azam, 1996). Ceruloplasmin is a copper-dependent enzyme responsible for the transport of hepatic copper to the tissues, as well as the acute phase response and oxygen radical scavenging (Linder & Hazegh-Azan, 1996). This reduction in hepatic iron flow is thought to contribute to the anemia found with copper deficiency, although impaired heme production and other factors likely play a part in this response as well (Linder & Hazegh-Azan, 1996). In addition, a recent study in weanling, male Wistar rats found an improvement in copper status in rats fed 240 ppm zinc in comparison to rats fed 60 or 120 ppm zinc (Iskandar, et al. 2005). This improvement was directly correlated to the up-regulation of several intestinal zinc transporters, including MT, ZnT-1, and ZnT-2, which may mediate the antagonism between zinc and copper at high dietary intakes (Iskandar, et al. 2004). Thus, the degree of interference with copper absorption likely depends on animal age, whole diet composition, and length of the feeding trial (Reeves, 1995).

Lead Exposure & Copper Status

Also of interest, lead treatment resulted in a modest decrease (13%) in hepatic copper concentration (Figure 21). Several published studies in the past have documented clear copper-lead interactions (Petering, 1980). In general, copper deficiency has been shown to aggravate lead toxicity while increased dietary copper was found to be protective (Petering, 1980). Additionally, lead administration reportedly decreased both serum and tissue copper concentrations (Petering, 1980), which is in agreement with the hepatic copper concentrations found in this study.

However, a similar study by Cerklewski & Forbes (1977) reported contradictory findings with respect to lead toxicity and dietary copper. Weanling, male, albino rats were fed 1, 5, or 20 ppm copper with and without 200 ppm lead for 4 weeks. Interestingly, the severity of lead toxicity increased as dietary copper increased, as evidenced by increased urinary ALA excretion and increased renal lead deposition. The disparity in results was explained by the authors through differences in experimental protocol between the studies previously described (Petering, 1980), including a higher lead dose (5000 and 500 ppm vs. 200 ppm), longer study period (8 weeks vs. 4 weeks), and variable dietary components such as manganese. It would appear that the role of copper in lead toxicity, the interaction of copper and zinc, and their effect on lead in the body are in need of further investigation.

To the best of my knowledge, the effect of lead on whole body copper status has not been studied. Lead may be decreasing copper absorption through competition at the gastrointestinal level or causing a re-distribution of copper within the body stores. From the limited evidence available, lead does appear to decrease mineral availability within the body.

Lead Exposure & Iron Status

Interactions between lead and iron metabolism have been previously described (Mahaffey, 1981). For example, iron-deficient rats consuming water with 200 ppm PbAc have elevated renal and femur lead concentrations when compared to rats on the same lead treatment with an iron-adequate diet (Mahaffey & Rader, 1980). Conversely, iron supplementation at only 3 times the recommended level in rats significantly decreased renal, femur, and blood lead concentrations over a 10 week study period (Mahaffey & Rader, 1980).

Liver iron concentration is generally considered a reliable index of nutritional status for iron (Mahaffey & Rader, 1980). In the present study, hepatic iron concentration was not affected by marginal zinc, supplemental zinc, or dietary restriction (Figure 20). However, lead exposure resulted in higher hepatic iron concentrations at all levels of dietary zinc, with an overall increase of 16%. Intraperitoneal lead administration has been shown to increase hepatic iron by as much as 24% (Tandon et al. 1994). This response was attributed to an increased iron uptake or retention upon exposure to lead, suggesting that iron uptake may accompany lead absorption through a common gastrointestinal pathway (Tandon et al. 1994). In fact, recent cell culture work has revealed that the major intestinal ferrous iron transport protein, DMT1, transports both iron and lead with similar affinity in yeast cells (Bannon, 2002). In addition, the transport of lead was completely inhibited in the presence of 25-fold iron. Interestingly, lead transport by DMT1 may also explain the correlation between low dietary iron intake and increased lead absorption in animal models, as DMT1 mRNA is sharply up-regulated when an iron-deficient diet is provided (Bannon et al. 2002). Increased lead absorption may be the result of increased DMT1 expression or the lack of dietary iron to compete with lead for transport (Bannon et al. 2002). Although there is indirect evidence of

zinc transport by DMT1, zinc and iron are not thought to compete for DMT1. Enhanced DMT1 expression and the functional disruption of DMT1 do not affect zinc absorption (Kordas & Stoltzfus, 2004). Two recently identified families of zinc transporter proteins are now thought to be responsible for cellular zinc influx and efflux. There is no evidence that these zinc transporter proteins are involved in iron uptake and/or transport, but competitive inhibition between these two minerals may also be occurring at a post-absorptive stage (Kordas & Stoltzfus, 2004). At the present time there are no published studies on the capacity of zinc transport proteins to transport lead.

Zinc and Lead Concentrations

Kidney

Renal zinc concentration was affected by both MZ and SZ treatment in this study (Figure 26). MZ-fed rats had a 17% lower renal zinc concentration than C rats. While still a significant reduction, it is considerably small in comparison to the 70% reduction reported in weanling, male SD rats fed <1 ppm zinc for 15 days (Szczurek, 2000). Renal zinc concentrations of PW and C rats were not different, suggesting that malnutrition was not a factor in renal zinc accumulation. This is also in agreement with the Szczurek (2000) study. SZ-fed rats had a slightly, but significantly, higher renal zinc concentration (6%) than C rats, implying that the SZ treatment increased zinc absorption and/or renal accumulation. Similarly, Reeves (1995) found that kidney zinc concentrations were elevated in rats fed 350 ppm zinc diets from day 3 until day 28, after which they declined to the same level as control rats. Of note, on day 21, renal zinc concentrations were elevated by approximately 7-8% in comparison to control rats.

Renal zinc concentration was also 5% lower in lead treated animals than in animals not

exposed to lead (Figure 26), which is in agreement with previous studies (Ashraf & Fosmire, 1985; Mahaffey, et al. 1981), although the biochemical and physiological consequences of this effect are not known. This response may be due to a redistribution of zinc among the tissues or an increase in zinc excretion, as a 200 ppm lead treatment has been shown to increase zinc excretion by more than 2-fold (Victery et al. 1987). However, in the Victery et al (1987) study, a drop in tissue zinc was only seen in bone and to a lesser degree in the testes. There was no decrease in kidney, liver, brain, pancreas, plasma, or RBC zinc concentration. Zinc homeostasis in these tissues is likely maintained at the expense of the labile zinc pool present in bone (Victery et al. 1987). This study differs from the present study in that the diet was based on the AIN-76 recommendations and tap water, rather than deionized water, was provided to the animals. Of note, lead and zinc excretion were found to be highly correlated, suggesting competition for a common re-absorptive or secretory pathway along the renal tubule (Victery et al, 1987).

It has been well documented that the kidney accumulates the highest concentrations of lead present in soft tissues (Ashraf & Fosmire, 1985; Kishi et al. 1983; Miller & Massaro, 1983), a finding also confirmed by the present study. The kidney had higher a lead concentration and higher lead content than the liver (Figure 17). In terms of dietary treatment, MZ-fed rats had 35% higher renal lead concentrations than C rats, whereas SZ-fed rats had 33% lower renal lead concentrations than the C (Figure 16). However, the renal lead concentrations of MZ and PW rats were not significantly different, suggesting that the lead accumulation in soft tissues may be at least partially due to undernutrition.

Liver

Hepatic zinc concentration was not strongly affected by dietary zinc in the present study. MZ-fed rats had only a 10% lower hepatic zinc concentration in comparison to PW rats, although the PW and MZ rats were not different than either the C or SZ rats (Figure 23). Other studies have reported that pair-fed rats have significantly higher hepatic zinc concentrations than both zinc deficient and control rats (Szczureck et al. 2000; Wallwork et al. 1981). However, these animals were pair-fed to rats on <1 ppm zinc diet, which is a much more severe restriction than the conditions employed in the present study. Dietary restriction, as seen with pair-weighing and pair-feeding, results in periods of fasting. Fasting triggers a transfer of zinc from the peripheral tissues to the liver (Panemangalore & Bebe, 1996) and therefore likely is responsible for these results.

Hepatic zinc concentration is thought to respond to short term rather than long term changes in dietary zinc, in young growing rats. Wallwork et al (1980) have shown that plasma and hepatic zinc vary with the cyclic feeding pattern of severely zinc deficient rats, but stabilize over a 15 day period of zinc deficiency. The period of marginal zinc deficiency in this study was 22 days, thus the hepatic zinc concentration was likely stabilized by this time.

In contrast to hepatic zinc concentration, dietary zinc had a profound effect on hepatic lead concentration. MZ-fed rats had a 52% higher hepatic lead concentration than C animals (Figure 24). Furthermore, SZ-fed rats had a 63% lower hepatic lead concentration than C animals, indicating that the SZ diet was more protective than an adequate zinc diet in terms of hepatic lead accumulation. Similar to the situation with the kidney, hepatic lead concentration was not different between the MZ and PW groups, suggesting that malnutrition may be involved in the greater lead accumulation, rather than zinc deficiency *per se*. Although, the liver is not a target site for lead

accumulation in the body, lead can remain in the blood and soft tissues for several weeks before it is deposited in bone (WHO, 1995).

Intestine

In contrast to a previous study in the same lab, dietary zinc had a pronounced effect on both duodenal and jejunal zinc concentration. MZ-fed rats had 30-36% lower intestinal zinc concentrations than C rats, while SZ-fed rats had 36-38% higher intestinal zinc concentration (Figures 32 & 34). Therefore, zinc concentration in the intestine appeared to be responsive to dietary zinc levels in this study. As the marginal zinc deficiency in this study was less severe than the <1 ppm zinc diet used in the Szczurek (2000) study, this discrepancy is surprising. However, the Szczurek (2000) study lasted for 15 days whereas the present study was 22 days long. As intestinal zinc concentration is not commonly reported in zinc deficiency and supplementation studies, comparison of these results to other work is limited.

In contrast to the renal and hepatic zinc results, PW rats had similar intestinal zinc concentrations as C rats and the concentration was significantly higher than MZ-fed rats. This suggests that the effect of marginal zinc deficiency on intestinal zinc concentration was not due to undernutrition, but rather was a direct or indirect consequence of zinc deficiency *per se*.

Despite, the pronounced effect of dietary zinc on intestinal zinc concentration, there was no effect of dietary zinc on intestinal lead accumulation (Figures 33 & 35). While there was a trend towards greater intestinal lead concentration with increasing dietary zinc, the variability within the treatments was too high for the results to achieve statistical significance. At the present time, the intestinal lead concentration of lead-exposed rats appears not to have been reported.

Femur

As previously discussed under Zinc Status, femoral zinc concentration was reflective of dietary zinc intake. MZ-fed rats had a mean of 62% less femoral zinc than C rats (Figure 14), which was on par with the 70% lower femoral zinc in severely zinc restricted rats of the same age and sex, in comparison to their controls, reported by Hosea et al (2004), as well as other studies (Zhou et al. 1993; El-Gazaar et al. 1978) Additionally, SZ-fed rats had a mean of 26% higher femoral zinc concentration than C rats in the present study.

Femoral lead concentration was also reflective of dietary zinc intake, but the lead concentration was reduced as dietary zinc increased, as discussed under Lead Status. MZ-fed rats had 62% higher femoral lead concentration than C rats, while the SZ treatment resulted in a 65% lower femoral lead concentration (Figure 15). Therefore, the SZ treatment was more effective than the adequate zinc diet in reducing femoral lead deposition. Furthermore, as lead deposition was also reduced in the liver and kidney with SZ treatment and there was no change in the intestine, it seems likely that there was a decrease in lead absorption at the gastrointestinal level and not simply a redistribution of lead among the tissues.

Feed restriction has been shown to increase the retention of orally ingested lead in rats (Quarterman et al. 1976). Therefore, the use of pair-fed or PW animals is important in order to determine the relative importance of malnutrition and zinc deficiency in this type of study. Previous rat studies examining the interaction of dietary zinc and lead toxicity have either not reported or not controlled for the effect of malnutrition. In terms of femoral lead concentration, malnutrition did not have an effect (Figure 15), as the PW and C groups were not different from each other. However, in the case of renal and hepatic lead, the PW rats had tissue lead concentrations that were

intermediate to the MZ and C rats (Figures 16 & 24), suggesting that the reduction in feed intake and body weight played a role in organ lead deposition. However, soft tissue lead levels are transient whereas bone lead is indicative of whole body lead burden. In fact, the human skeletal system stores over 95% of the lead body burden of adults and may provide an index of the cumulative effects of lead exposure over extended periods of time (Peraza et al. 1998). Therefore, the effect of dietary zinc and dietary restriction on bone lead concentration is a more meaningful outcome in terms of long term health than soft tissue lead concentrations.

Intestinal Metallothionein & Cysteine-rich Intestinal Protein mRNA

There was no effect of lead treatment on MT mRNA expression in the intestine (Figure 41). While previous studies have observed hepatic, but not renal, MT induction and low affinity binding of lead to MT, the intestinal response was either not measured or reported (Nolan & Shaikh, 1992). However, the ability of lead to induce hepatic MT protein synthesis *in vivo* has been modest (Waalkes, 1984). The lack of a transcriptional response to lead in the intestine was reflected in MT immunostaining (Figures 36 & 37), as lead treatment did not affect the amount or distribution of MT. MT genes are transcriptionally regulated by metals, including zinc and cadmium. Transcription is stimulated, in the case of zinc, by occupancy of MTF-1, which binds specifically to a MRE sequence in the MT gene promoter (Davis & Cousins, 2000). Therefore, it would seem that lead does not bind to the MRE of MT, through MTF-1 or another transcription factor and does not induce MT synthesis.

Conversely, MT expression was responsive to dietary zinc. MT mRNA levels were lower in MZ rats than all other treatments in both the duodenum and jejunum (Figures 41 & 43), reflecting the low zinc status of these animals. MT synthesis is down-regulated during zinc deficiency to

enhance absorption (Davis & Cousins, 2000). This result is supported by previous findings in which severely zinc deficient rats (<1 ppm zinc) displayed a 57% lower intestinal (jejunal) MT concentration than *ad libitum*-fed controls (Szczurek, 2000). In comparison, MT mRNA levels were 67-89% lower in MZ rats than C rats in the present study.

Dietary restriction had no effect on intestinal MT transcription. Severe dietary restriction is known to induce MT synthesis, however, the degree of restriction in the present study was not severe. Therefore, a strong effect on MT synthesis was not expected. Zinc supplementation resulted in higher levels of MT mRNA compared to control animals, although the effect only reached significance in the jejunum. MT is thought to be induced during periods of excess intake, as a means of limiting absorption (Davis & Cousins, 2000). However, there appears to be a metabolic adaptation to chronic, high doses of zinc over time, allowing for the down-regulation of MT synthesis. Reeves (1995) fed 8 week old SD rats either the AIN-93G diet as a control or a modified diet with 350 ppm zinc for 3, 7, 14, 21, 28, 35, and 42 days. The positive induction of MT by the high zinc diet was found to only last for the initial 14 days of the experiment, after which concentrations fell to that of the control by day 28 (mRNA levels were not measured). Thus, during chronic high zinc consumption, there is likely an alternative mechanism to reduce the amount of zinc present in the body that does not involve MT (Reeves, 1995). Reeves (1995) speculated that the mechanism of this response is likely an increased rate of elimination via increased intestinal zinc secretion and/or increased rate of intestinal cell turnover. However, decreased zinc absorption and/or increased elimination may also be responsible (Reeves, 1995). In the present study, MT induction by SZ was still evident after 21 days, at least in the jejunum.

Of note, the higher levels of MT transcription in PW, C and SZ animals, compared with MZ,

were reflected by MT immunostaining in the intestine, although the magnitude of differences among these three treatments was moderate (Figures 36 & 37).

These results confirm a previous study with growing rats fed 5, 30, and 180 ppm zinc, which showed, by qualitative northern blot analysis, MT transcription to be proportional to zinc supply in the kidney and liver, and to a lesser degree in the intestine, with much lower band intensities seen with the low zinc treatment (Cousins & Lee-Ambrose, 1992).

Intestinal CRIP mRNA levels were not affected by dietary zinc, lead, or their interaction (Figures 42 & 44). Although it had been suggested that CRIP may be involved in heavy metal detoxification (Fernandes et al. 1997) and zinc absorption (Hempe & Cousins, 1992), there was no evidence of involvement in either role in the duodenum or jejunum. Unfortunately, it was not possible to visualize intestinal CRIP levels by immunohistochemistry in the present study.

Metallothionein Immunostaining & Zinc Concentrations

Duodenum & Jejunum

Immunolocalization of MT in the duodenum and jejunum were similar in terms of intensity and distribution among the various treatments, with the exception that villi epithelial staining tended to be stronger in the duodenum than in the jejunum (Figures 36 & 37). While there were obvious differences in MT immunostaining between low and adequate or high dietary zinc, there was no apparent effect of lead.

MT immunostaining was weak to nil in the MZ and MZ+Pb groups and only found in the Paneth cells of the intestinal crypts (Figures 36, 37 & 38). This is reflective of the low zinc status of these animals and is on par with the Sczcurek (2000) study, which found no detectable MT

immunostaining in the intestine of severely zinc deficient rats. It is also in agreement with the lower zinc concentrations found in the duodenum and jejunum of MZ and MZ+Pb groups (Figures 32 & 34), as MT and zinc tissue concentrations are highly correlated (Davis & Cousins, 2000).

In all other treatments, MT immunostaining was strong to moderate in the Paneth cells of intestinal crypts and weak to nil in villi epithelial cells (Figures 36, 37 & 38), with one exception. The villi epithelia of SZ and SZ+Pb treatments had strong MT immunostaining in the duodenum (Figure 36). Thus, there was little variability between C rats and PW or SZ rats in this study, despite the fact that pair-weighing and zinc supplementation are known to induce MT synthesis. However, this is not that surprising as the pair-weighing restrictions employed were not that severe and consequently, the PW animals were likely not overly stressed. Also, the MT induction by zinc supplementation was likely higher during the initial days of the study, but after two weeks, there is an adaptation which allows for the down-regulation of MT synthesis. Despite this adaptation, jejunal MT mRNA levels were 62% higher in SZ rats than C rats (Figure 43). Therefore, either the increase in transcription may not have been proportional to the increase in protein synthesis or the immunohistochemical analysis was not sensitive enough to detect this difference.

Generally, MT was localized within the proliferative region of the villi including the crypt and basal portion of the villi, rather than the apical tip of the villi (Figures 36, 37 & 40). This supports previous findings (Sczcurek, 2000) and provides further evidence for the role of MT in cellular proliferation and mucosal turnover. In contrast, a direct role in zinc absorption does not appear likely, as MT was not concentrated along the apical tip of the villi. MT may also be functioning in short-term zinc storage in villi epithelia, as high dietary zinc is thought to induce MT, which then acts to sequester the excess zinc within the enterocyte (Hinskens et al. 2000). Additionally, MT

may function as a zinc reservoir to aid in homeostatic regulation, as MT-null mice are more sensitive to both zinc deficiency and toxicity (Kelly et al. 1996).

The response of MT in Paneth cells to changes in dietary zinc supply supports the role of MT in protection from zinc toxicity, and potentially a role in gut immunity. Paneth cells have been implicated in mucosal host defense, as well as an elimination route for heavy metals (Fernandes et al. 1997). However, there is no evidence within the present study to support a role for MT or Paneth cells in lead detoxification in the intestine. Additionally, the Paneth cell associated protein CRIP did not appear to be involved in lead absorption or detoxification processes.

Despite the lack of evidence linking MT and lead detoxification or elimination in the present study, the possibility of such a relationship has not been excluded. A recent study by Qu et al (2002) found sub-cellular changes in the kidneys of MT-null mice exposed to lead when histological sections were analyzed by electron microscopy, rather than light microscopy. This study will be further discussed in the following section.

Kidney

Immunolocalization of MT in the kidney followed a similar pattern in terms of intensity, as seen in the intestine. The response in MZ and MZ+Pb rats was moderate to weak in the renal cortex and pelvis, whereas PW, PW+Pb, C, C+Pb, SZ, and SZ+Pb rats showed a strong to moderate response (Figures 27 & 29). Of note, the PW and PW+Pb rats had a stronger response in the epithelial cells of the collecting ducts in the renal pelvis than the other treatments (Figure 28). In addition, the C and C+Pb rats had a stronger response in the epithelial cells of the proximal convoluted tubules in the renal cortex than SZ and SZ+Pb rats (Figure 27). All treatment groups

had some staining present in the lumina of the renal tubules (Figures 27-29). Lead treatment had no apparent effect on the intensity or distribution of MT at any level of zinc (Figures 27-28).

While there were obvious differences in staining intensity, there were no apparent differences in distribution among the various levels of dietary zinc. Staining was generally present throughout the entire cortex and into the medulla. This contradicts the Sczcurek (2000) study, in which the weak staining seen in severely zinc deficient rats was restricted only to the outer rim of the cortex. This difference likely reflects the moderate zinc deficiency state employed in the present study in comparison to severe zinc deficiency.

The presence of MT in renal tubular epithelial cells and the lumina of renal tubules is indicative of the participation of MT in zinc excretion and/or reabsorption. These observations are consistent with studies on rats treated with dietary zinc (Sczcurek 2000), as well as $CdCl_2$ (Danielson et al. 1982). The localization of MT within the epithelia of collecting ducts was also reported by Sczcurek (2000), although the reason for this observation remains unclear. The primary function of the collecting duct is osmotic equilibration of the ultrafiltrate, thus a potential role in zinc homeostasis is not clear.

Urinary zinc excretion accounts for only 2-10% of total endogenous zinc excretion, as the major route of excretion is through the gastrointestinal tract (King & Keen, 1999). In addition, urinary zinc losses tend to remain stable over a wide range of zinc intakes unless dietary zinc is severely restricted or given in great excess (King & Keen, 1999). Urinary zinc mainly originates from the ultrafilterable portion of plasma zinc through proximal tubule secretion (Vallee and Falchuk, 1993) and up to 95% of filtered zinc appears to be reabsorbed in the distal parts of the renal tubule (King & Keen, 1999). Thus, the strong concentration of MT in the proximal convoluted

tubules in C, C+Pb, PW, PW+Pb, SZ, and SZ+Pb treatments may be contributing to zinc secretion. The weak and limited MT staining in the distal convoluted tubules in the present study suggests that zinc reabsorption is also limited in these treatments or MT is not involved (Figures 27 & 28).

The lower intensity of renal MT immunostaining in MZ fed rats was reflective of the lower zinc concentration found in their kidneys (Figure 26). However, the SZ-fed rats had a higher renal zinc concentration than C rats, which was not reflected in MT staining intensity. This can likely be attributed to the adaptive response in MT expression seen after 2 weeks of dietary zinc supplementation discussed in the previous section (Reeves, 1995).

The moderate dietary restriction employed in the present study did not have a great impact on MT expression, as reported by Sczcurek (2000). Renal MT immonostaining intensity was greater in the renal pelvis in PW rats than C and SZ fed rats, although similar staining intensity was seen in the renal cortex among all of these treatments. This difference is also likely reflective of the less severe restrictions used in the present study than in the Sczcurek (2000) study.

As was found in the intestine, MT expression in the kidney did not appear to be involved in lead detoxification or elimination (Figures 27 & 29). However, the Qu et al (2002) study has recently implicated MT in a lead detoxification process. MT-null mice have an inability to form renal nuclear and cytoplasmic lead inclusion bodies, which are pathogenic features of lead intoxication (Qu et al. 2002). The inclusion bodies are lead-protein complexes, thought to be involved in the mitigation of lead toxicity (Qu et al. 2002). In addition to not forming inclusion bodies, MT-null mice were more sensitive to lead-induced nephromegaly and had depressed renal function compared to wild type mice. However, the mechanism of how MT may be involved in inclusion body formation is not currently known. If MT does contribute to the mitigation of lead toxicity on a sub-cellular level,

this was not detectable by the methodologies employed in the present study.

Skeletal Growth

Skeletal growth was assessed by DEXA scans of femoral and whole body skeletal area, as well as morphometric analysis and weight of excised femurs. Skeletal growth is a fundamental component and the principal stimulator of somatic growth (Ronis et al. 2001). Therefore, the changes in skeletal growth seen in this study are largely reflected in the changes in body weight previously discussed.

Skeletal growth was retarded by MZ deficiency and diet restriction (PW) in terms of dry femoral weight and whole body skeletal area (Figures 45 & 57). Femoral area was only lower in MZ-fed rats compared to C and SZ rats. This inhibition of skeletal growth ranged from 4-7%, whereas body weights were lower by 10-11% in comparison to C rats. Though there is little data available on marginally zinc deficient animals, severe zinc deficiency and pair-feeding result in smaller femurs that weigh less (Hosea et al. 2003; Rossi et al. 2001). Weanling, male, SD rats on a 1 ppm zinc diet for 28 days had lower mean values in ponderal growth rate, femur length and weight, serum IGF-1 concentration, breaking stress, and decreased growth plate thickness of the femur (Rossi et al. 2001). Morphometric abnormalities in the growth plate observed in zinc deficiency are thought to be due to the role of zinc in cell division, differentiation, and apoptosis and may also explain the inhibition seen in linear bone growth (Rossi et al. 2001). Rossi et al (2001) also speculated that the reduction in circulating IGF-1 may be the mechanism responsible for impaired growth plate activity. Thus, the results in the present study in terms of skeletal growth, while not as damaging as seen with models of severe zinc deficiency, do support the proposition that marginal zinc status and moderate dietary restriction are also of concern in growing animals.

The Hosea et al (2003) study reported a decrease in knee width in zinc deficient and pair-fed animals in comparison to adequately nourished rats. While this finding was not replicated in the present study, there was an interaction of lead and dietary zinc for femoral knee width (Figure 49). Knee width was reduced by lead exposure in the MZ rats, but not in any other treatment group, supporting the hypothesis that lead toxicity and marginal zinc deficiency would have additive, detrimental effects. However, no other outcomes of skeletal analysis produced lead and zinc interactions. Nonetheless, this is an important finding as a decrease in epiphyseal width may reflect abnormal growth plate function and/or trabecular bone development.

