THE EFFECTS AND INTERACTIONS OF COPPER, ZINC, AND CHROMIUM ON PLASMA LIPIDS, GLUCOSE, AND TISSUE AND BONE MINERALS IN MALE JAPANESE QUAIL

By

REBECCA GAIL NIHISER

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Thesis Approved:

Janice R. Hermann
Thesis Adviser

Barbara J. Stoecker

Christie F. Hanson

Lee L. Ebro

Thomas C. Collins
Dean of the Graduate College

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CHAPTER I

RESEARCH PROBLEM

Introduction to Topic

Diet has been associated with disease throughout history. Prior to the 1940's, diseases such as rickets, pellagra, scurvy, beriberi, xerophthalmia, and goiter were common throughout the world (1). Societies have evolved, however, and nutritional deficiencies are no longer the sole nutritional concern. Diseases of dietary excesses and imbalances have also become linked to illness and death (1). More recently, epidemiologic data have linked many trace elements to the incidence of chronic diseases such as cancer, coronary heart disease, and hypertension (2). However, much of the epidemiologic data have been inconclusive and incomplete and more research is recommended (2).

Coronary heart disease has consistently been a leading cause of death in America for several decades. More than 500,000 Americans die of coronary heart disease each year, 1 million Americans suffer from heart attacks and over 6 million Americans live with symptoms of coronary heart disease. Elevations in plasma lipids and cholesterol

values are strongly associated with the development of coronary heart disease (3).

Both diets low in saturated fat and cholesterol, and increased exercise are beneficial in lowering plasma lipids (4). However, other dietary factors including trace minerals may also affect plasma lipids and lipoprotein profiles. Low intakes of several minerals, including copper, zinc, and chromium have been implicated in the incidence of coronary heart disease (5-7). These minerals may have potential for lowering heart disease risk. This is of great importance because trace mineral intakes are low in typical American diets (8). However, no currently published research has dealt with effects of the interrelationship of these minerals on plasma lipids.

Osteoporosis and age related bone loss are also common afflictions, particularly in the elderly population (9,10). Much of the research in this area has focused on low intakes of dietary calcium and/or vitamin D as the major contributing factor (9,11). However, additional nutrients, including trace minerals are related to bone loss and have an impact on bone mineral content (11).

Significance of the Problem

In 1975, Klevay began investigating correlations between the trace mineral status of zinc and copper and coronary heart disease incidence (5). Zinc and copper are antagonistic for absorption (12), and a higher zinc intake

relative to copper may suppress copper absorption resulting in hypercholesterolemia induced by copper deficiency (13). Klevay theorized that a high zinc to copper ratio contributed to hypercholesterolemia, and therefore increased the risk of coronary heart disease (5).

Independent copper deficiency also is recognized as a causal agent in the precipitation of hypercholesterolemia (13). Copper deficiency has been reported to increase plasma cholesterol concentrations from 30% to as high as 185% (13). Copper deficiency also has been reported to increase tissue cholesterol concentrations and decrease tissue and organ copper concentrations (5,13-15).

Zinc also is a trace mineral that appears to have an effect on plasma cholesterol values (16). This effect, however, as previously discussed, has been attributed to copper deficiency induced by excessive zinc intake. A human study investigating the effect of excessive zinc supplementation (300 mg zinc per day), reported an increase in low density lipoprotein (LDL) cholesterol and a decrease in high density lipoprotein (HDL) cholesterol (16). Moderate zinc intakes also may affect plasma cholesterol. Moderate zinc intakes in humans (50-75 mg zinc per day) also have been reported to depress HDL cholesterol (16). In this study serum copper concentrations were not significantly affected by zinc intake, thus indicating that the effect of zinc intake on plasma cholesterol may be independent of copper status.

In addition, dietary chromium also may influence serum cholesterol concentrations. Chromium deficiency in rats has resulted in increased serum cholesterol values, and chromium supplementation in various forms has returned serum cholesterol values to normal (17,18). "Controlled" human studies have shown decreases in serum cholesterol concentrations and higher HDL levels upon supplementation with chromium-rich yeast (19,20).

A recent study using rabbits as the experimental animal also illustrated an effect of chromium on cholesterol concentrations (19). In this study, the rabbits consumed chromium in their water in the form of potassium chromate or chromium chloride. The chromium-treated rabbits had a significantly lower cholesterol concentration "per-unit length of aorta," in comparison with the non-chromium treated rabbits.

The potential influences of trace mineral intakes on plasma cholesterol are numerous and diverse. Dietary fat consumption is no longer the singular etiological link to cholesterol values. Regulation and metabolism of cholesterol is far more complex than once thought.

In addition to the increased recognition of associations between trace minerals and disease states, tissue and bone mineral concentrations may also provide valuable information in regard to diagnosing and assessing deficiency and disease states. Copper is an important factor in osteoporosis (10). Copper deficiency and the

resultant lysyl oxidase deficiency are responsible for defects in collagen and elastin which leads to connective tissue abnormalities (10). Osteoporosis is the probable consequence. Assessment of tissue and bone copper concentrations could be valuable in interpreting the extent of this condition. However, the inadequacy of knowledge concerning tissue copper concentrations limits the extent to which these values can be interpreted (10). Therefore, more research in the area of tissue and bone copper concentrations and their relationship to disease is warranted.

Zinc also could serve as an indicator or marker, providing information on the extent or nature of deficiency or disease states. A retrospective