Conversely, SZ feeding showed a trend towards an increase in each parameter of skeletal growth although these effects failed to reach a level of significance, as occurred in terms of body weight gain. However, with respect to femoral length and area, there was a significant difference between both MZ and PW rats versus the SZ-fed rats (Figures 48 & 54).

Zinc supplementation has been shown to stimulate bone growth and strength in weanling rats (Ovesen, 2001). Male, Wistar, weanling rats were fed 2, 47, or 60 ppm zinc diets for 4 weeks. Final body weight, femoral length, and femoral cross-sectional area were higher in the zinc supplemented group (60 ppm zinc) than the control group (47 ppm zinc). Femoral weight was not reported. Notably, the improvements in bone mass did not have a negative effect on bone quality, as determined by biomechanical testing (Ovesen, 2001). In the present study, the only significant increase in skeletal or ponderal growth between C and SZ rats was in weight gain (Figure 9). While a zinc level of 2 times the recommended level for growing rats stimulates bone growth and quality, ingestion of 10 times the recommended level may be less beneficial. If longitudinal growth outpaces mineralization, bone quality will be compromised. Additionally, there may be indirect

effects on bone quality, as pharmacological levels of zinc are thought to impede copper absorption and copper is an important trace element in bone metabolism (Roughead & Lukaski, 2003).

There were no differences between the MZ and PW treatments in any parameter of skeletal growth, with the exception of femoral weight to body weight ratio. When calculated as a percentage of body weight, femoral weight was lower in the MZ rats than PW, C, and SZ treatments (Figure 45). This finding, in conjunction with the lower femoral area found in MZ rats (Figure 53), suggests that longitudinal bone growth inhibition may be more severe with marginal zinc deficiency than malnutrition, as the growth inhibition of PW rats was proportional to their body size.

Lead treatment also resulted in retardation of skeletal growth, as indicated by lower femoral weight, length, femoral head width, and whole body skeletal area (Figures 45, 48, 50 & 56). The lower femoral area seen with lead treatment approached significance ($p=0.0675$), as well. It has been well established that lead exposure results in growth retardation in animal models (Rossi et al. 2001; Escribano et al. 1997; Hamilton & O'Flaherty, 1994), as well as humans (Campbell et al. 2004; Pounds et al. 1991). Lead-induced growth inhibition in the present study ranged from 2-6% for skeletal measures and 7% for body weight. This is comparable to a study by Hammond et al (1989) who reported 17% less linear and ponderal growth in weanling, female rats exposed to 250 ppm lead for 23 days, which was largely accounted for by reduced feed intake. Of note, linear growth was measured by tail length rather than longitudinal bone growth. The greater response in the Hammond et al (1989) study than in the present study may be explained by the slightly higher lead dose and the use of female, rather than male rats. Hammond et al (1989) also reported a dose-dependent reduction in weight gain with lead given in drinking water at 0, 50, 250, and 500

ppm lead acetate, while there was no effect on feed efficiency, which confirmed previous observations (Kimmel et al. 1980). Similarly, lead did not affect feed efficiency in the present study (Figure 10).

The lower width of the femoral head in lead-exposed rats is an important finding as the active growth plate is found in this region (Figure 50). This is consistent with previous studies such as Gonzalez-Riola et al (1997). Weanling, female Wistar rats were supplemented with a diet containing 17 ppm lead for 50 days. Growth plate cartilage thickness was analyzed by histomorphometry and found to be higher in the control rats than in the lead-exposed rats (Gonzalez-Riola et al. 1997). However, whether the growth inhibition associated with lead exposure is due to a systemic effect, a specific effect on osteoblasts, or through effects on the epiphyseal growth plate cartilage remains unknown (Hicks et al. 1996). The shorter stature reported in lead-intoxicated children is indicative of growth plate chondrocyte inhibition, as longitudinal growth is secondary to endochondral bone development (Hicks et al. 1996). Growth plate chondrocytes are metabolically active and share several features in common with osteoblasts, including proliferation, differentiation, matrix production, and mineralization (Hicks et al. 1996). An *in vitro* study with isolated avian chondrocytes incubated with physiologically relevant lead concentrations (0.1 – 5 μM) resulted in suppression of ALP, and type II and type X collagen expression at the protein and mRNA level (Hicks et al. 1996). While still in the cell culture stage, this data suggests an inhibitory effect of lead on endochondral bone formation through the regulation of growth plate chondrocytes. Further research has revealed that lead alters the rate of chondrocyte maturation by targeting specific signaling pathways and growth factors (Zuscik et al. 2002). As well, lead is localized within the areas of bone mineralization and growth (Hamilton &

O'Flaherty, 1995) and incorporated into hydroxy-apatite crystals during the process of calcification, where it remains until the bone is resorbed (Hicks et al. 1996). Thus, the growth plate appears to be an important target of lead toxicity.

Skeletal Densitometry & Mineralization

Dietary Zinc

Densitometric analysis was performed with DEXA on the whole body and specific regions of rat carcasses *in situ*, as well as excised femurs. High resolution scans of the whole body revealed that skeletal area and BMC were lower in MZ-fed rats than C rats by 7% each (Figures 56-58). Similar reductions were also found in the PW rats. The skeletons of MZ and PW rats were smaller and had less mineral content, thus it is not surprising that there was no difference in BMD among the various dietary zinc treatments (BMD was calculated as the ratio of BMC to area). However, it is possible that the treatments had specific effects on either cortical and/or trabecular bone which are not evident in a whole body scan.

Similarly, femoral area and BMC were lower by 6% and 9%, respectively, in MZ rats and BMC was lower by 8% in PW rats than C rats (Figure 53 & 54). In terms of BMD, however, PW and SZ rats had 6% lower BMD than C rats and there was no difference between MZ and C rats (Figure 55). These findings are explained by looking at the area of the femur in relation to the BMC. The PW rats had a slight, but not significantly, higher area and slightly, not significantly, lower BMC than MZ rats, thus, the BMD is lower. Similarly, the SZ rats had a slightly higher area and lower BMC than C rats, resulting in a lower BMD. BMD is a key indicator of bone mass, used to diagnose osteoporosis (Javaid & Cooper, 2002). As BMD decreases, the risk of osteoporotic fracture rises (Javaid & Cooper, 2002). Thus, although this data can not necessarily be

extrapolated to humans, a 6% decrease in BMD over only a 3 week dietary treatment in growing animals is a concern, as rats undergo rapid linear skeletal growth during the first 6 months of life. A comparable response in humans, such as a 7% increase in BMD in children, would be equivalent to 2 years of bone growth (Campbell et al. 2004).

As expected, the effect of dietary restriction on BMD in the present study (6% reduction) was less severe than reported in the Hosea et al (2003) study, in which pair feeding resulted in a 13% lower BMD than control animals (Figure 55). The BMD of MZ-fed rats was not affected, as seen with rats fed < 1 ppm zinc, which showed a 20% reduction. This is surprising given that MZ rats had 4% lower femoral calcium and phosphorus concentrations (Figures 46 & 47), and 62% lower femoral zinc concentrations than C rats (Figure 14), whereas the calcium, phosphorus, and zinc concentrations of PW rats were not different from C. On the other hand, the lead concentration of MZ femurs was 62% higher than C and 50% higher than PW rats (Figure 15). Although the DEXA is optimized to detect calcium as hydroxyapatite, the presence of lead may have caused a false elevation in BMC readings. However, even if this was the case, it is not likely to have a significant impact as lead constituted only 0.12% of the mineral concentration based on calcium and phosphorus concentrations analyzed by ICP spectrophotometry. Also, BMC as determined by DEXA has been shown to be highly correlated with bone calcium concentration ($r=0.89$) in small, excised rat bones (Kastl et al. 2002).

The lower BMD found in SZ rats was an unexpected outcome (Figure 55). The SZ treatment was more detrimental than the MZ treatment in terms of BMD. Zinc supplementation is known to stimulate skeletal growth and maturation, both *in vivo* and *in vitro* (Yamaguchi, 1998). However, this effect is undoubtedly dose-related. For example, the Ovesen et al (2001) study

previously found that zinc supplementation at 60 ppm had a stimulatory effect on growth rate and femoral mass, size, and strength. Histomorphometric analysis also revealed that zinc exerted its primary effect on the periosteal envelope, mainly increasing bone and tissue area, and thereby mimicking the effect of growth hormone and IGF-1 on bone (Ovesen et al. 2001). At the present time, and to the best of my knowledge, there are no published *in vivo* data on outcomes of bone growth at higher levels of zinc supplementation. However, the results of the present study support the proposition that zinc increases bone area. It appears, though, that in the case of a 300 ppm zinc diet, the process of mineralization was not able to keep pace with bone growth, resulting in a lower BMD. The poor copper status of the SZ rats may have caused or contributed to impaired bone mineral density. Copper is essential for collagen synthesis, a key bone protein in which hydroxyapatite crystallizes and hardens (Roughead & Lukaski, 2003).

Lead Exposure

Lead exposure during growth and development may reduce peak BMD, thereby predisposing an individual to osteoporosis later in life (Campbell et al. 2004). Lead exposed animals in this study had a lower femoral BMD than non-lead exposed animals, which was also mirrored by femoral and whole body BMC, as well as femoral and whole body area (although the effect on area did not reach significance, p=0.0675; Figures 53-58). However, previous studies examining lead-induced effects on bone mass accumulation and development have reported equivocal findings. Bagchi & Preuss (2005) found that growing, SD rats drinking 1% PbAc (10 ppm) for 40 days had a lower BMD over a year-long period. Escribano et al (1997) reported a lead-induced reduction in bone mass, as assessed by histomorphometry, but an increase in bone mass,

as determined by densitometry (DEXA). However, this study differed from the present study in that it was done in female rats for 50 days, with a dietary lead concentration of only 17 ppm. In children, skeletal impairments, including reduced stature and chest circumference, have been documented with blood lead concentrations less than 10 µg/dL (Pounds et al. 1991). A recent cross-sectional study in children also reported that children with high lead exposure (mean, 23.6 µg/dL blood) had significantly increased BMD compared to the low lead exposure (mean, 6.5 µg/dL blood) group in four of seventeen measured sites, including the head, and the third and fourth lumbar vertebrae (Campbell et al. 2004). The increased BMD was not thought to be a false reading due to the deposition of lead in bone and the results appeared to be clinically relevant as a 7% increase in lumbar vertebrae BMD is equivalent to about 2 years of bone growth (Campbell et al. 2004).

The mechanism of a lead-induced increase in childhood BMD is not known. However, lead inhibits PTHrP *in vitro* leading to premature chondrocyte maturation (Zuscik et al. 2002), which could result in elevated BMD (Campbell et al. 2004). In addition, the higher BMD associated with the inhibition of PTHrP is likely to be transient, based on studies in mice (Campbell et al. 2004). PTHrP acts on bone remodeling in adults, rather than endochondral ossification, by promoting osteoblast differentiation and impeding apoptosis, which may lead to increased bone loss in adults (Campbell et al. 2004). Alternatively, early lead exposure may result in the attainment of a lower peak bone mass in young adulthood, which would predispose to osteoporosis later in life (Campbell et al. 2004). While this association has limited and equivocal evidence in humans, it should also be noted that these studies have been relatively short in duration (18-47 months) and relied upon blood lead, rather than bone lead, as an indicator of body lead burden (Campbell et al. 2004).

In general, lead appears to have a detrimental effect on skeletal development, whether it occurs through inhibition of bone formation or accelerated maturation. Premature achievement of peak bone mass and a reduction in peak bone mass will likely both predispose towards osteopenia and osteoporosis later in life. There was no evidence of an interaction between lead and dietary zinc in terms of skeletal densitometry or mineralization.

Skeletal Formation & Resorption

Serum osteocalcin and serum C-terminal telopeptides of type I collagen (Ratlaps) were not affected by lead but responded differentially in terms of dietary zinc (Figures 59 & 60). MZ rats had lower osteocalcin concentrations than either C or SZ rats, but not PW rats, suggesting that bone formation was less in marginally zinc deficient animals. This is consistent with the results for growth, as MZ rats had lower body weight, femur length, femur weight, and femur area than C and SZ rats. PW rats had an osteocalcin concentration that was intermediate to the MZ and C rats, but not different than either. However, this response was also reflected in femur length and area, suggesting that the inhibitory effects in skeletal growth seen with the MZ treatment may be in part due to anorexia and reduced weight gain. PW rats had lower a osteocalcin concentration than SZ rats, which was also paralleled by reduced femur length and weight.

It has been reported that lead is a potent inhibitor of vitamin D_3-induced osteocalcin synthesis in rat osteosarcoma (ROS 17/2.8) cells (Long et al. 1990). Further research has shown that calcium is readily displaced from osteocalcin by lead and that this further impedes osteocalcin binding to hydroxyapatite (Pounds et al. 1991). In lead-intoxicated children, plasma osteocalcin is reported to be depressed and returns to normal within a few weeks following chelation therapy

(Pounds et al. 1991). The lack of a lead effect in the present study is most likely due to the relatively low dose of lead given over only a three week period.

Serum Ratlaps concentrations were lower in PW rats than C and SZ rats, suggesting that bone resorption was lower with dietary restriction. Thus, the skeleton of PW rats may be undergoing less modeling for growth and/or have less of a need to release minerals for other tissues. This is supported by the lower body and femur weight seen in PW animals versus C and SZ animals and the lower BMD versus C animals. In contrast, the serum Ratlaps concentration of MZ rats was not different from either C or SZ rats. This is surprising when considered with respect to growth, however, in terms of feed efficiency and growth rate the PW rats fell well below the rates of all other treatments over the final week of the study. The growth rate of MZ rats, while depressed after week 2, was able to recover to that of C and SZ rats by week 3. Thus, the growth of PW rats appears to have been more severely hindered than MZ rats during the final week of the study.

In light of the fact that the MZ and PW rats had similar reductions in bone mass, it seems likely that the net achievement in bone mass occurred through different mechanisms. The low bone mass achievement with marginal zinc deficiency appears to be the result of a decline in bone growth, which is thought to be mediated by growth plate dysfunction (Rossi et al. 2001). In contrast, PW rats appear to undergo less modeling for growth, thus the size of the bone is not changing as quickly to accommodate for ponderal growth.

VI. CONCLUSIONS

Growth during marginal zinc deficiency, dietary restriction & zinc supplementation.

- Marginal zinc deficiency and dietary restriction (PW treatment) resulted in moderate growth retardation, but only marginal zinc deficiency resulted in less adipose tissue.
- Marginal zinc deficiency impaired the growth of the kidney, intestine, and femur as compared to the control group, while dietary restriction impaired the growth of the liver and femur as compared to the control group. In addition, femoral weight, when calculated as a percentage of body weight, was only lower with marginal zinc deficiency. Thus, marginal zinc deficiency was more severe than dietary restriction in terms of growth inhibition.
- Marginal zinc deficiency impaired feed efficiency and growth rate initially, but both measures recovered by the end of the study. Dietary restriction severely impaired feed efficiency and growth rate only during the final week of the study.
- Zinc supplementation stimulated growth, in terms of weight gain over 3 weeks. Body weight was also greater than control after 2 weeks, but not by the end of the study.

Growth during lead exposure.

- Lead exposure resulted in moderate growth retardation, although lower organ and adipose fat weights were proportional to the lower body weights of lead exposed rats.
- The effects of lead exposure and marginal zinc deficiency were not additive, in terms of growth.

Zinc Status

- The marginally zinc deficient diet (8 ppm zinc) produced significant reductions in serum and femur zinc in comparison to the control group, indicating a state of marginal to moderate zinc deficiency. Dietary restriction had no effect on serum or femur zinc concentration, indicating that zinc status was not affected by reduced feed intake. Thus, outcomes of zinc deficiency can be separated from outcomes of reduced feed intake by comparing these treatment groups.

- The zinc supplemented diet (300 ppm zinc) resulted in higher serum and femur zinc concentrations than the control group.

Lead Status

- Tissue lead accumulation was reflective of dietary zinc intake in the kidney, liver, and bone, with an adequate zinc diet resulting in less tissue lead than the marginal zinc diet and the supplemental zinc diet having less tissue lead than both control and marginally zinc deficient diets.
- Dietary restriction resulted in a femoral lead concentration that was not different from the control group.
- Zinc supplementation appeared to be protective against tissue lead accumulation, based on calculations of the percentage of total measured tissue lead content in the femur, intestine, kidney, and liver. As dietary zinc increased, the percentage of lead deposited in the femur was less, while the percentage of lead accumulating in the intestine was greater.

General Micronutrient Status

- Copper status was moderately impaired in both zinc-supplemented and lead exposed animals, as evidenced by lower hepatic copper concentrations, although there was no evidence of anemia in these animals.
- Lead exposure was associated with higher iron accumulation in the liver, which may reflect a common absorptive pathway between lead and iron, such as the intestinal transporter, DMT1.
- Lead exposure resulted in less hepatic copper and less renal and femur zinc concentrations, suggesting that lead intoxication impairs mineral absorption and/or availability.

Tissue Zinc & Lead Concentrations

- Tissue zinc concentration was reflective of dietary intake in the serum, kidney, duodenum, jejunum, and femur. Hepatic zinc concentration was conserved during marginal zinc deficiency.
- As dietary zinc increased, tissue lead concentration decreased in the liver, kidney, and femur, suggesting a protective effect of adequate and supplemental zinc on lead absorption.

Metallothionein mRNA Levels & Immunolocalization

- MT gene expression was responsive to dietary zinc intake. MT mRNA levels were lower in the duodenum and jejunum of MZ rats than all other treatments and this difference was reflected in MT protein levels as visualized by immunostaining. MT mRNA levels were higher in SZ rats than C rats in the jejunum, although this difference was not obvious when visualized by immunohistochemistry.
- MT immunostaining was localized within intestinal Paneth cells and villi epithelia and was weak in marginally zinc deficient groups, suggesting that MT does not have a main role in zinc absorption but may function in zinc storage, protection from metal toxicity, and mucosal turnover.
- MT immunostaining in the kidney was localized within the proximal convoluted tubules of the renal cortex, supporting a role in zinc secretion. Therefore, weak immunostaining in these cells in the marginally zinc deficient rats suggests renal conservation of zinc.
- MT gene expression was not responsive to lead treatment based on mRNA transcription or immunohistochemistry. Thus, while supplemental zinc was effective in reducing renal and femoral lead deposition, MT does not appear to be directly involved in this attenuation.

Skeletal Growth

- Marginal zinc deficiency impaired skeletal growth in terms of femoral weight, femoral area, and whole body area. This was also supported by a lower rate of bone formation, as indicated by a lower serum osteocalcin concentration.

- Dietary restriction impaired skeletal growth in terms of femoral weight and whole body area. Bone resorption was impaired in diet restricted animals, as seen by a lower serum Ratlaps concentration. Thus, the mechanisms of skeletal growth inhibition in zinc deficiency and dietary restriction appear to be different.
- Lead exposure impaired skeletal growth in terms of femoral weight, femoral length, femoral head width, and whole body area. Femoral weight, relative to body weight, was impaired by zinc deficiency *per se*, and not dietary restriction.
- Lead exposure altered the femoral knee, head, and length, but not the diaphysis, suggesting inhibition of the growth plate.
- The effects of lead exposure and dietary zinc were not additive in terms of skeletal growth, except in the case of femoral knee width. Lead exposure resulted in a lower knee width only in marginally zinc deficient rats. Thus, marginal zinc deficiency may exacerbate lead toxicity on skeletal targets, such as the growth plate or regions with trabecular bone.

Skeletal Quality

- Marginal zinc deficiency and dietary restriction resulted in less femoral and whole body bone mineral content, but only marginal zinc deficiency was associated with less femoral calcium, phosphorus, and zinc. Conversely, diet restricted animals had less bone mineral density than control and marginally zinc deficient animals. Thus, zinc deficiency was more detrimental to bone mineralization than dietary restriction, but dietary restriction was more damaging to bone quality.
- Supplemental zinc-fed rats had a lower bone mineral density than control and marginally zinc deficient rats, suggesting that zinc-induced growth in bone size outpaced bone mineralization, leading to impaired bone quality.
- Lead exposure impaired bone quality in terms of femoral bone mineral content, femoral bone mineral density, and whole body bone mineral content. These effects may be occurring at the growth plate of long bones. Bone mineralization may have been impaired by direct toxic effects of lead or indirectly through impaired mineral availability, especially zinc and copper.

- The effects of lead exposure and dietary zinc were not additive in terms of skeletal quality. Thus, the negative effects of lead and inadequate or excess zinc on bone development may be acting through different mechanisms.

VII. SUMMARY

Marginal zinc deficiency and chronic lead exposure adversely affect growth and development. While the molecular mechanisms of lead toxicity have not been well defined, the symptoms of several mineral deficiencies are comparable to the toxic effects of lead. Therefore, lead is thought to interact with essential trace elements in the body. However, it is not known whether mineral deficiencies worsen lead toxicity or whether lead exposure produces symptoms of mineral deficiencies.

Zinc deficiency appears to contribute to tissue lead deposition and zinc supplementation was more effective than an adequate zinc diet in impeding lead accumulation. However, while zinc supplementation was protective against bone and organ lead accumulation, MT and CRIP do not appear to be directly involved. In addition, the high zinc dose used in the present study had detrimental effects on bone quality. Therefore, the optimal level of zinc supplementation to reduce lead absorption and support growth and development requires further investigation. Lead appeared to target the growth plate region of the long bone and the effect was additive with marginal zinc deficiency in one location. However, MZ deficiency generally did not intensify other measures of lead toxicity, despite exacerbating tissue lead deposition.

VIII. STRENGTHS & LIMITATIONS

This study was strengthened by the use of a marginal zinc deficiency and a low-level of lead exposure, which are more reflective of current health issues in human populations. Rat models of zinc deficiency are often severely restricted (< 1 ppm zinc), while human zinc deficiencies tend to be only moderate to marginal in degree. Similarly, acute lead intoxication is not as pervasive a problem as chronic exposure to relatively low environmental levels.

In contrast, the high zinc dose used in the present study was not reflective of human intakes. The 300 ppm zinc supplement was a pharmacological dose. A zinc supplement of 60-80 ppm would be a more appropriate dose in terms of physiological concentrations.

The factorial design of the present study was beneficial in that it allowed for the examination of the main effects of lead and dietary zinc, as well as the effect of their interaction. However, due to the large number of treatment groups (8), it was statistically more difficult to obtain a significant interaction effect. Thus, the addition of the PW group to the study was both a strength and drawback. It was necessary to interpret what was an effect of zinc deficiency *per se* versus what was an effect of reduced feed intake and weight gain, but it required the addition of 2 more treatment groups to the experiment.

This study extended previous knowledge on the relationship between dietary zinc and lead ingestion by ruling out two important zinc binding proteins (MT and CRIP) from a role in lead absorption. It also contradicted previous reports that marginal zinc deficiency (8 ppm zinc) and low level lead exposure (200 ppm) do not result in growth inhibition in growing rats after 3 and 7 weeks of treatment. In addition, to the best of my knowledge, interaction effects of lead and dietary zinc on bone growth and quality have not been investigated.

While the results of the present study are not directly comparable to humans, the SD rat is considered a good model for zinc deficiency, as well as human osteopenias. However, the use of a semi-purified diet may have affected mineral absorption by increasing transit time. In addition, rats are thought to have higher rates of paracellular mineral absorption than humans. These factors limit the ability to extrapolate our results to human gastrointestinal absorption of lead and zinc.

SD rats are also the most commonly used model for lead intoxication, although they are relatively insensitive to lead toxicity in comparison to humans. Thus, much higher levels of exposure are required for rat studies, in order to see comparable toxic effects, such as hematological outcomes. Nevertheless, the lead dose used in the present study (200 ppm lead) is a minimally toxic dose for growing rats.

The incorporation of lead into the drinking water provided more uniformity in the lead dosage. However, human lead exposure derives from a variety of sources, thus the use of a single exposure route is a limitation.

Finally, the interpretation of the results of this study are complicated by potential interactions of lead with other dietary minerals and feed components. For example, it is not possible to exclude other mineral interactions as a root cause, when making conclusions regarding the effect of dietary zinc on lead absorption and toxicity.

IX. FUTURE DIRECTIONS

- Studies on lead and zinc competition for zinc transporters, especially hZIP4 and ZnT1 in the enterocyte plasma membrane
- Studies to determine the intestinal trans-membrane transport protein for zinc
- Studies of lead and iron competition for DMT1 in the enterocyte
- Investigation of sub-cellular metallothionein localization in response to varying dietary intakes of zinc and the role of MT in lead-inclusion body formation
- Studies to determine zinc supplementation levels to optimize bone growth and bone quality
- Further investigation of the effect of zinc supplementation on copper status, especially with respect to bone strength and quality
- Studies examining the effect of multiple mineral deficiencies, especially iron, calcium, zinc, and copper, on lead absorption and toxicity
- Randomized, controlled clinical trials investigating the relationship between nutrition and bone lead accumulation, in addition to blood lead concentration. Growth outcomes and measures of bone quality should also be included, especially when zinc is given as a supplement.
- Examination of long term mineral supplementation on the deposition and mobilization of bone lead

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XI. APPENDIX A

Key to Tables & Figures

Keys to Figures

The following letter codes were used to identify the different treatment groups:

MZ = Marginal Zinc Deficiency

PW = Pair-Weighed to MZ

C = Control

SZ = Supplemental Zinc

Table 1A. Body weights at day 0, 7, 14, and 21 of the study. [1,2]

	Treatment Group							
	MZ	**MZ + Pb**	**PW**	**PW + Pb**	**C**	**C + Pb**	**SZ**	**SZ + Pb**
Body Weight **Day 0** (g)	107[a] ± 3	105[a] ± 2	106[a] ± 4	108[a] ± 2	105[a] ± 3	105[a] ± 2	107[a] ± 3	104[a] ± 3
Body Weight **Day 7** (g)	162[a] ± 3	**147[b] ± 4**	167[a] ± 6	165[a] ± 3	167[a] ± 5	160[a] ± 4	170[a] ± 5	166[a] ± 4
Body Weight **Day 14** (g)	205[bc] ± 4	**181[d] ± 5**	214[abc] ± 5	**201[c] ± 4**	208[ab] ± 6	210[bc] ± 6	229[a] ± 6	220[ab] ± 4
Body Weight **Day 21** (g)	**255[c] ± 5**	**229[d] ± 6**	**248[c] ± 4**	**229[d] ± 3**	272[ab] ± 8	263[bc] ± 8	285[a] ± 6	273[ab] ± 5

Table 1B[1,2]. Main effect of lead on weekly body weight.

DAY	0 Pb	+ Pb
0	106 ± 2	106 ± 1
7	166 ± 2	**159 ± 2***
14	217 ± 3	**203 ± 3***
21	265 ± 4	**248 ± 5***
p-value		**<0.0001**

Table 1C[1,2]. Main effect of zinc on weekly body weight.

DAY	MZ	PW	C	SZ
0	106 ± 2[a]	107 ± 2[a]	105 ± 2[a]	105 ± 2[a]
7	**154 ± 3[b]**	166 ± 3[a]	164 ± 3[a]	168 ± 3[a]
14	**193 ± 4[c]**	**208 ± 3[b]**	**214 ± 4[b]**	224 ± 4[a]
21	**241 ± 5[b]**	**239 ± 3[b]**	268 ± 6[a]	279 ± 4[a]
p-value				**<0.0001**

[1] Values are means ± SEM for n=8 (A), n=32 (B),and n=16 (C). Means within rows with different letters or an asterisk are significantly different as determined by Duncan's multiple range test.

[2] Body weight for the various time points was analyzed by repeated measures and significant main effects were found for lead and zinc over time ($p < 0.0001$), but not the interaction of lead and zinc over time ($p=0.1571$).

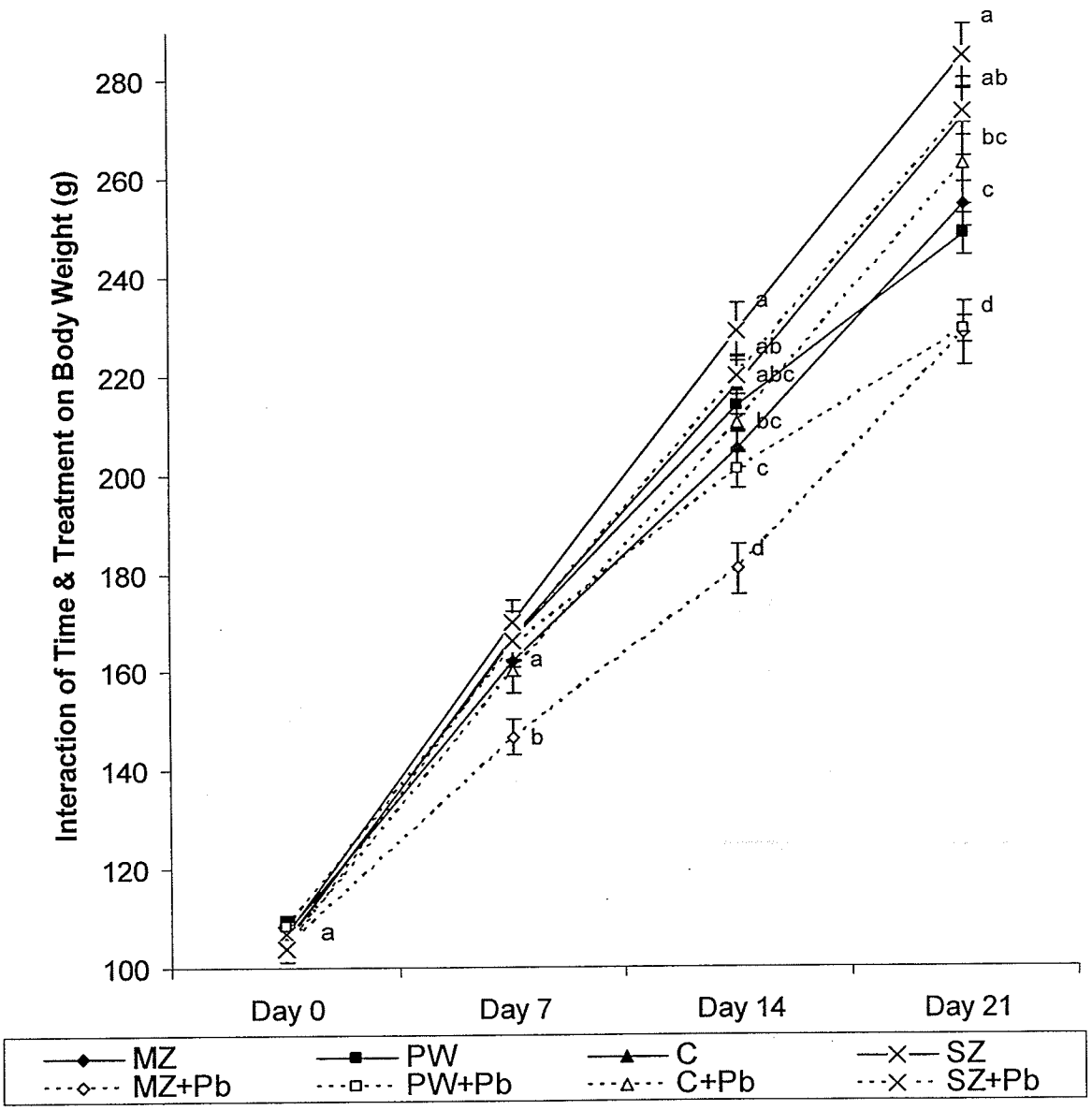

Figure 1. Effect of dietary zinc and lead exposure on body weight at days 0, 7, 14, and 21 of the study. Data points represent means for n=8. Data points with different lower case letters are significantly different within the same week as determined by repeated measures. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The overall effect of time^{x}lead^{x}zinc was not significant ($p=0.1571$), but there were significant main effects of lead and zinc after weeks 1, 2, and 3 ($p<0.0001$).

Table 2. Total feed intake (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	382 ± 6	383 ± 6	412 ± 15	440 ± 10	**404 ± 6**	
+ Pb	319 ± 12	349 ± 8	392 ± 16	408 ± 9	**367 ± 8***	
MAIN EFFECTS ZINC	**351 ± 10[b]**	**366 ± 6[b]**	**402 ± 11[a]**	**423 ± 8[a]**		**<0.0001**
p value					**<0.0001**	0.2459

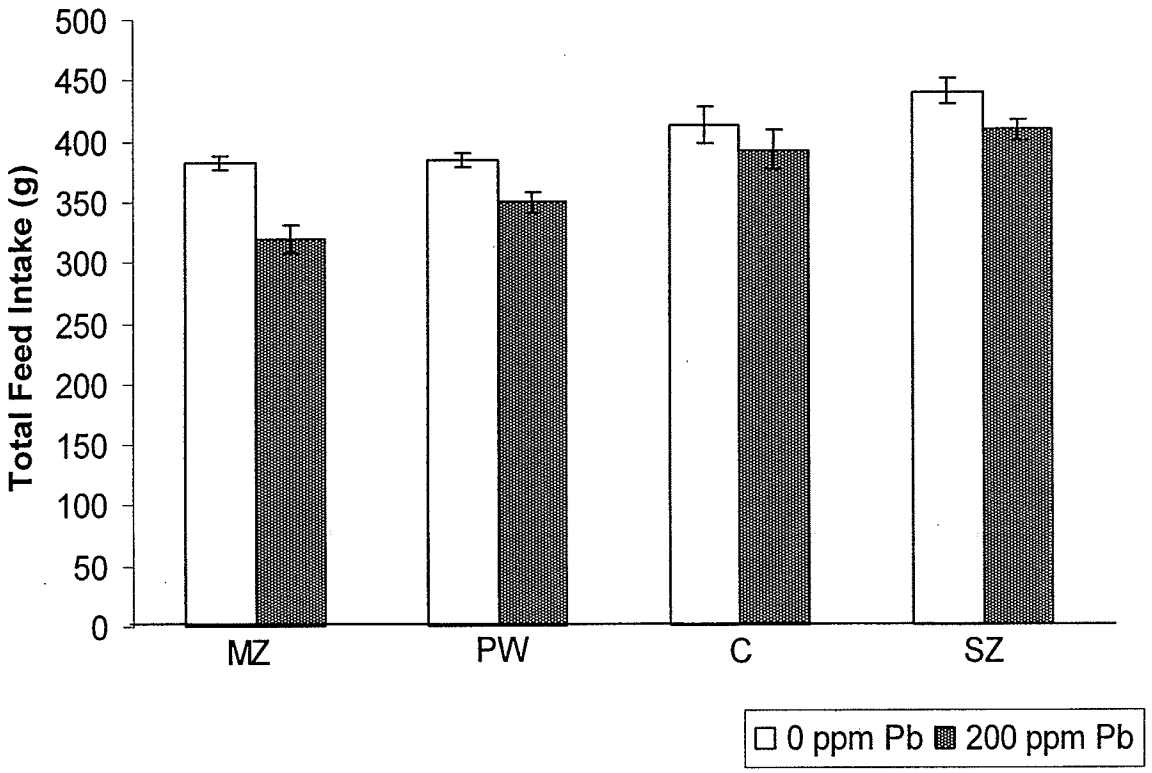

Figure 2. Effect of dietary zinc and lead exposure on total feed intake. Columns represent group means ± SEM for n=8. The main effect of lead was significant ($p < 0.0001$) and the main effect of zinc was significant at ($p < 0.0001$). The interaction of lead and dietary zinc was not significant ($p=0.1054$).

Table 3. Final body weight (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	254 ± 5	248 ± 4	272 ± 8	285 ± 6	**264 ± 4**	
+ Pb	228 ± 6	229 ± 3	263 ± 8	273 ± 5	**248 ± 5***	
MAIN EFFECTS ZINC	**241 ± 5[b]**	**239 ± 3[b]**	**268 ± 6[a]**	**279 ± 4[a]**		**<0.0001**
p value					**<0.0002**	0.4887

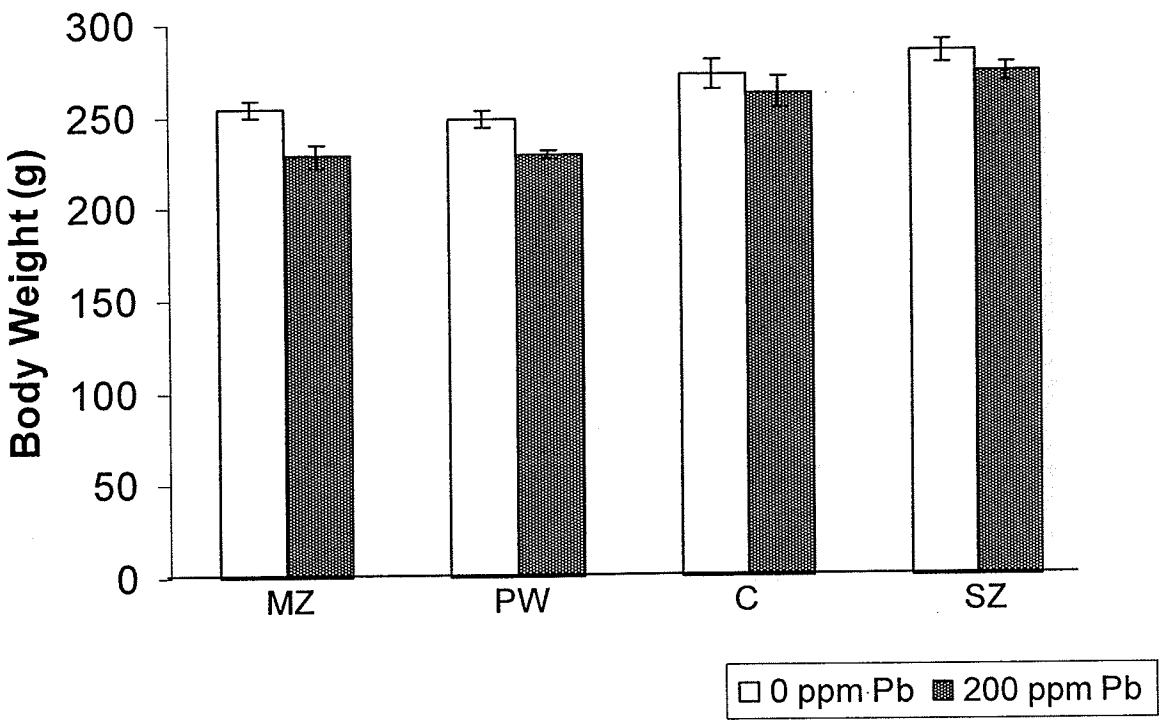

Figure 3. Effect of dietary zinc and lead exposure on final body weight. Columns represent group means ± SEM for n=8. The main effect of lead (p=0.0002) and the main effect of zinc (p< 0.0001) were significant. The interaction of lead and dietary zinc was not significant (p=0.4877)

Table 4. Weight gain (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	147 ± 3	143 ± 3	167 ± 5	178 ± 4	**159 ± 3**	
+ Pb	124 ± 6	121 ± 3	158 ± 7	169 ± 5	**143 ± 5***	
MAIN EFFECTS ZINC	**135 ± 4[c]**	**132 ± 3[c]**	**162 ± 4[b]**	**174 ± 3[a]**		**<0.0001**
p value					**<0.0001**	**0.2134**

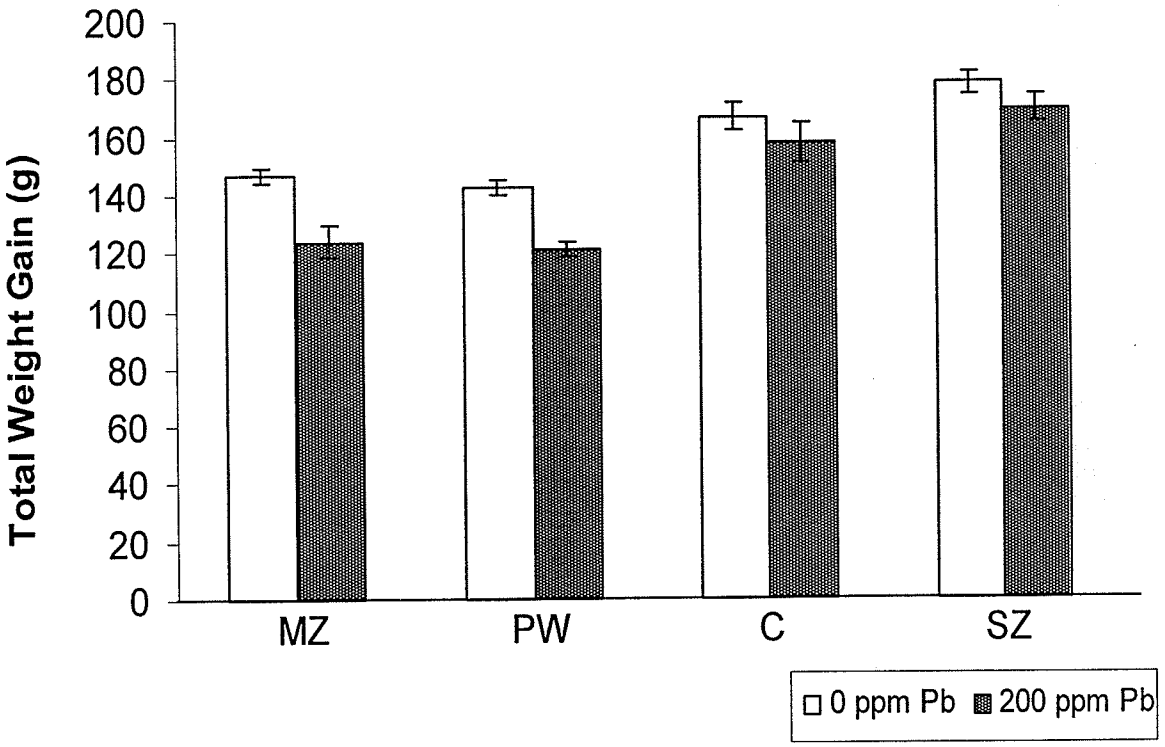

Figure 4. Effect of dietary zinc and lead exposure on total weight gain. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant ($p<0.0001$). The interaction of lead and dietary zinc was not significant ($p=0.2134$).

Table 5A. Feed efficiency calculated as the average weight gain (g) over the average feed intake (g) on week 1, 2, and 3. [1,2]

	Treatment Group							
	MZ	MZ + Pb	PW	PW + Pb	C	C + Pb	SZ	SZ + Pb
Feed Efficiency **Week1** (g/g)	0.49^{bc} ± 0.01	0.46^{c} ± 0.02	0.54^{a} ± 0.02	0.50^{ab} ± 0.01	0.53^{ab} ± 0.01	0.50^{ab} ± 0.01	0.52^{ab} ± 0.02	0.54^{a} ± 0.02
Feed Efficiency **Week 2** (g/g)	0.36^{a} ± 0.03	0.34^{a} ± 0.01	0.38^{a} ± 0.03	0.34^{a} ± 0.02	0.38^{a} ± 0.02	0.39^{a} ± 0.02	0.40^{a} ± 0.01	0.40^{a} ± 0.01
Feed Efficiency **Week3** (g/g)	0.34^{a} ± 0.02	0.38^{a} ± 0.01	0.24^{b} ± 0.02	0.21^{b} ± 0.03	0.34^{a} ± 0.01	0.35^{a} ± 0.01	0.34^{a} ± 0.01	0.34^{a} ± 0.01

Table 5B[1,2].
Main effect of lead on weekly feed efficiency.

Week	0 Pb	+ Pb
1	0.52 ± 0.01	0.50 ± 0.01
2	0.38 ± 0.01	0.37 ± 0.01
3	0.31 ± 0.01	0.32 ± 0.01
p-value		0.3663

Table 5C[1,2].
Main effect of zinc on weekly feed efficiency.

Week	MZ	PW	C	SZ
1	**0.47^{b} ± 0.01**	0.52^{a} ± 0.01	0.52^{a} ± 0.01	0.53^{a} ± 0.01
2	**0.35^{b} ± 0.01**	0.36^{ab} ± 0.02	0.39^{ab} ± 0.01	0.40^{a} ± 0.01
3	0.36^{a} ± 0.01	**0.23^{b} ± 0.02**	0.34^{a} ± 0.01	0.34^{a} ± 0.01
p-value				**<0.0001**

[1]Values are means ± SEM for n=8 (A), n=32 (B),and n=16 (C). Means within rows with different letters or an asterisk are significantly different as determined by Duncan's multiple range test.

[2] Data for the various time points was analyzed by repeated measures and significant main effects were found for dietary zinc over time ($p<0.0001$), but not lead over time ($p= 0.3663$) or the interaction of lead and zinc over time ($p=0.5761$).

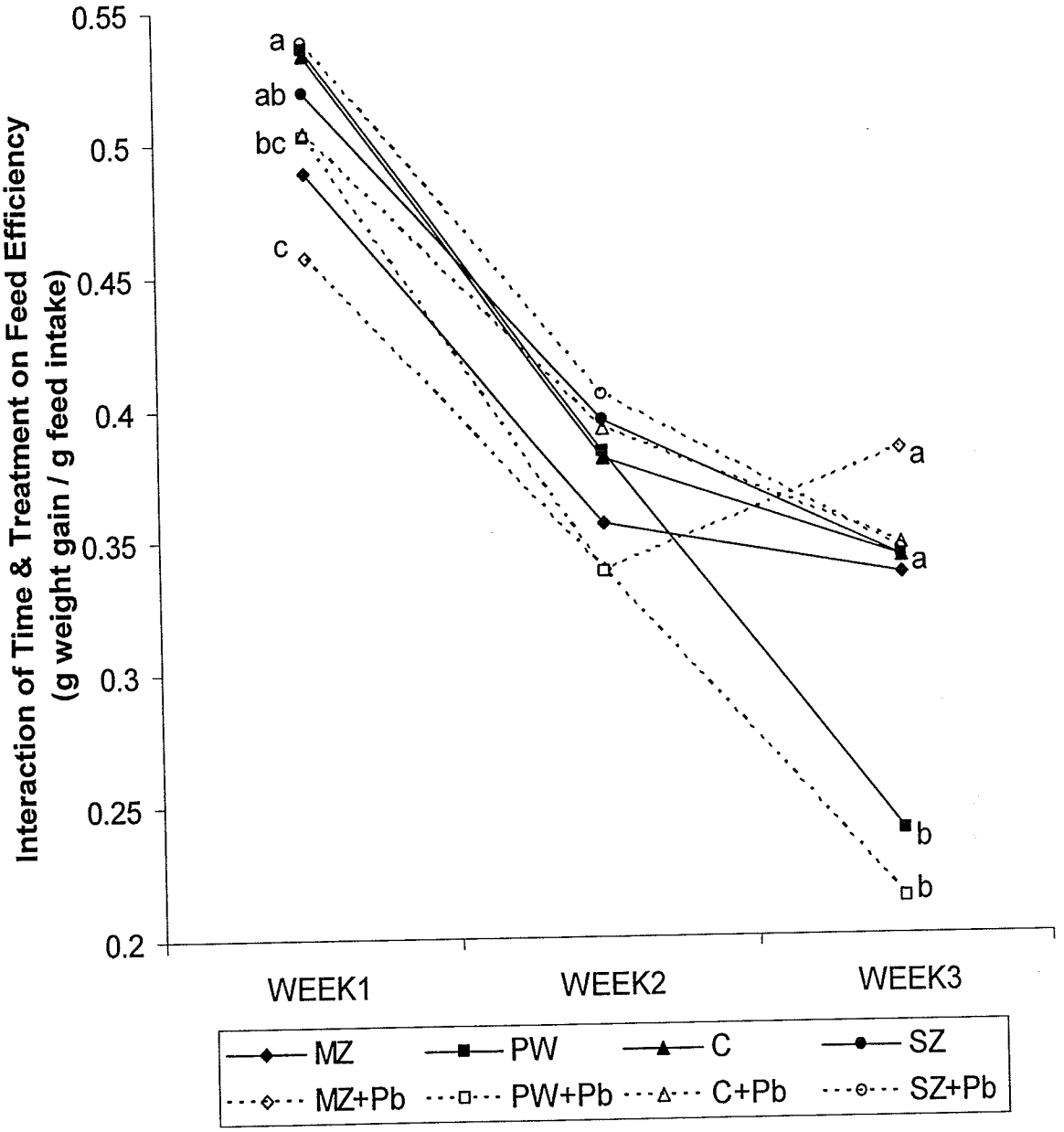

Figure 5. Effect of dietary zinc and lead exposure on feed efficiency at days 0, 7, 14, and 21 of the study. Data points represent means for n=8. Data points with different lower case letters are significantly different within the same week as determined by repeated measures. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The overall effect of time^{x}lead^{x}zinc was not significant ($p=0.5761$), but there was a significant main effect of zinc ($p<0.0001$).

Table 6 A. Growth rate calculated as the average daily weight gain (g) on week 1, 2, and 3. [1,2]

	Treatment Group							
	MZ	MZ + Pb	PW	PW + Pb	C	C + Pb	SZ	SZ + Pb
Growth Rate **Week1** (g/dau)	7.8 [c] ± 0.2	6.0 [d] ± 0.5	8.7 [abc] ± 0.4	8.1 [abc] ± 0.2	8.8 [ab] ± 0.3	7.9 [bc] ± 0.4	9.0 [a] ± 0.3	8.9 [a] ± 0.3
Growth Rate **Week 2** (g/day)	6.2 [cd] ± 0.5	4.9 [e] ± 0.4	6.8 [bc] ± 0.6	5.1 [de] ± 0.3	7.3 [abc] ± 0.4	7.2 [abc] ± 0.4	8.4 [a] ± 0.3	7.7 [ab] ± 0.3
Growth Rate **Week3** (g/day)	7.0 [a] ± 0.5	6.8 [a] ± 0.2	4.9 [b] ± 0.5	4.0 [b] ± 0.6	7.7 [a] ± 0.4	7.5 [a] ± 0.4	8.0 [a] ± 0.2	7.6 [a] ± 0.2

Table 6B[1,2].
Main effect of lead on weekly growth rate.

Week	0 Pb	+ Pb
1	8.6 ± 0.2	7.7 ± 0.3
2	7.2 ± 0.3	6.2 ± 0.3
3	6.9 ± 0.3	6.5 ± 0.3
p-value		0.3923

Table 6C[1,2].
Main effect of zinc on weekly growth rate.

Week	MZ	PW	C	SZ
1	**6.9 [b] ± 0.3**	8.4 [a] ± 0.2	8.3 [a] ± 0.3	9.0 [a] ± 0.2
2	**5.5 [b] ± 0.4**	**6.0 [b] ± 0.4**	7.2 [a] ± 0.3	8.1 [a] ± 0.2
3	**6.9 [b] ± 0.3**	**4.5 [c] ± 0.4**	7.6 [ab] ± 0.3	7.8 [a] ± 0.2
p-value				**<0.0001**

[1] Values are means ± SEM for n=8 (A), n=32 (B),and n=16 (C). Means within rows with different letters or an asterisk are significantly different as determined by Duncan's multiple range test.

[2] Data for the various time points was analyzed by repeated measures and a significant effect was found for dietary zinc over time ($p<0.0001$), but not lead over time ($p=0.3923$) or the interaction of lead and dietary zinc ($p=0.4411$).

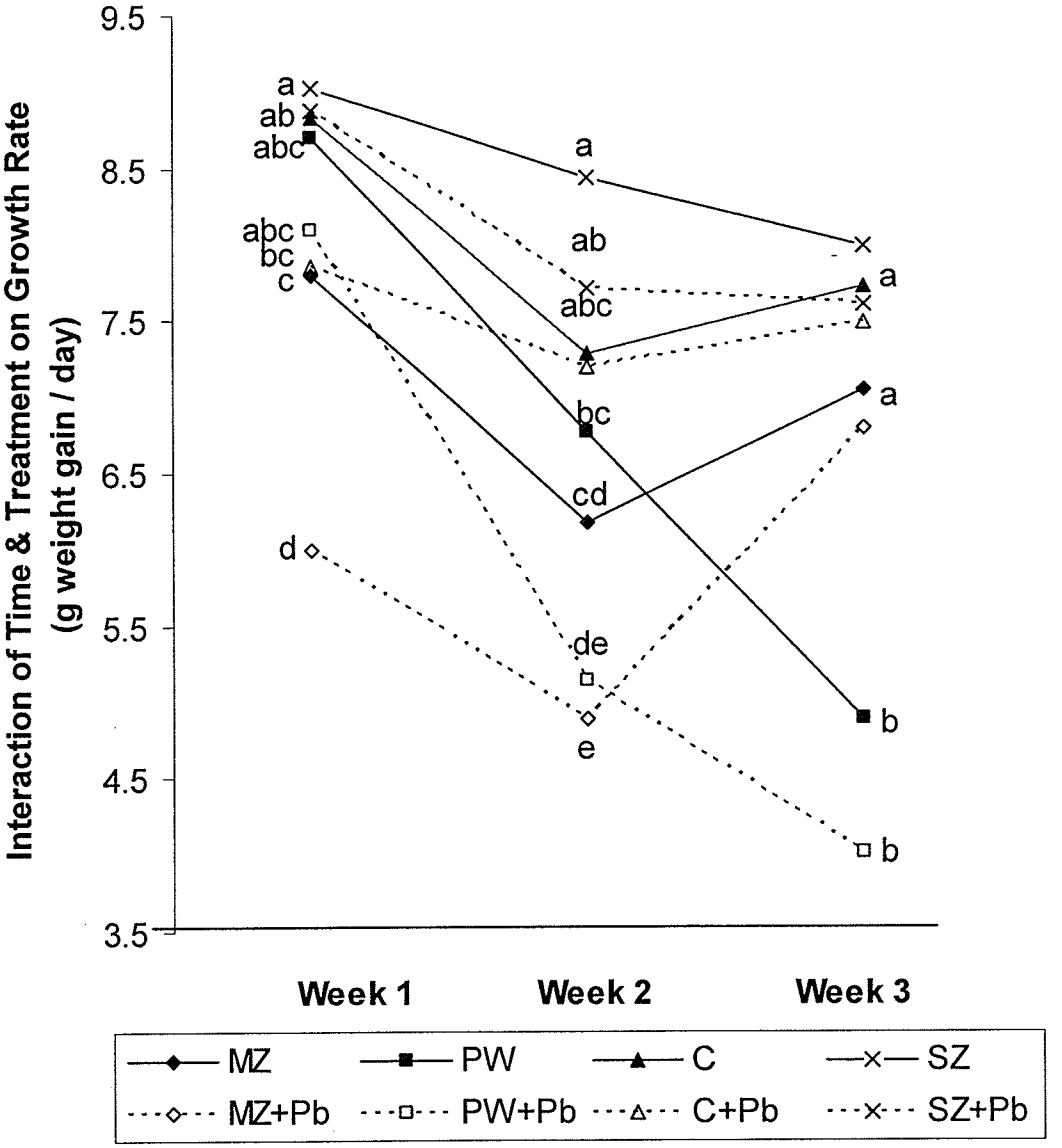

Figure 6. Effect of dietary zinc and lead exposure on growth rate at days 0, 7, 14, and 21 of the study. Data points represent means for n=8. Data points with different lower case letters are significantly different within the same week as determined by repeated measures. For overlapping data points, the same letter of significance is used to indicate that data points are not significantly different from each other. The overall effect of time×lead×zinc was not significant ($p=0.4411$), but there was a main effect of zinc ($p<0.0001$).

Table 7. Epididymal fat pad weight (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	2.6 ± 0.23	3.1 ± 0.12	3.3 ± 0.26	3.6 ± 0.18	**3.1 ± 0.11**	
+ Pb	2.3 ± 0.13	2.5 ± 0.25	3.1 ± 0.26	3.1 ± 0.22	**2.8 ± 0.13***	
MAIN EFFECTS ZINC	**2.4 ± 0.13^{c}**	**2.8 ± 0.15^{bc}**	**3.2 ± 0.18^{ab}**	**3.4 ± 0.15^{a}**		**0.0177**
p value					**<0.0001**	0.9037

Table 8. Epididymal fat pad weight to body weight ratio.

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.010 ± 0.0008	0.012 ± 0.0006	0.012 ± 0.0007	0.013 ± 0.0007	0.012 ± 0.0004	
+ Pb	0.010 ± 0.0005	0.011 ± 0.0001	0.012 ± 0.0007	0.012 ± 0.0006	0.011 ± 0.0004	
MAIN EFFECTS ZINC	**0.010 ± 0.0005^{b}**	**0.012 ± 0.0006^{a}**	**0.012 ± 0.0005^{a}**	**0.012 ± 0.0005^{a}**		**0.0204**
p value					0.1441	0.8589

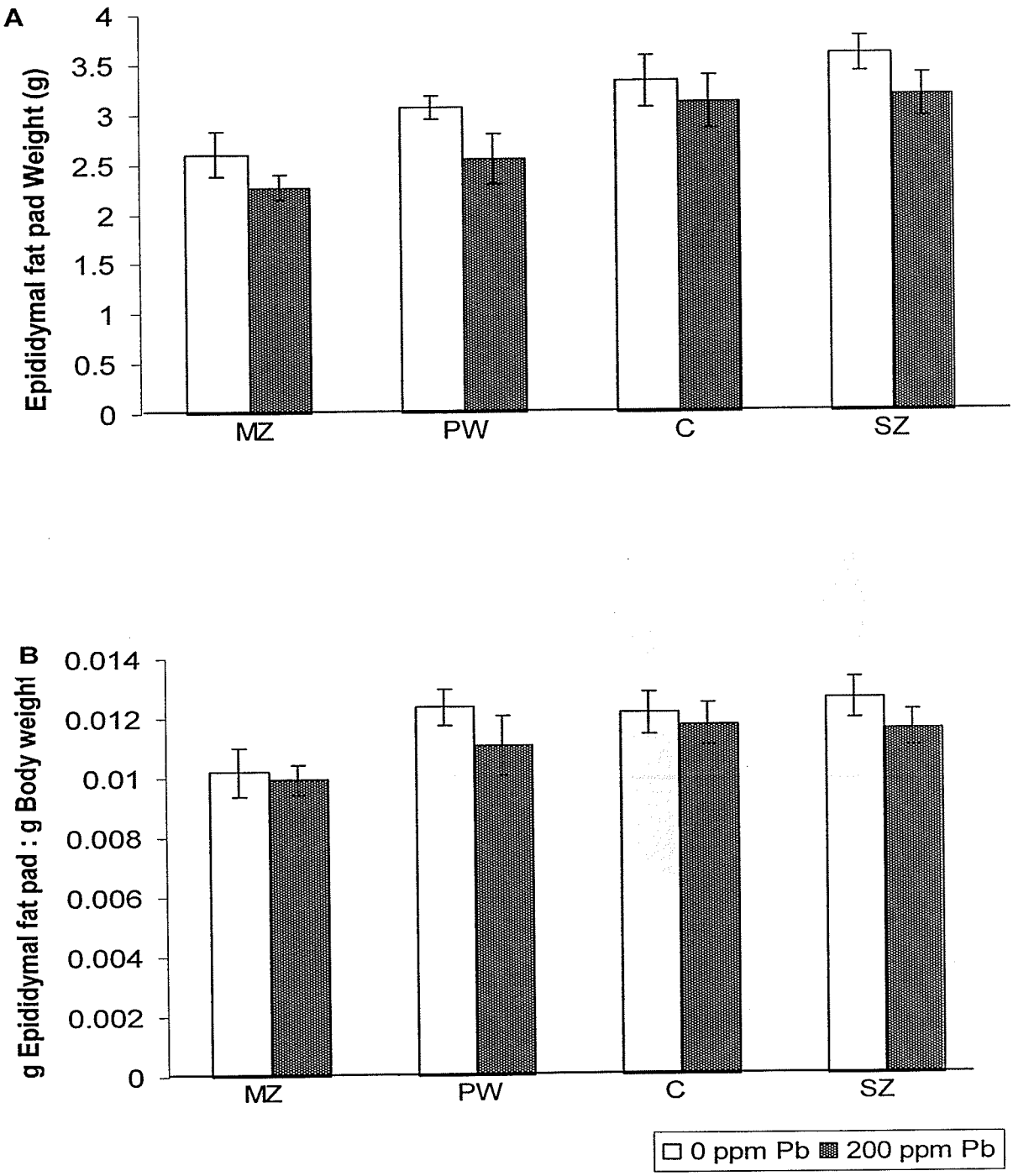

Figure 7. Effect of dietary zinc and lead exposure on epididymal fat pad weight (A) and epididymal fat pad to body weight ratio (B). Columns represent group means ± SEM for n=8. The main effect of lead was significant at p=0.0177(A) and the main effect of zinc was significant at p<0.0001 (A) and p=0.0204 (B). The interaction of lead and zinc was not significant (A, p=0.9037; B, p=0.8589).

Table 9. Serum Zinc Concentration (µmol / L).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	14.64 ± 1.45[c]	27.91 ± 1.50[b]	31.35 ± 1.35[b]	43.26 ± 2.18[a]	29.17 ± 2.11	
+ Pb	11.66 ± 0.91[c]	33.26 ± 2.31[b]	30.01 ± 1.58[b]	41.07 ± 2.30[a]	28.61 ± 2.15	
MAIN EFFECTS ZINC	**13.05 ± 0.89[c]**	**30.59 ± 1.50[b]**	**30.59 ± 1.05[b]**	**42.17 ± 1.55[a]**		**<0.0001**
p value					0.8203	0.0729

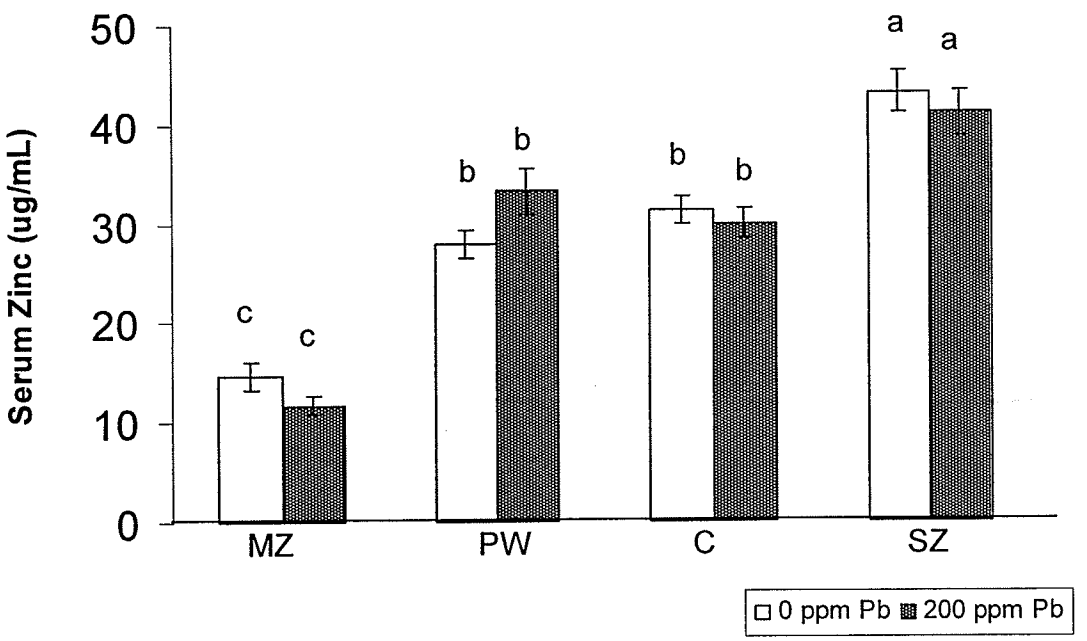

Figure 8. Effect of dietary zinc and lead exposure on serum zinc concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p<0.0001). The main effect of lead was not significant (p=0.8203). The interaction of lead and dietary zinc was significant (p=0.0729) overall but there was no effect of lead at any level of zinc, according to Duncan's multiple range test.

Table 10. Femur Zinc Concentration (umol zinc / g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.84 ± 0.07^{e}	4.93 ± 0.08^{c}	4.85 ± 0.06^{c}	6.65 ± 0.08^{a}	**4.57 ± 0.31**	
+ Pb	1.72 ± 0.05^{e}	4.52 ± 0.08^{d}	4.42 ± 0.11^{d}	5.95 ± 0.19^{b}	**4.15 ± 0.28***	
MAIN EFFECTS ZINC	**1.78 ± 0.05^{c}**	**4.73 ± 0.08^{b}**	**4.64 ± 0.08^{b}**	**6.30 ± 0.13^{a}**		**<0.0001**
p value					**<0.0001**	**0.0556**

Table 11. Femur lead content (µmol lead / g dry weight).

	MZ	PW	C	SZ	p value
+ Pb	1.74 ± 0.25^{a}	0.87 ± 0.10^{b}	0.67 ± 0.13^{b}	0.23 ± 0.04^{c}	**<0.0001**

Table 12. Renal lead content (µmol lead / g dry weight).

	MZ	PW	C	SZ	p value
+ Pb	0.23 ± 0.04^{a}	0.20 ± 0.03^{ab}	0.15 ± 0.02^{b}	0.10 ± 0.03^{c}	**0.0002**

Table 13. Percentage of total tissue lead (%).

	MZ	PW	C	SZ	p value
Femur	81.2 ± 1.2^{a}	68.8 ± 3.4^{b}	63.7 ± 4.5^{b}	50.4 ± 4.5^{c}	**<0.0001**
Kidney	11.3 ± 0.7^{b}	16.8 ± 1.6^{a}	16.3 ± 1.3^{a}	18.9 ± 1.5^{a}	**0.0025**
Liver	1.2 ± 0.1^{ab}	1.5 ± 0.2^{a}	1.3 ± 0.2^{ab}	0.9 ± 3.7^{b}	**0.0419**
Intestine	6.3 ± 0.8^{c}	12.9 ± 2.9^{b}	18.6 ± 3.7^{ab}	29.8 ± 5.4^{a}	**0.0006**

Table 14. Hepatic ALAD Activity (mmol PBG / L).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.519 ± 0.034	0.801 ± 0.148	0.741 ± 0.082	0.925 ± 0.080	0.746 ± 0.05	
+ Pb	0.540 ± 0.026	0.772 ± 0.073	0.682 ± 0.042	0.801 ± 0.097	0.699 ± 0.04	
MAIN EFFECTS ZINC	**0.529 ± 0.021^{b}**	**0.786 ± 0.080^{a}**	**0.711 ± 0.045^{a}**	**0.863 ± 0.063^{a}**		**0.0011**
p value					0.4093	0.8401

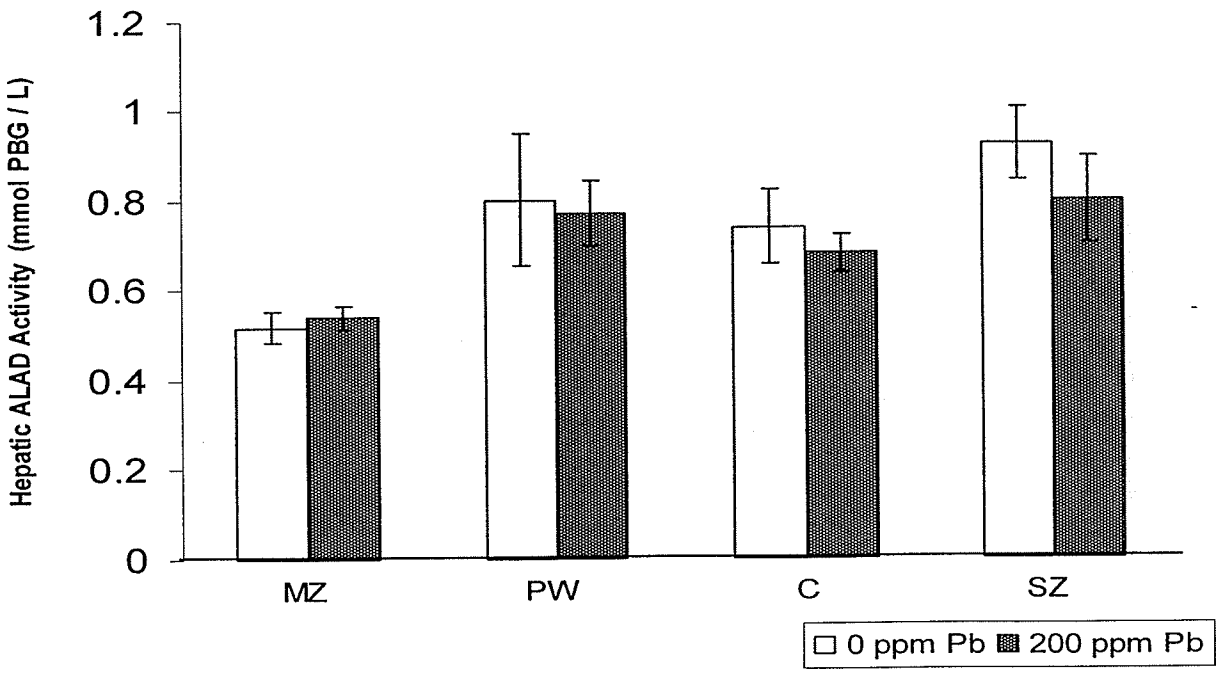

Figure 9. Effect of dietary zinc and lead exposure on hepatic ALAD activity. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0011). The main effect of lead and the interaction of lead and dietary zinc were not significant (p=0.4093 and p=0.8401, respectively).

Table 15. Hematocrit analysis (%).

					MAIN EFFECTS	
	MZ	PW	C	SZ	LEAD	p value
0 Pb	37.4 ± 1.7	36.8 ± 1.7	35.5 ± 2.0	37.2 ± 2.5	36.7 ± 1.0	
+ Pb	37.6 ± 1.8	36.5 ± 1.9	38.6 ± 2.4	36.9 ± 1.7	37.4 ± 0.9	
MAIN EFFECTS ZINC	37.5 ± 1.2	36.6 ± 1.2	37.1 ± 1.6	37.1 ± 1.5		0.6288
p value					0.9771	0.7930

Table 16. Hepatic Iron Concentration (µmol iron / g dry weight).

					MAIN EFFECTS	
	MZ	PW	C	SZ	LEAD	p value
0 Pb	6.00 ± 0.48	5.64 ± 0.40	5.04 ± 0.27	4.77 ± 0.64	**5.36 ± 0.24**	
+ Pb	6.77 ± 0.52	6.69 ± 1.09	5.58 ± 0.44	6.52 ± 0.44	**6.39 ± 0.33***	
MAIN EFFECTS ZINC	6.39 ± 0.36	6.16 ± 0.58	5.31 ± 0.26	5.64 ± 0.44		0.2500
p value					**0.0160**	0.7512

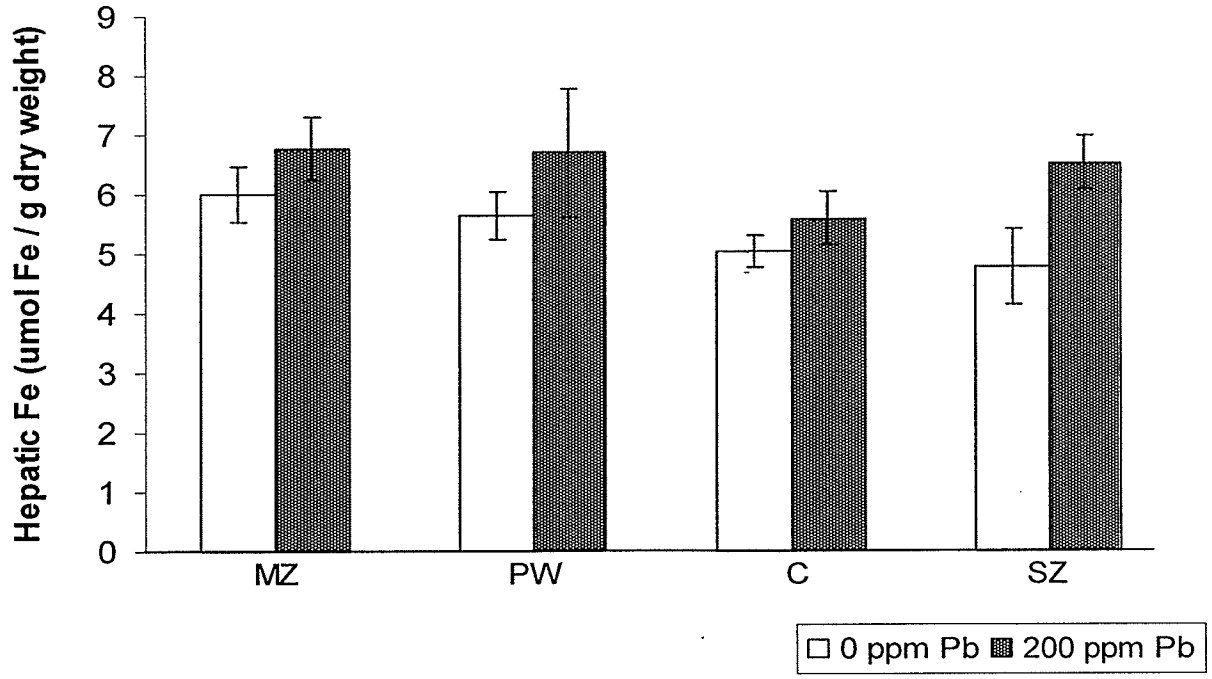

Figure 10. Effect of dietary zinc and lead exposure on hepatic iron concentration. Columns represent group means ± SEM for n=8. The main effect of lead was significant (p=0.0160). The main effect of zinc and the interaction of lead and zinc were not significant (p=0.2500 and p=0.7512, respectively).

Table 17. Hepatic Copper Concentration (nmol copper / g dry weight).

					MAIN EFFECTS	
	MZ	**PW**	**C**	**SZ**	**LEAD**	**p value**
0 Pb	177.5 ± 5.9	183.2 ± 4.8	184.3 ± 9.3	136.5 ± 12.3	**170.4 ± 5.4**	
+ Pb	153.8 ± 9.7	186.7 ± 7.8	157.6 ± 10.1	96.82 ± 8.9	**148.7 ± 7.3***	
MAIN EFFECTS ZINC	**165.6 ± 6.3[b]**	**184.9 ± 4.4[a]**	**171.0 ± 7.5[ab]**	**116.7 ± 9.0[c]**		**<0.0001**
p value					**0.0011**	0.1122

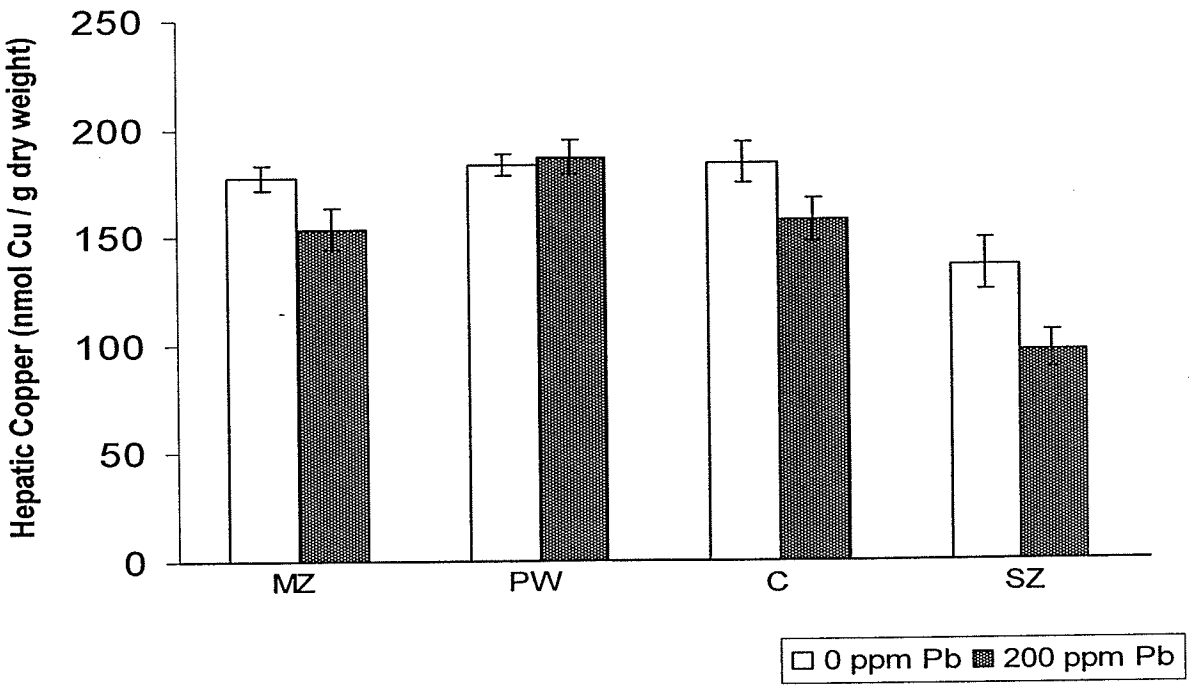

Figure 11. Effect of dietary zinc and lead exposure on hepatic copper concentration. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant at p=0.0011 and p< 0.0001, respectively. The interaction of lead and dietary zinc was not significant (p=0.1122).

Table 18. Hepatic wet weight (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	12.53 ± 0.42	11.56 ± 0.31	12.97 ± 0.82	14.02 ± 0.57	**12.77 ± 0.31**	
+ Pb	10.89 ± 0.40	10.60 ± 0.27	12.31 ± 0.40	13.5 ± 0.68	**11.82 ± 0.30***	
MAIN EFFECTS ZINC	**11.71 ± 0.35b[c]**	**11.08 ± 0.24[c]**	**12.64 ± 0.45[b]**	**13.76 ± 0.44[a]**		**<0.0001**
p value					**0.0125**	0.7083

Table 19. Hepatic wet weight to body weight ratio.

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.049 ± 0.002	0.047 ± 0.002	0.047 ± 0.002	0.049 ± 0.001	0.048 ± 0.001	
+ Pb	0.047 ± 0.001	0.046 ± 0.001	0.047 ± 0.001	0.049 ± 0.002	0.048 ± 0.001	
MAIN EFFECTS ZINC	0.048 ± 0.001	0.046 ± 0.001	0.047 ± 0.001	0.049 ± 0.001		0.2798
p value					0.5721	0.9419

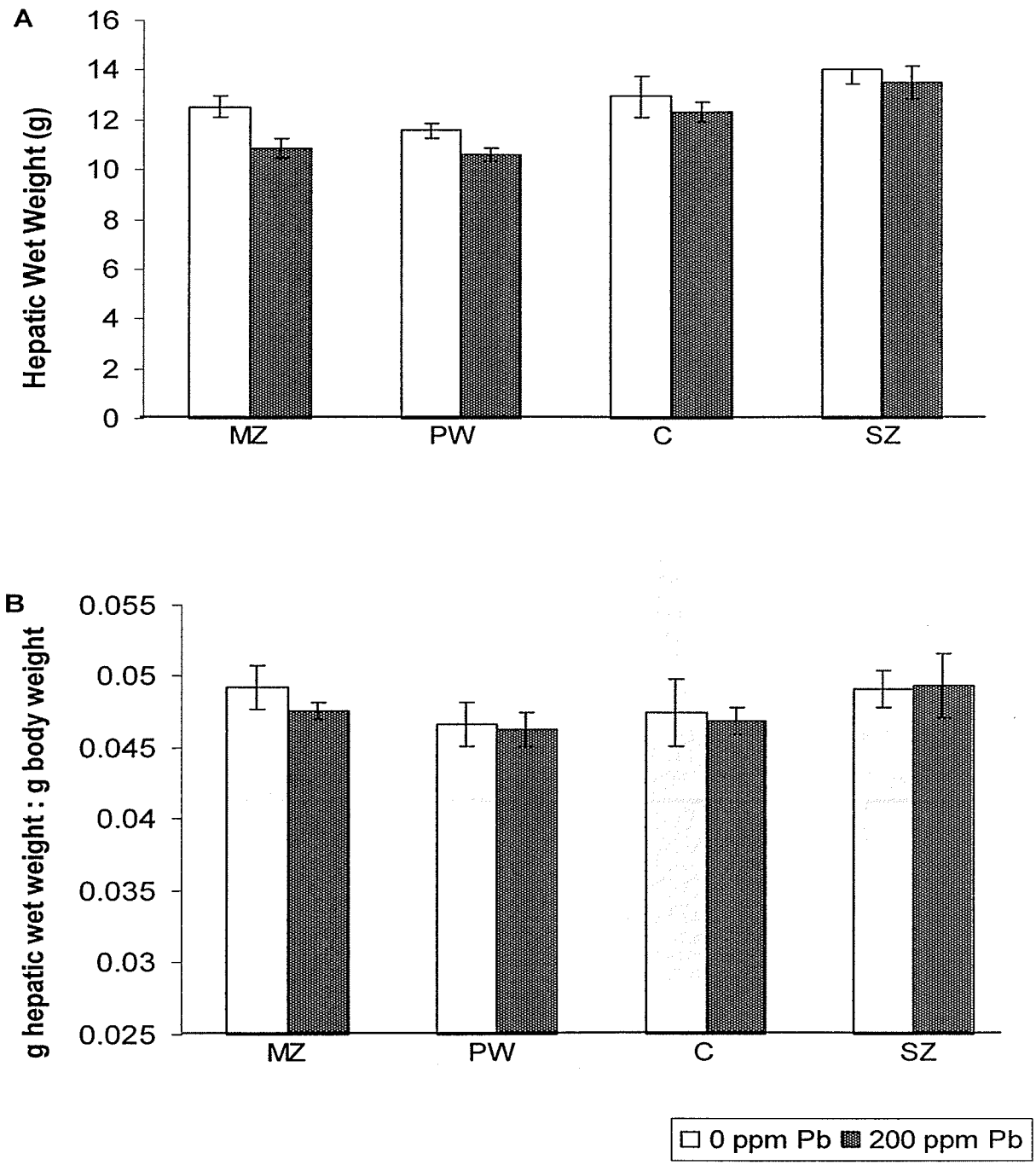

Figure 12. Effect of dietary zinc and lead exposure on hepatic wet weight (A) and hepatic wet weight to body weight ratio (B). Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant ($p=0.0125$ and $p<0.0001$, respectively), but the interaction of lead and dietary zinc was not significant ($p=0.7083$) for hepatic wet weight. No significant main effects for lead and zinc or their interaction were found for hepatic wet weight to body weight ratio ($p=0.5721$; $p=0.2798$; $p=0.9419$, respectively). Data (A&B) was log transformed to obtain normality and homogeneity of variance.

Table 20. Hepatic Zinc Concentration (µmol zinc / g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.59 ± 0.04	1.72 ± 0.06	1.69 ± 0.06	1.68 ± 0.05	1.67 ± 0.03	
+ Pb	1.06 ± 0.03	1.19 ± 0.04	1.13 ± 0.02	1.07 ± 0.05	1.65 ± 0.03	
MAIN EFFECTS ZINC	**1.58 ± 0.03^{b}**	**1.75 ± 0.04^{a}**	**1.68 ± 0.03^{ab}**	**1.63 ± 0.05^{ab}**		**0.0191**
p value					0.6366	0.6351

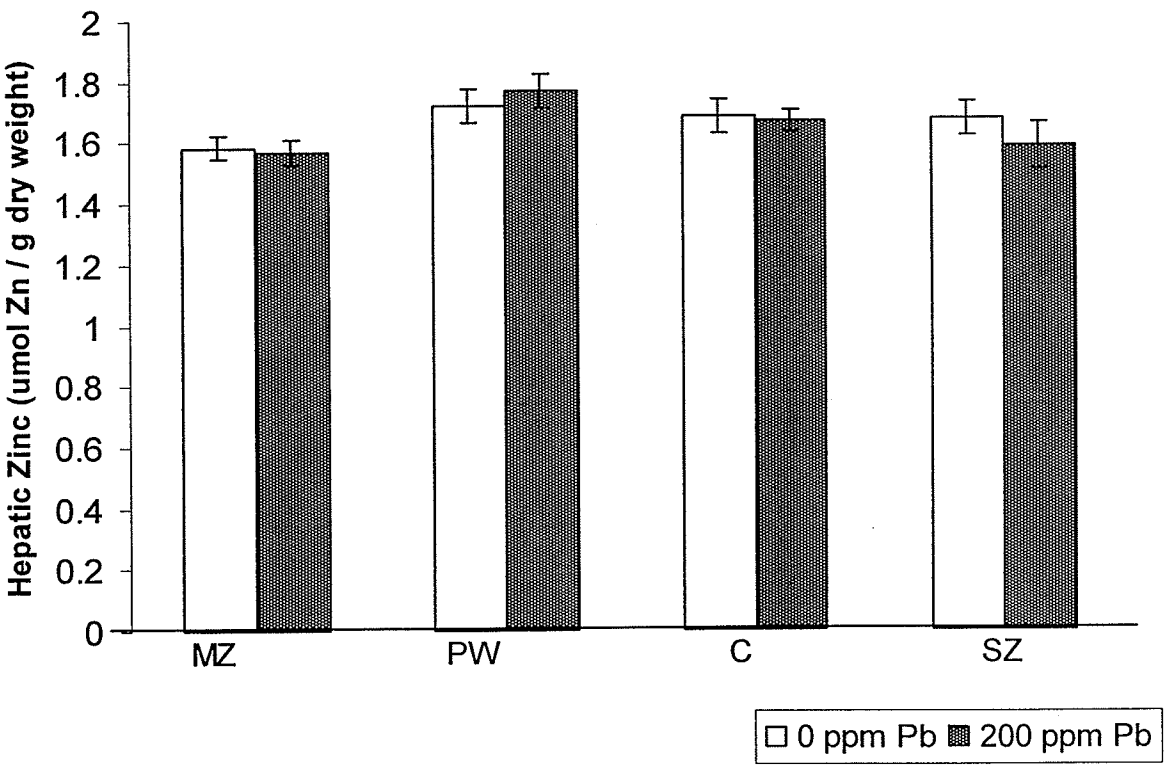

Figure 13. Effect of dietary zinc and lead exposure on hepatic zinc concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0191). The main effect of lead and the interaction of zinc and lead were not significant (p=0.6366; p=0.6351, respectively).

Table 21. Hepatic lead concentration (nmol / g dry weight).

	MZ	PW	C	SZ	p value
+ Pb	26.9 ± 5.6[a]	19.0 ± 2.7[ab]	13.0 ± 2.1[b]	4.8 ± 1.1[c]	0.0006

Table 22. Renal wet weight (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	2.60 ± 0.23	3.06 ± 0.12	3.32 ± 0.26	3.59 ± 0.18	3.14 ± 0.12	
+ Pb	2.26 ± 0.12	2.54 ± 0.25	3.12 ± 0.26	3.19 ± 0.22	2.78 ± 0.13*	
MAIN EFFECTS ZINC	2.43 ± 0.13[c]	2.80 ± 0.15[bc]	3.22 ± 0.18[ab]	3.39 ± 0.15[a]		<0.0001
p value					0.0177	0.9037

Table 23. Renal wet weight to body weight ratio.

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.013 ± 0.0008	0.012 ± 0.0006	0.012 ± 0.0007	0.010 ± 0.0007	0.011 ± 0.0004	
+ Pb	0.010 ± 0.0005	0.011 ± 0.0010	0.012 ± 0.0007	0.012 ± 0.006	0.012 ± 0.0004	
MAIN EFFECTS ZINC	0.012 ± 0.0005[a]	0.012 ± 0.0005[a]	0.012 ± 0.0006[a]	0.010 ± 0.0005[b]		0.0204
p value					0.1441	0.8580

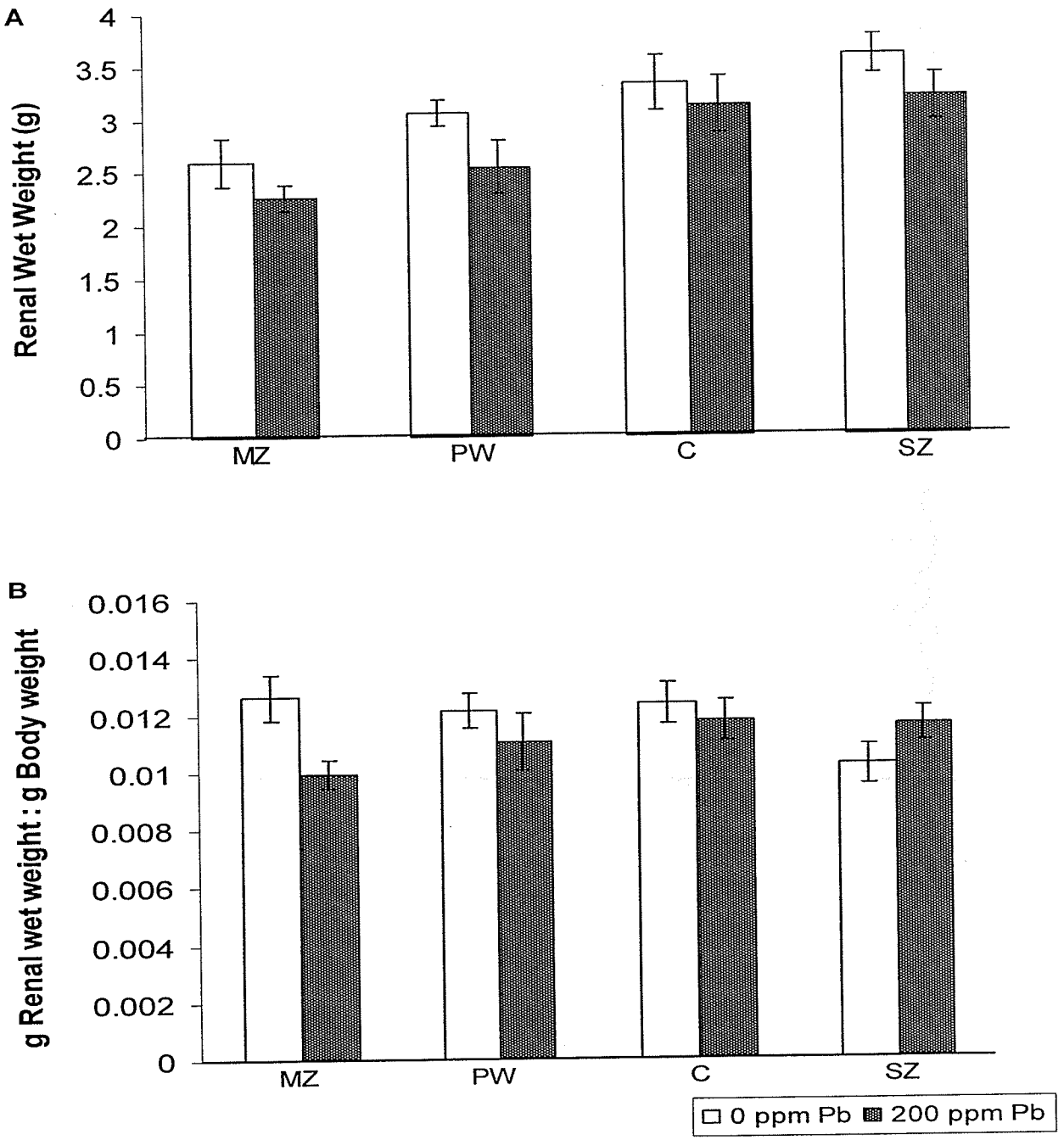

Figure 14. Effect of dietary zinc and lead exposure on renal wet weight (A) and renal wet weight to body weight ratio (B). Columns represent group means ± SEM for n=8. The main effect of lead and main effects of zinc were significant (A, p=0.0177; A, p<0.0001; B, p=0.0204, respectively). The interaction of lead and zinc were not significant (A, p=0.9037; B, p=0.8580).

Table 24. Renal zinc concentration (µmol zinc / g dry weight).

					MAIN EFFECTS	
	MZ	PW	C	SZ	LEAD	p value
0 Pb	1.25 ± 0.05	1.57 ± 0.02	1.54 ± 0.03	1.59 ± 0.06	**1.49 ± 0.03**	
+ Pb	1.25 ± 0.02	1.46 ± 0.04	1.41 ± 0.02	1.54 ± 0.03	**1.41 ± 0.02**	
MAIN EFFECTS ZINC	**1.25 ± 0.02^{c}**	**1.51 ± 0.03^{ab}**	**1.48 ± 0.02^{b}**	**1.57 ± 0.03^{a}**		**<0.0001**
p value					**<.0031**	0.2053

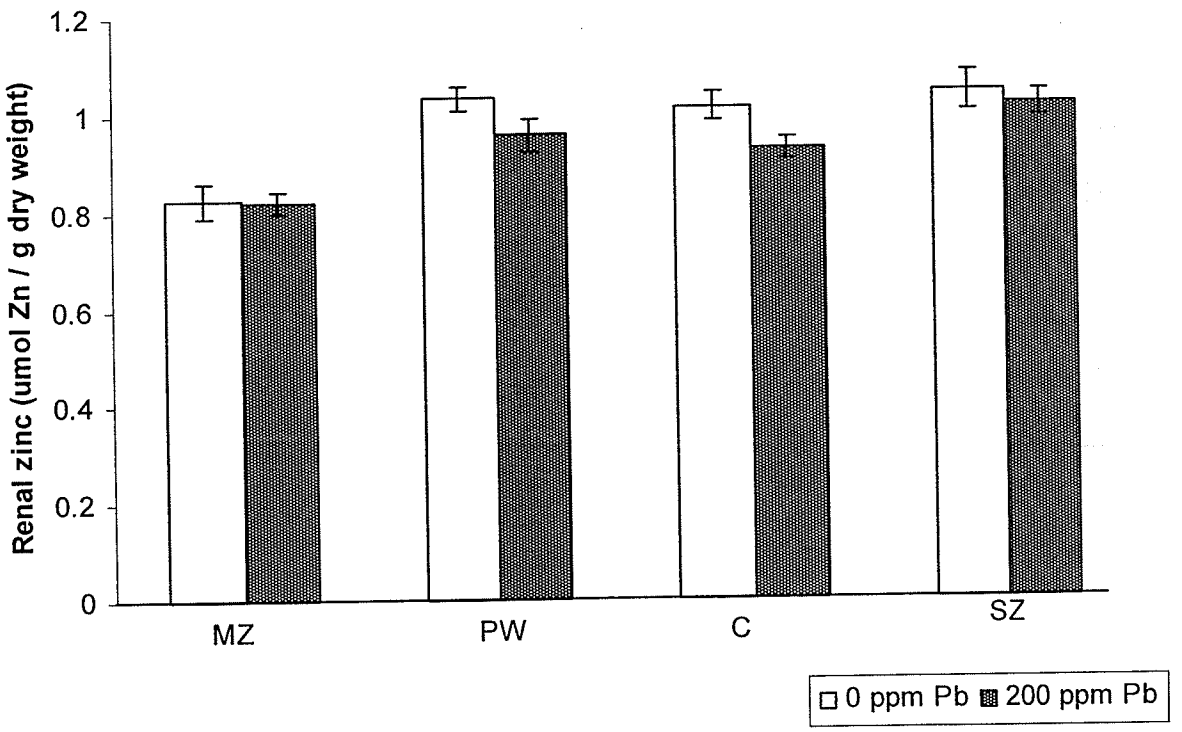

Figure 15. Effect of dietary zinc and lead exposure on renal zinc concentration. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant (p=0.0031; p<0.0001, respectively). The interaction of lead and zinc was not significant (p=0.2053).

Table 25. Intestinal weight (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	5.68 ± 0.25	6.09 ± 0.39	6.17 ± 0.36	6.66 ± 0.29	6.15 ± 0.17	
+ Pb	5.22 ± 0.17	5.34 ± 0.30	6.21 ± 0.34	6.52 ± 0.18	5.82 ± 0.16	
MAIN EFFECTS ZINC	**5.45 ± 0.16^{c}**	**5.72 ± 0.26^{bc}**	**6.19 ± 0.24^{ab}**	**6.59 ± 0.16^{a}**		**0.0015**
p value					0.1225	0.5612

Table 26. Intestinal weight to body weight ratio.

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.022 ± 0.0010	0.025 ± 0.0017	0.023 ± 0.0008	0.023 ± 0.0009	0.023 ± 0.0006	
+ Pb	0.023 ± 0.0011	0.023 ± 0.0011	0.024 ± 0.0008	0.024 ± 0.0008	0.023 ± 0.0005	
MAIN EFFECTS ZINC	0.023 ± 0.0007	0.024 ± 0.0010	0.023 ± 0.0010	0.024 ± 0.0006		0.6404
p value					0.7937	0.6975

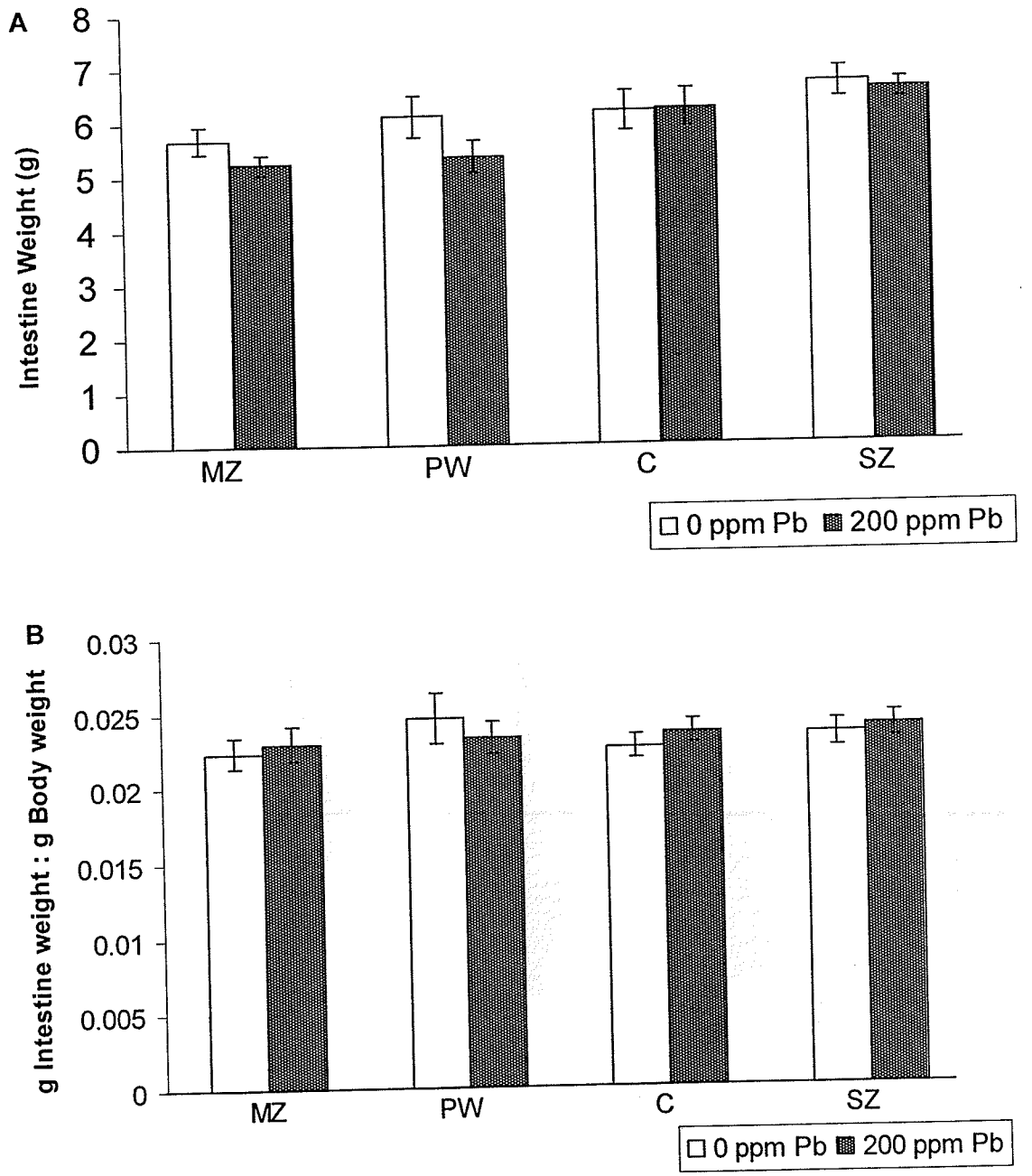

Figure 16. Effect of dietary zinc and lead exposure on intestinal wet weight (A) and intestinal wet weight to body weight ratio (B). Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0015) for intestinal weight, but the main effect of lead and the interaction of lead and dietary zinc were not significant (p=0.1225, p=0.5612, respectively). There were no significant differences in intestinal weight when calculated as a percentage of body weight (lead effect, p=0.7937; zinc effect p=0.6404; leadxzinc, p=0.6975).

Table 27. Intestinal length (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	5.68 ± 0.25	6.09 ± 0.39	6.17 ± 0.36	6.66 ± 0.29	106.09 ± 0.95	
+ Pb	5.22 ± 0.17	5.34 ± 0.30	6.21 ± 0.34	6.52 ± 0.18	104.88 ± 0.90	
MAIN EFFECTS ZINC	**102.56 ± 0.95^{b}**	**104.56 ± 1.10^{ab}**	**106.75 ± 1.53^{a}**	**108.06 ± 1.25^{a}**		**0.0125**
p value					0.3214	0.2950

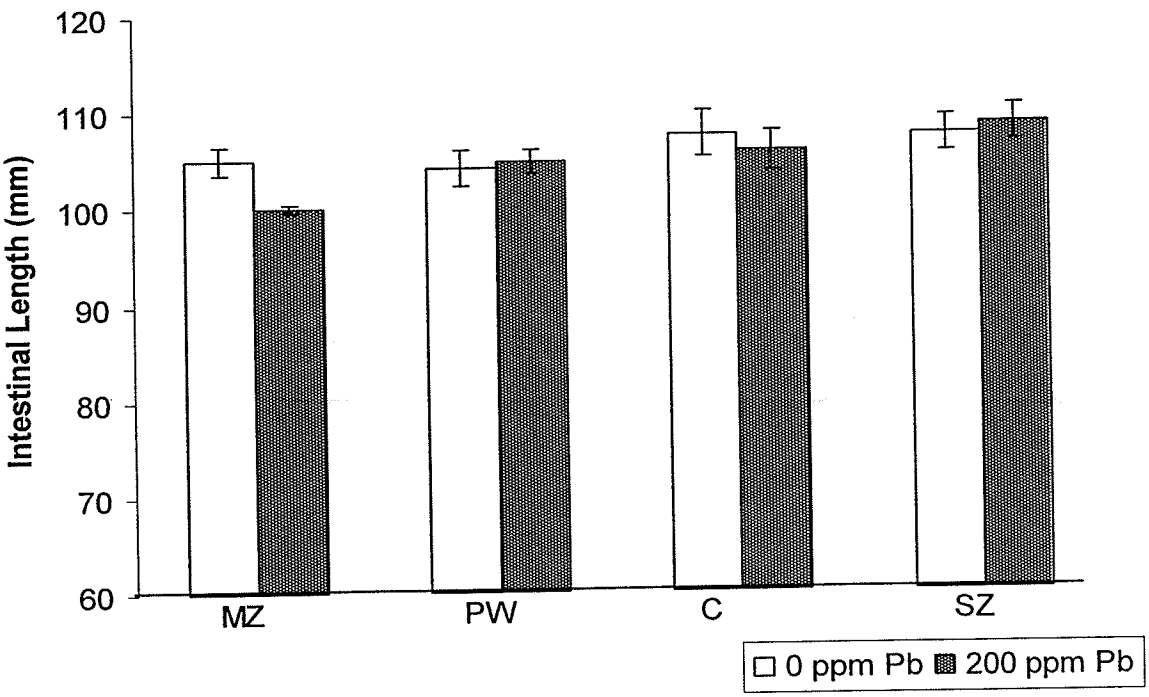

Figure 17. Effect of dietary zinc and lead exposure on intestinal length. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0125). The main effect of lead and the interaction of lead and zinc were not significant (p=0.3214 and p=0.2950, respectively).

Table 28. Duodenum zinc concentration (µmol/g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.47 ± 0.03	1.93 ± 0.06	1.91 ± 0.05	3.30 ± 0.02	2.17 ± 0.14	
+ Pb	1.42 ± 0.03	1.86 ± 0.05	2.20 ± 0.02	3.30 ± 0.04	2.19 ± 0.17	
MAIN EFFECTS ZINC	**1.44 ± 0.02[c]**	**1.89 ± 0.04[b]**	**2.05 ± 0.12[b]**	**3.30 ± 0.22[a]**		**<0.0001**
p value					0.7596	0.7705

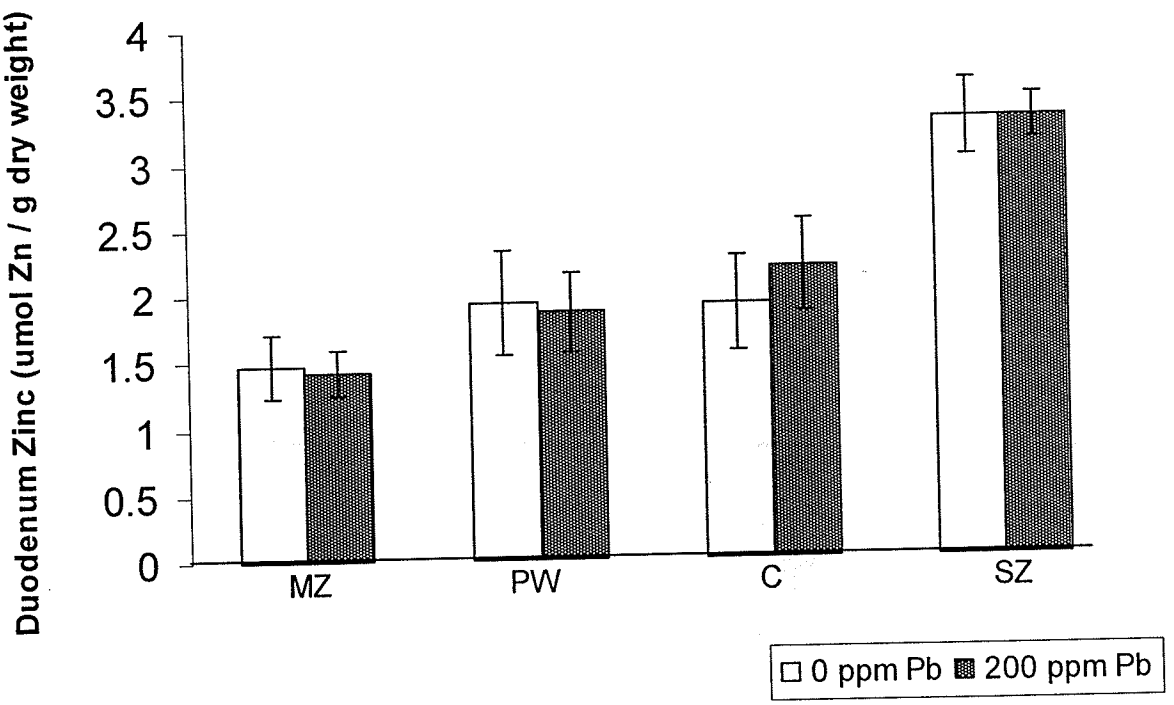

Figure 18. Effect of dietary zinc and lead exposure on duodenum zinc concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p< 0.0001). The main effect of lead and the interaction of lead and zinc were not significant (p=0.7596; p=0.7705, respectively).

Table 29. Duodenum lead concentration (µmol Pb / g dry weight).

	MZ	PW	C	SZ	p value
+ Pb	0.09 ± 0.01	0.13 ± 0.03	0.14 ± 0.03	0.09 ± 0.02	0.3485

Table 30. Jejunum zinc concentration (µmol Zn / g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.36 ± 0.04	2.19 ± 0.10	2.29 ± 0.05	3.52 ± 0.44	2.34 ± 0.18	
+ Pb	1.44 ± 0.03	2.11 ± 0.07	2.08 ± 0.12	3.30 ± 0.38	2.24 ± 0.16	
MAIN EFFECTS ZINC	**1.40 ± 0.03[c]**	**2.15 ± 0.06[b]**	**2.19 ± 0.07[b]**	**3.41 ± 0.28[a]**		**<0.0001**
p value					0.4987	0.8959

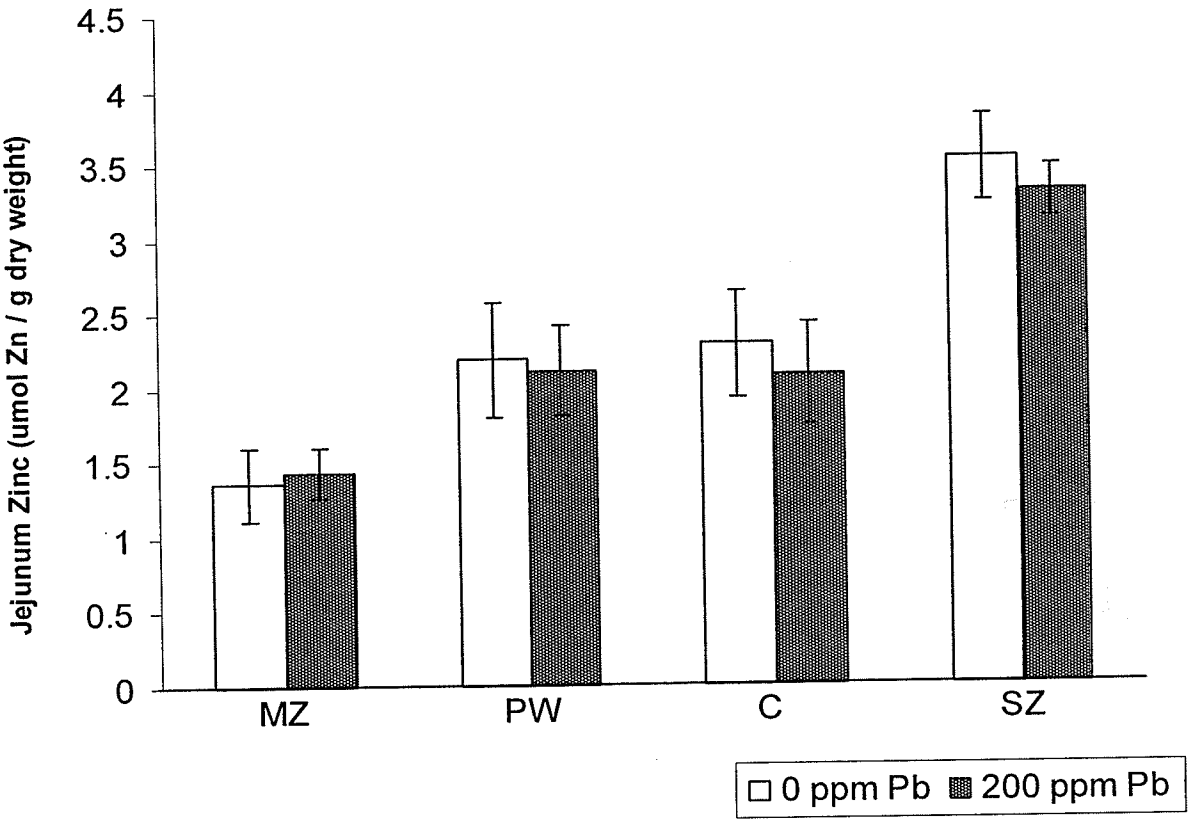

Figure 19. Effect of dietary zinc and lead exposure on jejunum zinc concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant ($p<0.0001$). The main effect of lead and the interaction of lead and zinc were not significant ($p=0.4987$; $p=0.8959$, respectively).

Table 31. Jejunum lead concentration (umol Pb / g dry weight).

	MZ	PW	C	SZ	p value
+ Pb	0.15 ± 0.02	0.19 ± 0.06	0.19 ± 0.03	0.23 ± 0.11	0.8594

Table 32. Duodenum MT mRNA ($2^{\Delta CT}$).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.04 ± 0.75	1.17 ± 0.28	1.35 ± 0.56	3.90 ± 0.75	1.99 ± 0.43	
+ Pb	0.11 ± 0.04	1.41 ± 0.68	2.15 ± 0.45	2.31 ± 0.98	1.50 ± 0.36	
MAIN EFFECTS ZINC	**0.57 ± 0.39[c]**	**1.29 ± 0.34[b]**	**1.75 ± 0.37[ab]**	**3.20 ± 0.63[a]**		**0.0040**
p value					**0.4240**	0.2499

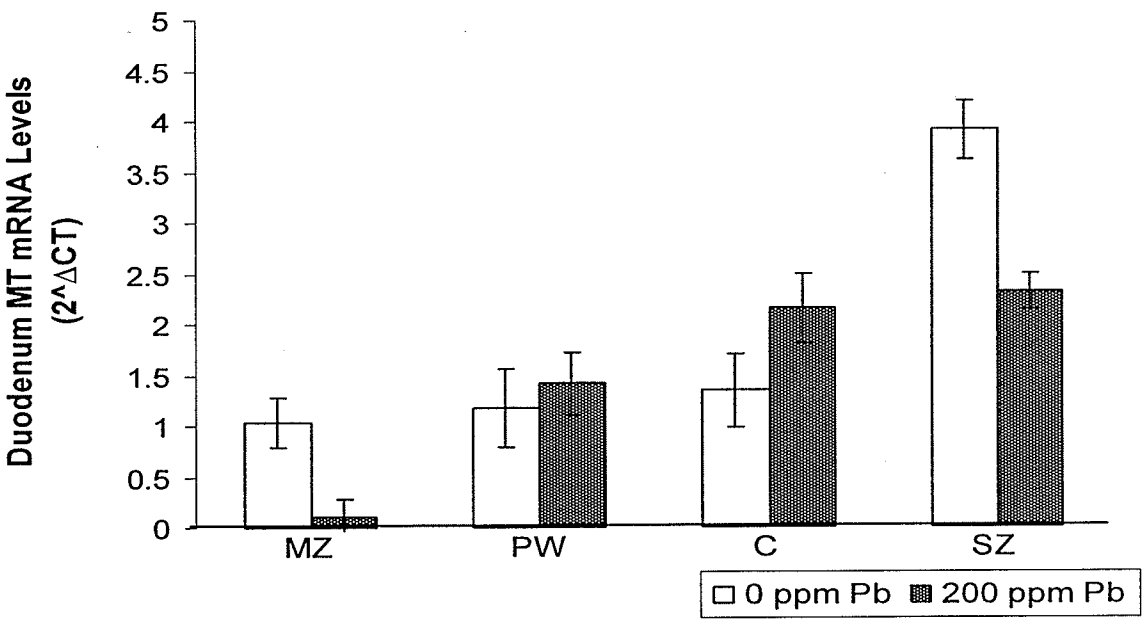

Figure 20. Effect of dietary zinc and lead exposure on duodenum MT mRNA levels. Columns represent group means ± SEM for n=4-5. The main effect of zinc was significant (p=0.0040). The main effect of lead and the interaction of lead and zinc were not significant (p=0.4240 and p=0.2499, respectively).

Table 33. Duodenum CRIP mRNA ($2^{\Delta CT}$).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.46 ± 0.40	1.39 ± 0.49	1.05 ± 0.18	1.43 ± 0.24	1.34 ± 0.16	
+ Pb	1.73 ± 0.33	1.72 ± 0.36	1.29 ± 0.56	0.99± 0.25	1.43 ± 0.19	
MAIN EFFECTS ZINC	1.59 ± 0.25	1.56 ± 0.29	1.17 ± 0.28	1.24 ± 0.18		0.5398
p value					0.7012	0.6701

Table 34. Jejunum MT mRNA ($2^{\Delta CT}$).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.24 ± 0.15	2.00 ± 0.77	1.34 ± 0.43	4.37 ± 1.89	1.82 ± 0.55	
+ Pb	0.11 ± 0.04	2.82 ± 0.70	1.91 ± 0.50	4.24 ± 1.56	2.18 ± 0.29	
MAIN EFFECTS ZINC	**0.17 ± 0.07^{c}**	**2.55 ± 0.52^{ab}**	**1.62 ± 0.33^{b}**	**4.30 ± 1.13^{a}**		**0.0005**
p value					0.6507	0.9301

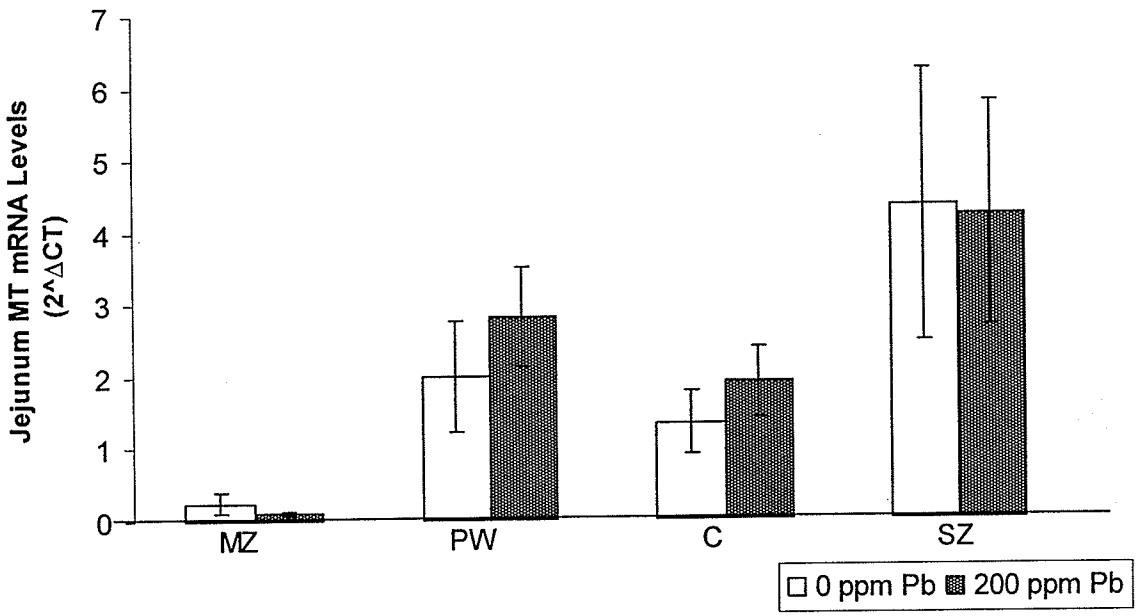

Figure 21. Effect of dietary zinc and lead exposure on jejunum MT mRNA levels. Columns represent group means ± SEM for n=3-6. The main effect of zinc was significant (p=0.0005). The main effect of lead and the interaction of lead and zinc were not significant (p=0.6507 and p=0.9301, respectively).

Table 35. Jejunum CRIP mRNA ($2^{\Delta CT}$).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.11 ± 0.12	1.19 ± 0.42	1.33 ± 0.39	1.26 ± 0.34	1.23 ± 0.15	
+ Pb	1.23 ± 0.35	2.04 ± 0.40	1.14 ± 0.18	1.16 ± 0.31	1.40 ± 0.17	
MAIN EFFECTS ZINC	1.18 ± 0.19	1.76 ± 0.32	1.23 ± 0.21	1.21 ± 0.22		0.5854
p value					0.4796	0.4693

Table 36. Femur dry weight (mg).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	341 ± 6	331 ± 10	340 ± 8	343 ± 8	**339 ± 4**	
+ Pb	303 ± 9	312 ± 7	336 ± 8	334 ± 7	**321 ± 4***	
MAIN EFFECTS ZINC	**322 ± 7[b]**	**322 ± 6[b]**	**338 ± 5[a]**	**339± 5[a]**		**0.0394**
p value					**0.0028**	0.1549

Table 37. Femur dry weight to body weight ratio.

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.010 ± 0.001	0.012 ± 0.001	0.012 ± 0.001	0.013 ± 0.001	0.012 ± 0.0004	
+ Pb	0.0099 ± 0.001	0.011 ± 0.001	0.012 ± 0.001	0.012 ± 0.001	0.011 ± 0.0004	
MAIN EFFECTS ZINC	**0.010 ± 0.001[b]**	**0.012 ± 0.001[a]**	**0.012 ± 0.001[a]**	**0.012 ± 0.001[a]**		**0.0204**
p value					0.1441	0.8580

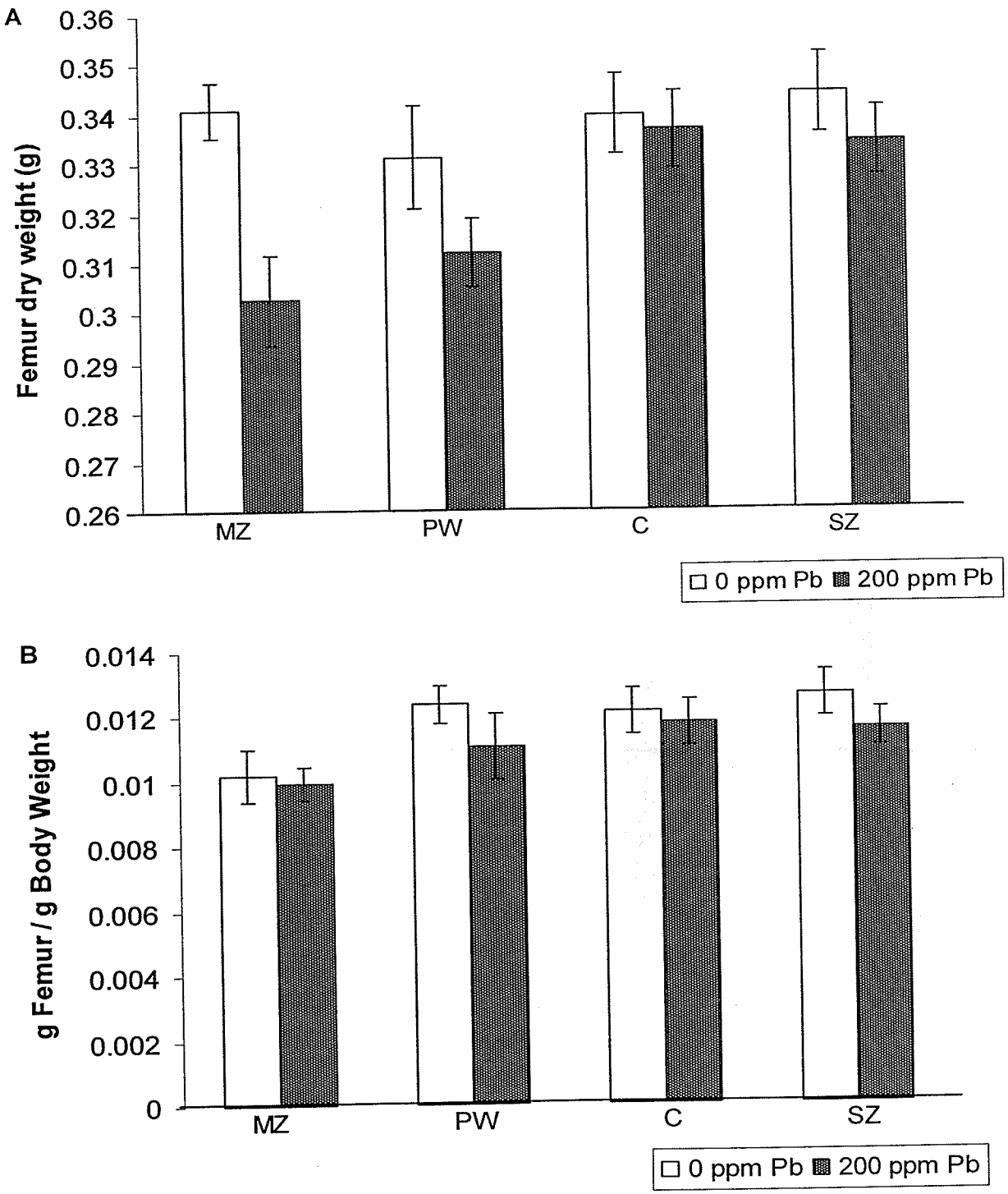

Figure 22. Effect of dietary zinc and lead exposure on femur dry weight (A) and femur dry weight to body weight ratio (B). Columns represent group means ± SEM for n=8. The main effects of zinc were significant (A, p=0.0394; B, p=0.0204), and the main effect of lead was significant (A, p=0.0028). The interactions of lead and zinc were not significant (A, p=0.1549; B, p=0.8580).

Table 38. Femur Calcium Concentration (mmol Ca / g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	5.14 ± 0.07	5.30 ± 0.09	5.36 ± 0.04	5.34 ± 0.06	5.29 ± 0.04	
+ Pb	5.11 ± 0.08	5.39 ± 0.08	5.32 ± 0.04	5.24 ± 0.09	5.26 ± 0.04	
MAIN EFFECTS ZINC	**5.13 ± 0.05[b]**	**5.35 ± 0.06[a]**	**5.34 ± 0.03[a]**	**5.34 ± 0.06[a]**		**0.0088**
p value					0.6765	0.5997

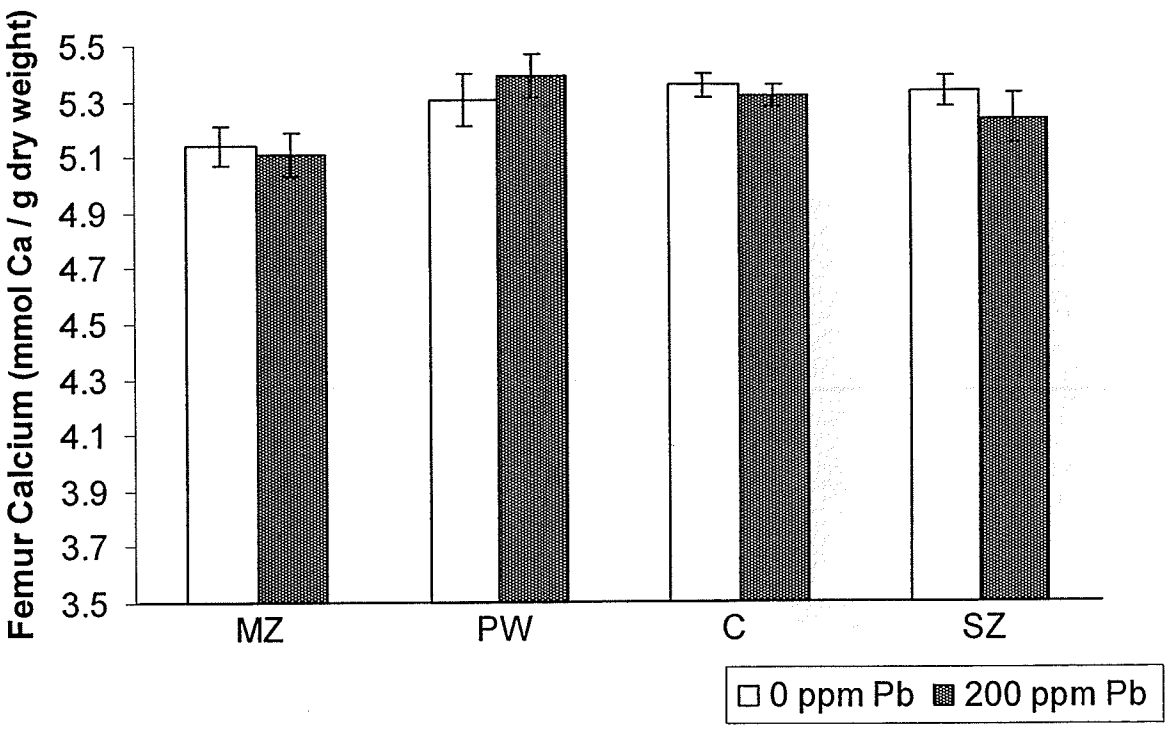

Figure 23. Effect of dietary zinc and lead exposure on femoral calcium concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0088), but the main effect of lead and the interaction of lead and zinc were not significant (p=0.6765; p=0.5997, respectively).

Table 39. Femur Phosphorus Concentration (mmol P / g dry weight).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	3.35 ± 0.04	3.45 ± 0.06	3.50 ± 0.02	3.49 ± 0.04	3.45 ± 0.02	
+ Pb	3.32 ± 0.05	3.51 ± 0.05	3.46 ± 0.03	3.42 ± 0.06	3.43 ± 0.03	
MAIN EFFECTS ZINC	**3.34 ± 0.03[b]**	**3.48 ± 0.04[a]**	**3.48 ± 0.02[a]**	**3.45 ± 0.03[a]**		**0.0052**
p value					0.6123	0.4981

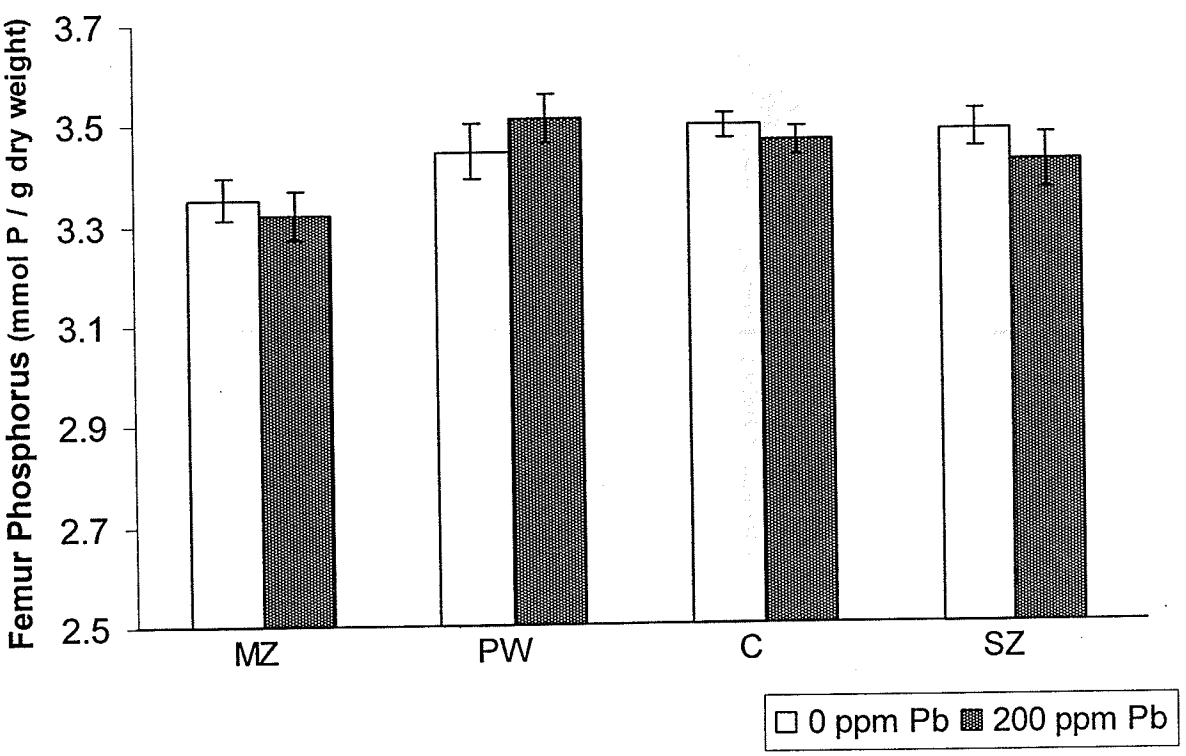

Figure 24. Effect of dietary zinc and lead exposure on femoral phosphorus concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0052), but the main effect of lead and the interaction of lead and zinc were not significant (p=0.6123; p=0.4981, respectively).

Table 40. Femur Length (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	32.11 ± 0.09	31.87 ± 0.11	32.07 ± 0.11	32.44 ± 0.10	**32.12 ± 0.12**	
+ Pb	31.16 ± 0.11	31.26 ± 0.10	31.78 ± 0.12	32.25 ± 0.10	**31.61 ± 0.15***	
MAIN EFFECTS ZINC	**31.64 ± 0.20[b]**	**31.56 ± 0.18[b]**	**31.93 ± 0.21[ab]**	**32.34 ± 0.16[a]**		**0.0145**
p value					**0.0064**	0.4375

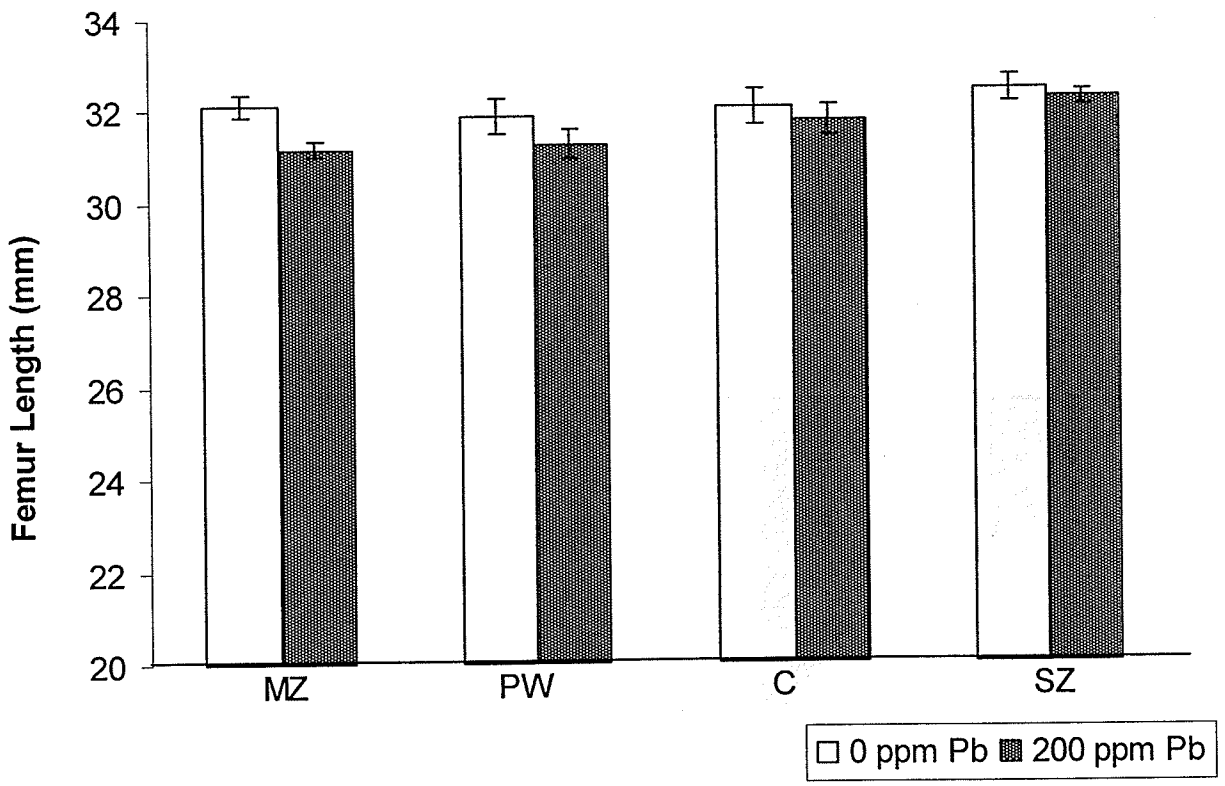

Figure 25. Effect of dietary zinc and lead exposure on femur length. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant (p=0.0064 and p=0.0145, respectively). The interaction of lead and zinc was not significant (p=0.4375).

Table 41. Femoral Knee Width (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	7.12 ± 0.04[a]	7.07 ± 0.06[a]	7.11 ± 0.05[a]	7.17 ± 0.04[a]	7.12 ± 0.03	
+ Pb	6.85 ± 0.06[b]	7.04 ± 0.04[ab]	7.11 ± 0.05[a]	7.21 ± 0.04[a]	7.05 ± 0.04	
MAIN EFFECTS ZINC	**6.98 ± 0.05[b]**	**7.05 ± 0.05[b]**	**7.11 ± 0.04[ab]**	**7.19 ± 0.03[a]**		**0.0074**
p value					0.1191	**0.0519**

Table 42. Femoral Head Width (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	4.42 ± 0.04	4.30 ± 0.06	4.37 ± 0.05	4.46 ± 0.04	**4.39 ± 0.03**	
+ Pb	4.22 ± 0.06	4.24 ± 0.05	4.38 ± 0.05	4.35 ± 0.05	**4.30 ± 0.03***	
MAIN EFFECTS ZINC	4.32 ± 0.05	4.27 ± 0.05	4.37 ± 0.04	4.40 ± 0.03		0.1372
p value					**0.0382**	0.3691

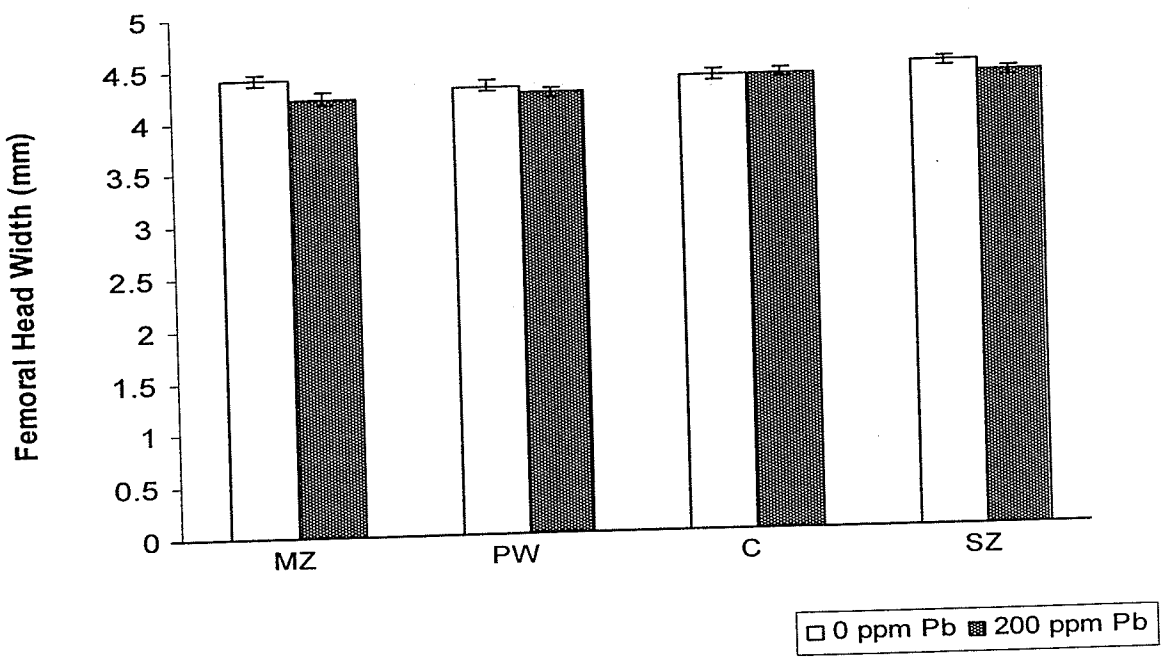

Figure 26. Effect of dietary zinc and lead exposure on femoral head width. Columns represent group means ± SEM for n=8. The main effect of lead was significant (p=0.0382), but the main effect of zinc and the interaction of lead and zinc were not significant (p=0.1372 and p=0.3691, respectively).

Table 43. Femoral Neck Width (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	2.03 ± 0.04	1.97 ± 0.04	2.00 ± 0.03	2.03 ± 0.04	2.01 ± 0.02	
+ Pb	1.97 ± 0.03	2.04 ± 0.04	2.01 ± 0.02	1.98 ± 0.03	2.00 ± 0.01	
MAIN EFFECTS ZINC	2.00 ± 0.02	2.00 ± 0.03	2.00 ± 0.01	2.01 ± 0.02		0.9999
p value					0.6246	0.1364

Table 44. Femoral Diaphysis Width (mm).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	3.89 ± 0.05	3.93 ± 0.07	3.91 ± 0.06	3.95 ± 0.05	3.92 ± 0.04	
+ Pb	3.77 ± 0.05	3.93 ± 0.04	3.94 ± 0.06	4.08 ± 0.05	3.93 ± 0.04	
MAIN EFFECTS ZINC	3.83 ± 0.05	3.93 ± 0.05	3.93 ± 0.05	4.02 ± 0.04		0.0908
p value					0.7953	0.3744

Table 45. Femoral Area (cm^2).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.39 ± 0.03	1.43 ± 0.04	1.45 ± 0.05	1.48 ± 0.03	1.44 ± 0.02	
+ Pb	1.31 ± 0.04	1.38 ± 0.03	1.43 ± 0.03	1.46 ± 0.02	1.39 ± 0.02	
MAIN EFFECTS ZINC	**1.35 ± 0.03^{b}**	**1.41 ± 0.03^{ab}**	**1.44 ± 0.03^{a}**	**1.47 ± 0.02^{a}**		**0.0058**
p value					0.0675	0.7484

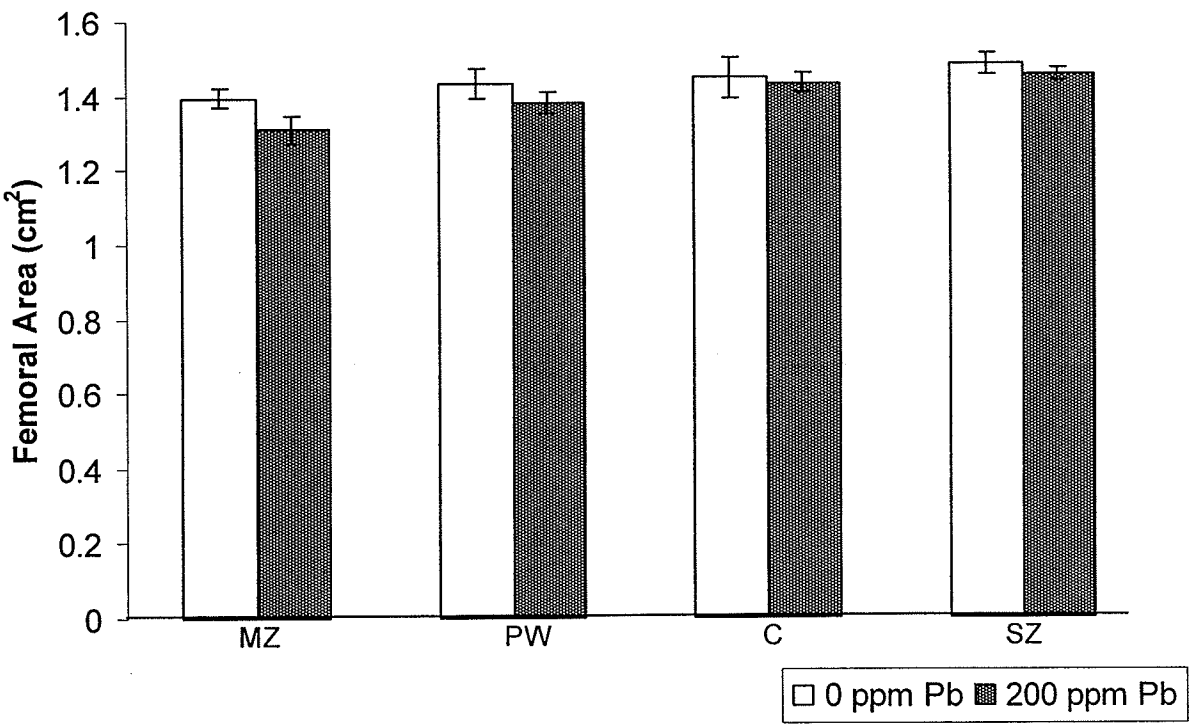

Figure 27. Effect of dietary zinc and lead exposure on femoral area. Columns represent group means ± SEM for n=5-8. The main effect of zinc was significant (p=0.0058). The main effect of lead and the interaction of lead and zinc were not significant (p=0.0675 and p=0.7484, respectively).

Table 46. Femur Bone Mineral Content (mg).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	197 ± 6	194 ± 7	209 ± 9	204 ± 7	**200 ± 4**	
+ Pb	176 ± 7	183 ± 5	202 ± 6	189 ± 7	**187 ± 4***	
MAIN EFFECTS ZINC	**186 ± 6^{b}**	**189 ± 5^{b}**	**205 ± 5^{a}**	**196 ± 5^{ab}**		**0.0484**
p value					**0.0085**	0.8139

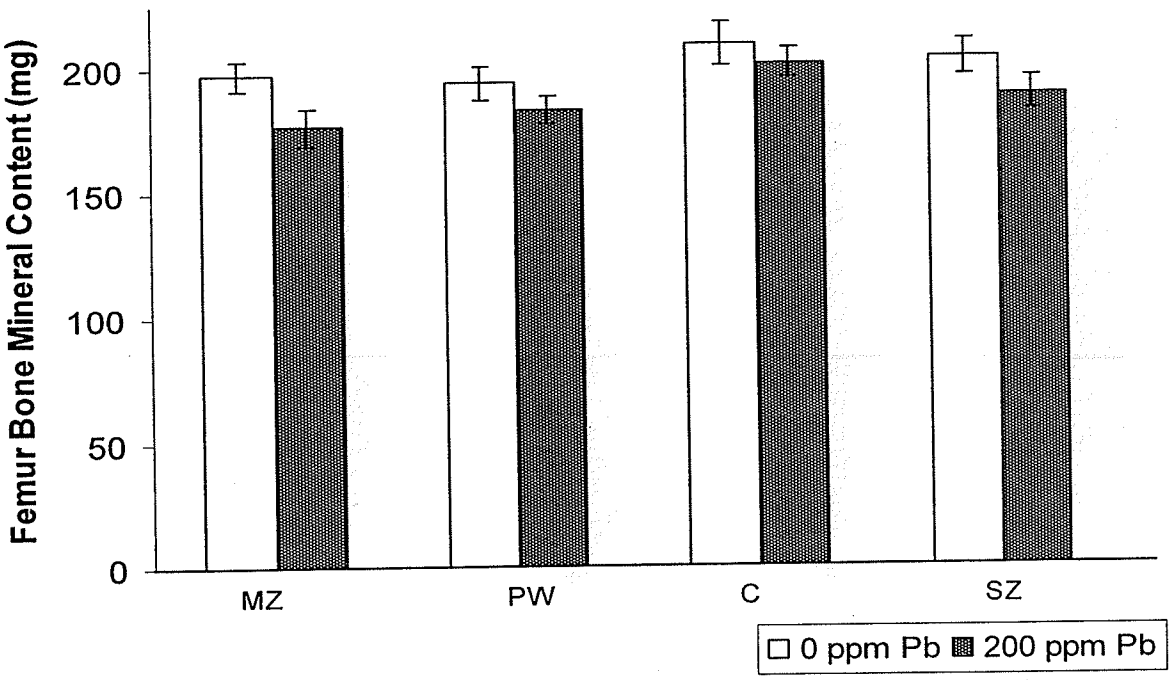

Figure 28. Effect of dietary zinc and lead exposure on femoral bone mineral content. Columns represent group means ± SEM for n=5-8. The main effects of lead and zinc were significant (p=0.0484 and p=0.0085, respectively), but the interaction of lead and zinc was not significant (p=0.8139).

Table 47. Femur Bone Mineral Density (mg / cm^2).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	141.4 ± 3.4	135.4 ± 2.0	144.52 ± 3.7	137.1 ± 3.0	**139.0 ± 1.5**	
+ Pb	134.7 ± 2.9	132.6 ± 3.1	140.7 ± 3.0	129.6 ± 3.4	**134.2 ± 1.7***	
MAIN EFFECTS ZINC	**137.8 ± 2.3^{ab}**	**134.1 ± 1.8^{b}**	**142.4 ± 2.3^{a}**	**133.3 ± 2.4^{b}**		**0.0191**
p value					**0.0209**	0.8304

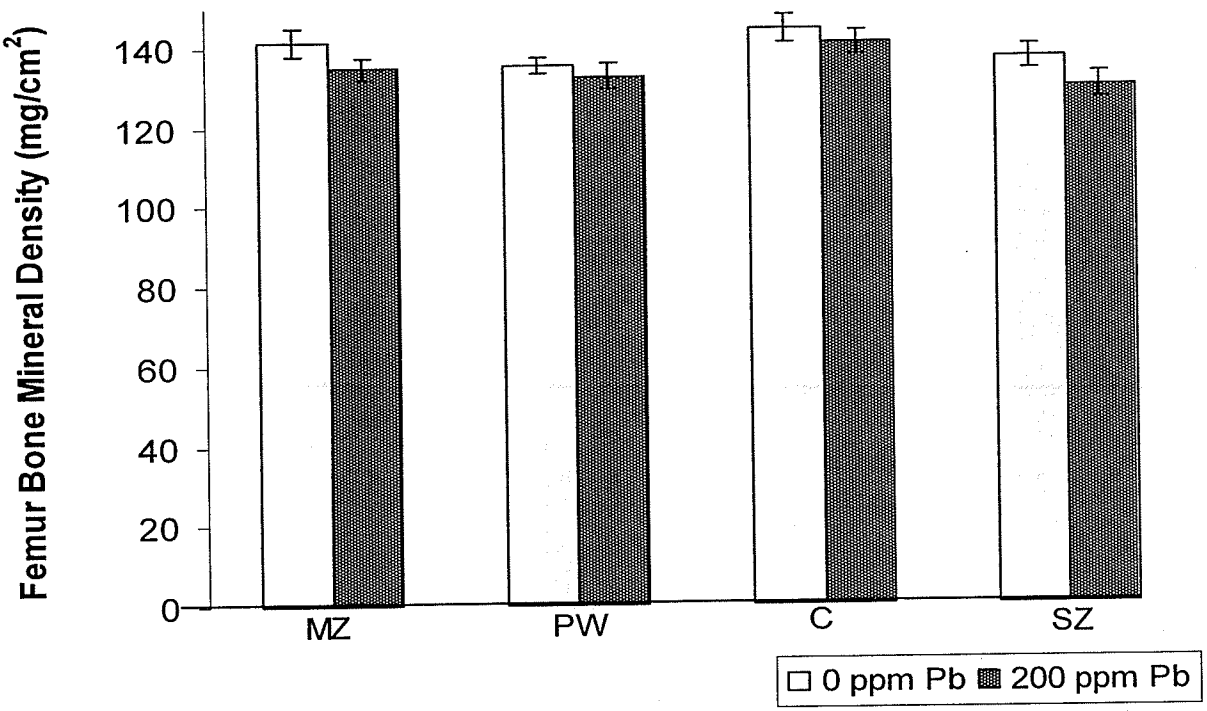

Figure 29. Effect of dietary zinc and lead exposure on femoral bone mineral density. Columns represent group means ± SEM for n=5-8. The main effects of lead and zinc were significant (p=0.0209 and p=0.0191, respectively), but the interaction of lead and zinc was not significant (p=0.8304).

Table 48. Whole body *in situ* high resolution scan of area (cm^2).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	50.0 ± 0.2	50.7 ± 0.2	52.0 ± 0.2	52.7 ± 0.2	**51.4 ± 0.5**	
+ Pb	45.5 ± 0.2	47.8 ± 0.2	51.0 ± 0.2	51.9 ± 0.2	**49.1 ± 0.6***	
MAIN EFFECTS ZINC	**47.8 ± 0.9[b]**	**49.3 ± 0.6[b]**	**51.5 ± 0.7[a]**	**52.3 ± 0.6[a]**		**<0.0001**
p value					**0.0004**	0.1183

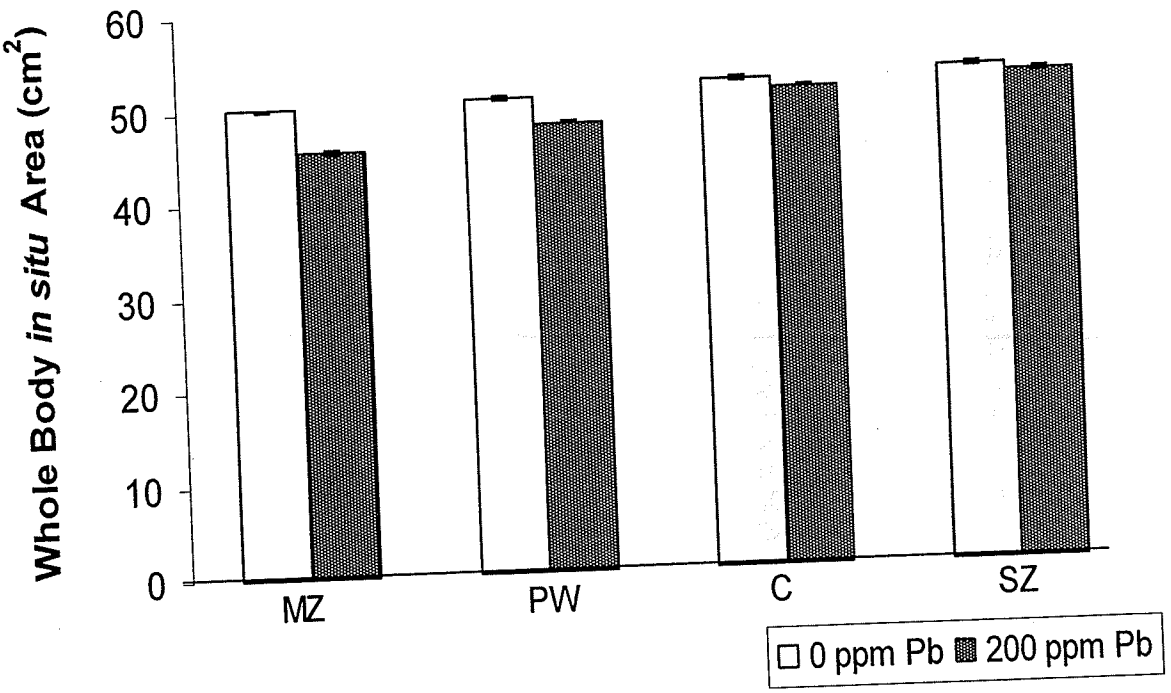

Figure 30. Effect of dietary zinc and lead exposure on whole body *in situ* area. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant (p=0.0004 and p<0.0001, respectively), but the interaction of lead and zinc was not significant (p=0.1183).

Table 49. Whole body *in situ* high resolution scan of Bone Mineral Content (g).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	5.861 ± 0.073	5.647 ± 0.069	5.974 ± 0.086	6.259 ± 0.080	**5.935 ± 0.076**	
+ Pb	5.224 ± 0.071	5.346 ± 0.062	5.939 ± 0.088	5.898 ± 0.066	**5.602 ± 0.082***	
MAIN EFFECTS ZINC	**5.543 ± 0.115[b]**	**5.500 ± 0.078[b]**	**5.956 ± 0.118[a]**	**6.078 ± 0.096[a]**		**<0.0001**
p value					**0.0007**	0.1604

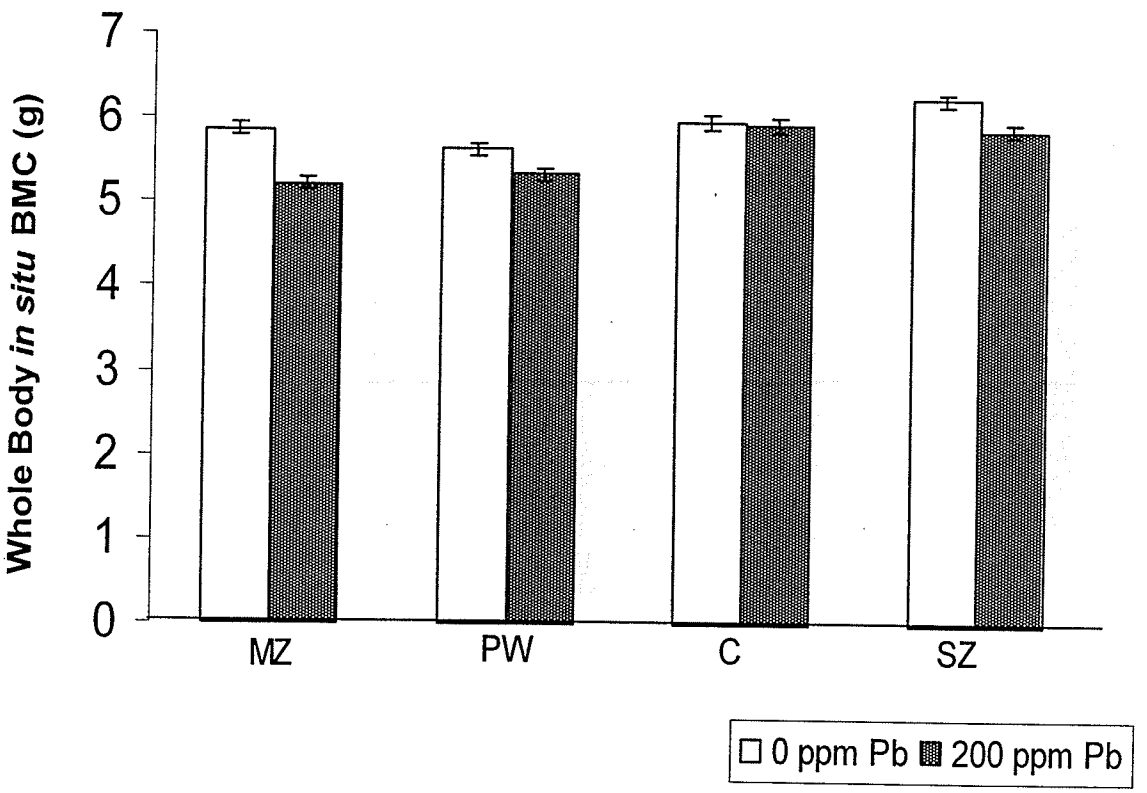

Figure 31. Effect of dietary zinc and lead exposure on whole body *in situ* BMC. Columns represent group means ± SEM for n=8. The main effects of lead and zinc were significant ($p=0.0007$ and $p<0.0001$, respectively), but the interaction of lead and zinc was not significant ($p=0.1604$).

Table 50. Whole body *in situ* high resolution scan of Bone Mineral Density (g / cm^2).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	0.117 ± 0.009	0.112 ± 0.009	0.115 ± 0.011	0.119 ± 0.011	0.116 ± 0.001	
+ Pb	0.115 ± 0.007	0.112 ± 0.010	0.116 ± 0.011	0.114 ± 0.010	0.114 ± 0.001	
MAIN EFFECTS ZINC	0.116 ± 0.001	0.112 ± 0.001	0.112 ± 0.002	0.116 ± 0.002		0.1248
p value					0.3705	0.4400

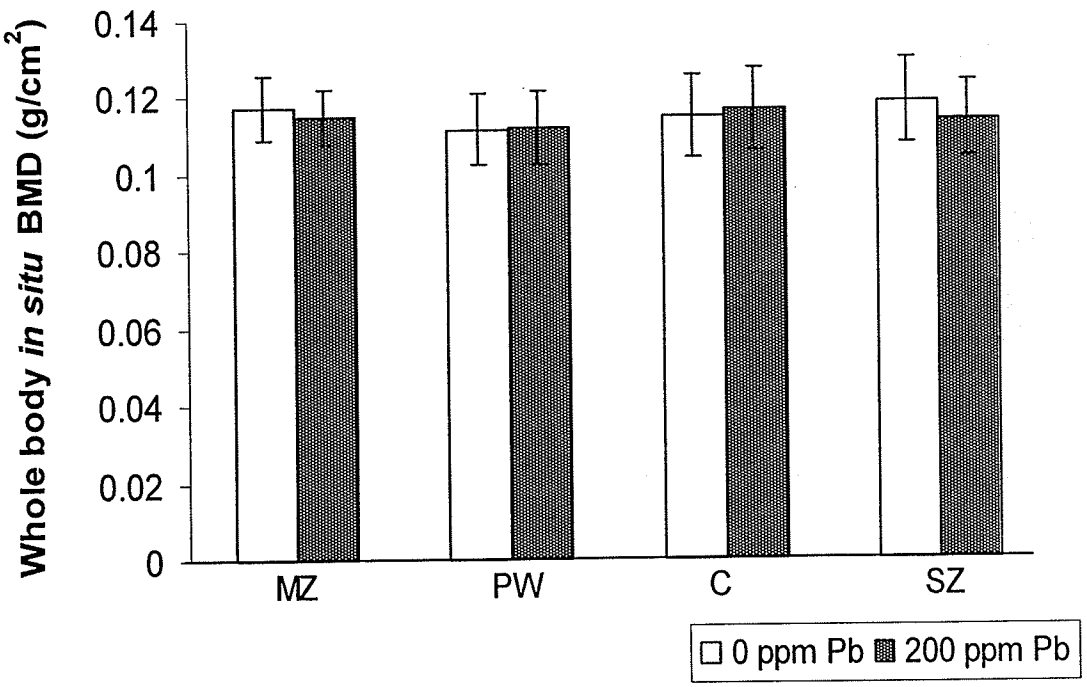

Figure 32. Effect of dietary zinc and lead exposure on whole body *in situ* BMD. Columns represent group means ± SEM for n=8. The main effects of lead and zinc and the interaction of lead and zinc were not significant (p=0.3705, p=0.1248, and p=0.4400, respectively).

Table 51. Spine *in situ* high resolution scans.

A) Area (cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.56 ± 0.04	1.57 ± 0.04	1.65 ± 0.03	1.78 ± 0.05	1.64 ± 0.03	
+ Pb	1.45 ± 0.03	1.59 ± 0.04	1.66 ± 0.04	1.67 ± 0.04	1.59 ± 0.02	
MAIN EFFECTS ZINC	**1.51 ± 0.03^{b}**	**1.58 ± 0.03^{b}**	**1.65 ± 0.02^{a}**	**1.72 ± 0.03^{a}**		**<0.0001**
p value					0.0775	0.1517

B) Bone Mineral Content (mg)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	315.21 ± 25.54	283.76 ± 25.30	316.93 ± 27.81	358.11 ± 23.35	**318.50 ± 8.5**	
+ Pb	279.09 ± 24.46	282.94 ± 21.60	319.34 ± 26.23	313.49 ± 22.38	**298.71 ± 6.9***	
MAIN EFFECTS ZINC	**297.15 ± 10.70^{bc}**	**283.35 ± 8.70^{c}**	**318.13 ± 11.30^{ab}**	**335.80 ± 9.90^{a}**		**0.0022**
p value					**0.0493**	0.2265

C) Bone Mineral Density (mg / cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	201.14 ± 13.39	180.88 ± 17.45	192.18 ± 20.66	201.78 ± 16.35	193.99 ± 3.60	
+ Pb	191.60 ± 18.27	178.58 ± 15.86	192.94 ± 20.00	187.99 ± 17.41	187.78 ± 3.70	
MAIN EFFECTS ZINC	196.37 ± 4.30	179.73 ± 4.30	192.56 ± 6.40	194.88 ± 4.80		0.0933
p value					0.2259	0.7336

Table 52. Humerus *in situ* high resolution scans.

A) Area (cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**0.84 ± 0.03^{a}**	**0.78 ± 0.03^{ab}**	**0.70 ± 0.02^{c}**	**0.83 ± 0.02^{a}**	**0.79 ± 0.02**	
+ Pb	**0.70 ± 0.03^{c}**	**0.74 ± 0.03^{bc}**	**0.74 ± 0.02^{bc}**	**0.81 ± 0.02^{ab}**	**0.75 ± 0.01***	
MAIN EFFECTS ZINC	**0.77 ± 0.03^{ab}**	**0.76 ± 0.02^{b}**	**0.72 ± 0.02^{b}**	**0.82 ± 0.02^{a}**		**0.0037**
p value					**0.0356**	**0.0077**

B) Bone Mineral Content (mg)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**155.50 ± 13.60^{a}**	**145.86 ± 8.60^{ab}**	**127.54 ± 10.10^{ab}**	**160.15 ± 6.20^{a}**	147.26 ± 5.20	
+ Pb	**116.80 ± 8.90^{b}**	**133.00 ± 12.90^{ab}**	**149.88 ± 5.90^{a}**	**137.34 ± 12.10^{ab}**	134.25 ± 5.40	
MAIN EFFECTS ZINC	136.15 ± 9.30	139.43 ± 7.70	138.71 ± 6.40	148.74 ± 7.20		0.6258
p value					0.0756	**0.0288**

C) Bone Mineral Density (mg / cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	183.83 ± 15.80	187.01 ± 11.40	183.38 ± 14.30	194.03 ± 4.70	187.059 ± 5.90	
+ Pb	165.89 ± 9.10	180.38 ± 19.90	203.90 ± 8.80	170.16 ± 15.30	180.08 ± 7.20	
MAIN EFFECTS ZINC	174.86 ± 9.10	183.69 ± 11.10	193.64 ± 8.50	182.09 ± 8.30		0.5644
p value					0.4581	0.3528

Table 53. Tibia *in situ* high resolution scans.

A) Area (cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**1.45 ± 0.04^{a}**	**1.37 ± 0.05^{abc}**	**1.25 ± 0.05^{cd}**	**1.45 ± 0.07^{a}**	**1.38 ± 0.03**	
+ Pb	**1.27 ± 0.04^{bcd}**	**1.21 ± 0.03^{d}**	**1.30 ± 0.05^{bcd}**	**1.39 ± 0.03^{ab}**	**1.29 ± 0.02***	
MAIN EFFECTS ZINC	**1.36 ± 0.04^{ab}**	**1.29 ± 0.03^{b}**	**1.28 ± 0.04^{b}**	**1.42 ± 0.04^{a}**		**0.0069**
p value					**0.0129**	**0.0563**

B) Bone Mineral Content (mg)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	266.05 ± 23.97^{a}	221.55 ± 24.12^{a}	234.60 ± 30.45^{a}	265.60 ± 24.69^{a}	246.95 ± 8.20	
+ Pb	248.14 ± 22.46^{a}	241.98 ± 23.08^{a}	264.69 ± 20.10^{a}	229.39 ± 24.91^{a}	246.05 ± 6.10	
MAIN EFFECTS ZINC	257.09 ± 8.70	231.76 ± 9.00	249.64 ± 11.70	247.49 ± 10.60		0.3231
p value					0.9267	**0.0630**

C) Bone Mineral Density (mg / cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	185.18 ± 22.45	162.84 ± 19.96	189.65 ± 29.12	183.81 ± 17.02	180.37 ± 6.20	
+ Pb	196.00 ± 21.63	200.14 ± 18.84	204.81 ± 16.86	165.95 ± 23.30	191.73 ± 5.30	
MAIN EFFECTS ZINC	190.59 ± 7.60	181.49 ± 7.6	197.23 ± 10.00	174.88 ± 7.10		0.2076
p value					0.1546	0.1140

Table 54. Femur *in situ* high resolution scans.

A) Area (cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	1.20 ± 0.03	1.14 ± 0.04	1.21 ± 0.05	1.27 ± 0.04	**1.20 ± 0.02**	
+ Pb	1.07 ± 0.01	1.10 ± 0.04	1.20 ± 0.05	1.22 ± 0.03	**1.15 ± 0.02***	
MAIN EFFECTS ZINC	**1.13 ± 0.02^b**	**1.12 ± 0.02^b**	**1.21 ± 0.03^a**	**1.25 ± 0.02^a**		**0.0012**
p value					**0.0236**	0.4638

B) Bone Mineral Content (mg)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**307.80 ± 11.70^{ab}**	**272.76 ± 12.50^b**	**270.66 ± 23.00^b**	**328.74 ± 11.90^a**	294.99 ± 8.60	
+ Pb	**283.21 ± 11.80^{ab}**	**299.56 ± 11.50^{ab}**	**291.61 ± 13.70^{ab}**	**283.21 ± 18.80^{ab}**	290.00 ± 6.90	
MAIN EFFECTS ZINC	296.70 ± 8.50	286.16 ± 8.90	281.14 ± 13.20	305.98 ± 12.3		0.3544
p value					0.6374	**0.0531**

C) Bone Mineral Density (mg / cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	252.78 ± 14.90	240.56 ± 11.40	227.43 ± 22.90	259.43 ± 9.50	245.05 ± 7.70	
+ Pb	267.14 ± 11.70	273.99 ± 9.70	248.00 ± 16.90	231.65 ± 14.90	255.19 ± 7.10	
MAIN EFFECTS ZINC	259.96 ± 9.40	257.28 ± 8.40	237.71 ± 14.00	245.54 ± 9.30		0.3956
p value					0.3301	0.1892

Table 55. Combined femur & fibia *in situ* high resolution scans.

A) Area (cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	2.64 ± 0.05	2.50 ± 0.06	2.46 ± 0.05	2.71 ± 0.07	**2.59 ± 0.04**	
+ Pb	2.34 ± 0.05	2.30 ± 0.05	2.49 ± 0.06	2.61 ± 0.04	**2.44 ± 0.04***	
MAIN EFFECTS ZINC	**2.49 ± 0.05^{b}**	**2.40 ± 0.05^{b}**	**2.48 ± 0.05^{b}**	**2.66 ± 0.05^{a}**		**0.0035**
p value					**0.0050**	0.1073

B) Bone Mineral Content (mg)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**573.14 ± 32.64^{ab}**	**493.76 ± 32.99^{b}**	**505.03 ± 40.80^{b}**	**592.96 ± 33.29^{a}**	541.22 ± 15.50	
+ Pb	**532.91 ± 31.20^{ab}**	**540.85 ± 31.46^{ab}**	**555.21 ± 25.77^{ab}**	**510.60 ± 37.21^{ab}**	534.89 ± 11.50	
MAIN EFFECTS ZINC	553.03 ± 16.60	517.31 ± 17.20	530.12 ± 20.60	551.78 ± 22.10		0.4520
p value					0.7325	**0.0306**

C) Bone Mineral Density (mg / cm^2)

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	**218.30 ± 22.96^{ab}**	**198.16 ± 20.80^{ab}**	**206.38 ± 26.99^{ab}**	**219.23 ± 18.54^{ab}**	210.52 ± 5.90	
+ Pb	**228.55 ± 22.44^{ab}**	**235.09 ± 19.34^{a}**	**224.38 ± 20.47^{ab}**	**196.66 ± 24.65^{b}**	221.17 ± 5.80	
MAIN EFFECTS ZINC	223.43 ± 8.10	216.63 ± 7.90	215.38 ± 9.50	207.94 ± 8.20		0.6096
p value					0.1940	**0.0827**

Table 56. Serum Osteocalcin Concentration (nmol / L).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	107 ± 8	117 ± 5	125 ± 5	129 ±5	119 ± 3	
+ Pb	107 ± 5	116 ± 3	119 ± 3	126 ± 3	117 ± 2	
MAIN EFFECTS ZINC	**107 ± 4^{c}**	**116 ± 3^{bc}**	**122 ± 3^{ab}**	**127 ± 3^{a}**		**0.0008**
p value					0.4913	0.9312

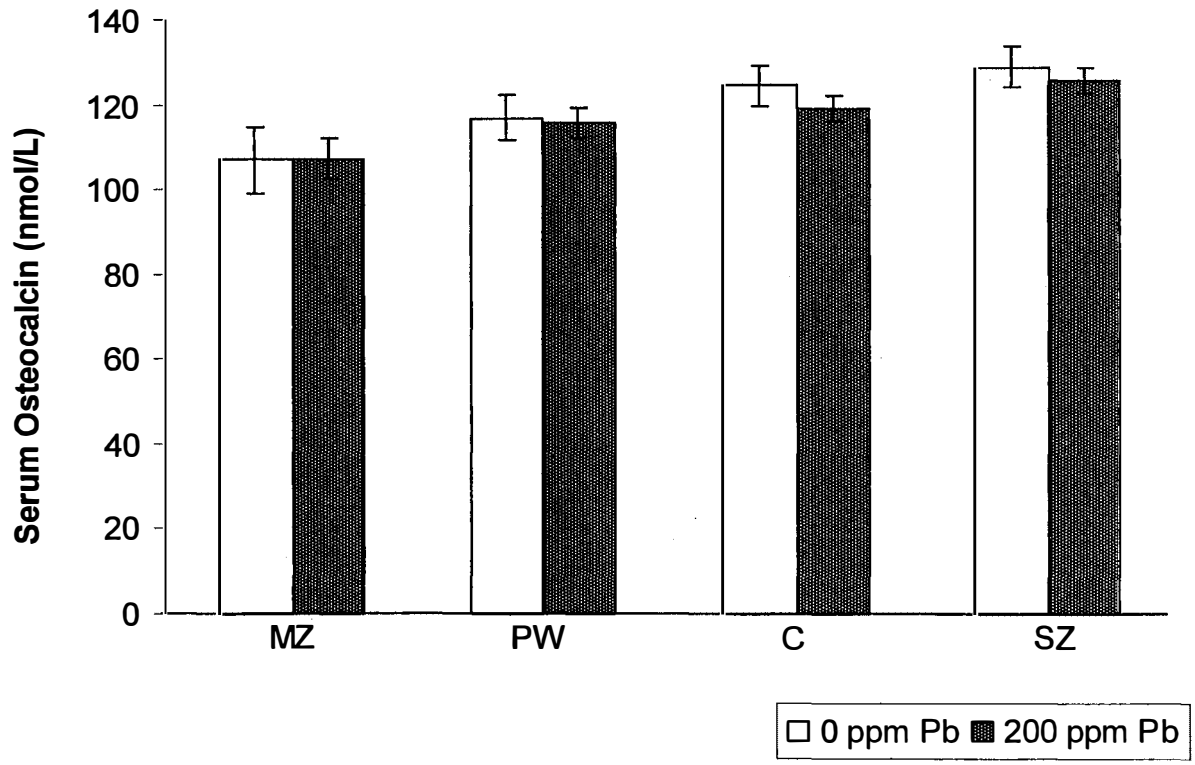

Figure 33. Effect of dietary zinc and lead exposure on serum osteocalcin concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0008), but the main effect of lead and the interaction of lead and zinc were not significant (p=0.4913 and p=0.9312, respectively).

Table 57. Serum Ratlaps Concentration (nmol / L).

	MZ	PW	C	SZ	MAIN EFFECTS LEAD	p value
0 Pb	114 ± 11	91 ± 9	109 ± 8	131 ± 12	111 ± 5	
+ Pb	105 ± 15	93 ± 8	131 ± 11	110 ± 11	110 ± 6	
MAIN EFFECTS ZINC	**109 ± 9^{ab}**	**92 ± 6^{b}**	**120 ± 7^{a}**	**121 ± 8^{a}**		**0.0394**
p value					0.9017	0.2433

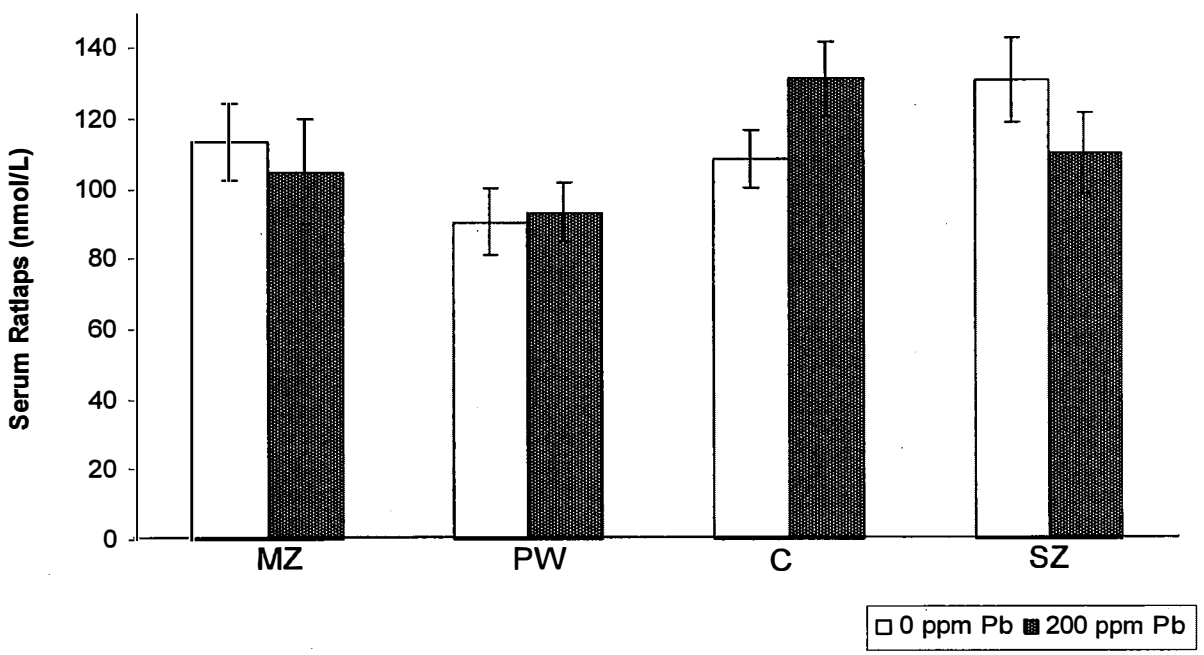

Figure 34. Effect of dietary zinc and lead exposure on serum osteocalcin concentration. Columns represent group means ± SEM for n=8. The main effect of zinc was significant (p=0.0394), but the main effect of lead and the interaction of lead and zinc were not significant (p=0.9017 and p=0.2433, respectively).

XII. APPENDIX B

ALAD ASSAY PROTOCOL.

$$\text{δ-ALA} + \text{δ-ALA} \xrightarrow[\text{δ-ALAD}]{} \textbf{PBG}\ \text{(Porphobilinogen)}$$

Preparation:

- Reserve floor model centrifuge (H503), N_2 tank, & spectrophotometer for use
- Set floor model centrifuge to 4°C
- Rotar should be stored in fridge overnight to chill to 4°C
- Turn on water bath to 37°C
- Check pH of pre-made buffers daily (assay is extremely sensitive to changes in pH)
- Chill labeled centrifuge tubes on ice

Tris Buffer:

May be made ahead of time and stored in fridge for 1-2 weeks.
0.25 M sucrose (FW= 57.05 g/mol) = 14.2625 g / L
10 mM Trizma acetate (FW=181.2 g/mol) = 1.812 g / L
Dissolve sucrose & Trizma acetate in double deionized water and pH to 6.8 with NaOH or Acetic Acid (0.5 – 1.0 M).

Sodium Acetate (NaAc) Buffer:

May also be made ahead of time and stored in fridge for 1-2 weeks [*but ALA & GSH must added only on the day of the assay].*
0.1 M NaAc·$3H_20$ (FW=136.08 g/mol) = 13.608 g/L
Dissolve NaAc in double deionized water and pH to 6.8 with Acetic Acid (0.5 – 1.0 M).
50 mL NaAc Buffer for daily use:
3.3 M ALA (δ-aminolevulinic acid hydrochloride; Sigma A-3785; FW=167.6 g/mol)=0.0276g
3.3 M GSH (reduced glutathione; Fisher BP25215; FW=307.3 g/mol) = 0.0507 g
Dissolve ALA and GSH in NaAc buffer, make up to 50 mL in volumetric flask.
Store buffer (on ice) & GSH powder (in fridge) under nitrogen & wrap bottle with parafilm.

Modified (2N) Ehrlich's Reagent (ER):

Must only be prepared on the day of the assay.
Prepare in fumehood. Store waste in labeled glass bottle.
$HClO_4$ (Perchloric Acid) is explosive when it dries. Ensure bottle is properly sealed & store in fumehood or solvent cabinet.
For 50 mL of ER (enough for 2 blocks of 8 samples):
Dissolve 1 g DMAB (dimethylaminobenzaldehyde) in ~30 mL Glacial Acetic Acid.
DMAB is light sensitive; turn out lights & wrap beakers with tin foil when working with it.
Add 9.333 mL of $HClO_4$, mix, then make up to 50 mL with Glacial Acetic Acid. Store in dark container or wrapped in tin foil in the fumehood.
When added to sample, colour takes 15 min to develop and is only stable for 10 min.

10% Trichloracetic Acid in 0.1 M Mercuric Chloride:

Prepare solution in fumehood; do not breathe in HgCl, wear respiratory mask to weigh.
For 50 mL solution:
Mix 5 g TCA & 1.3575 g HgCl (FW=271.5 g/mol) in ~40 mL double deionized water.
Make up to 50 mL in volumetric flask.

Tissue Preparation (Liver):

0.75 g wet weight in 20 mL of Tris buffer
Homogenize with Polytron PT2100 (30 seconds; speed 15)

Centrifuge to obtain Post-Mitochondrial Fraction:

Floor Model B-20 Centrifuge (Fisher Scientific)
Spin 4 samples at a time, with weight-balanced blanks.
3500 rpm (1000 g); 10 minutes
Remove supernatant to new, chilled tube
6000 rpm (3000 g); 10 minutes
Remove supernatant to new, chilled tube
11 000 rpm (13 000 g); 15 min
Store 0.5 mL aliquot of supernatant in -20°C freezer for protein assay

Reaction Mixture (1 mL):

Add 300 µL of supernatant & 700 µL NaAc buffer (with GSH & ALA) in a 1.5 mL microcentrifuge tube and put under nitrogen before closing each tube.
Prepare samples in triplicate and one blank for each sample with 300 µL Tris buffer + 700 µL µL NaAc buffer.
Vortex tubes and incubate in 37°C water bath for 90 minutes.

Reaction Termination:

Add 300 µL or TCA-HgCl solution to each tube
Vortex & Centrifuge 3250 rpm, 5 minutes.

NB – Turn on spectrophotometer to warm up at least 20 minutes before use.

Colourimetric Determination:

Prepare in fumehood in the dark.
Combine 750 µL reaction mixture with 750 µL ER.
Prepare one blank with 750 µL double deionized water & 750 µL ER.
Vortex tubes & store in the dark for 15 minutes.
Stagger addition of ER so each tube incubates for the same amount of time (about 45-60s).
Use the water/ER blank to zero the spectrophotometer.
Read samples & blanks at 553 nm in the order in which the ER was added.
Samples must all be read by 25 minutes from ER addition or colour will degrade.

NB - All waste from TCA-HgCl & ER must be stored in a labeled glass bottle in fumehood.

ALAD Activity = Rate of PBG formation

$$\text{Concentration} = \Delta A \text{ (mmol sample solution)} \left(\frac{V}{\epsilon \times d \times Vs}\right)$$

V = final volume (mL) = 1.5 mL
Vs = sample volume (mL) = 0.75 mL
d = light path (1 cm for Spectronic 3000 spectrophotometer)
ϵ = absorption coefficient at 553 nm (61 x (1 x $mmol^{-1}$ $cm^{-1})^3$
ΔA = absorbance difference of sample – blank

$$\text{Concentration} = \Delta A \left(\frac{1.5 \text{ mL}}{61 \times (1 \times \text{mmol}^{-1}\ \text{cm}^{-1})^3 \times 1 \text{ cm} \times 0.75 \text{ mL}}\right) = \text{mmol PBG / L}$$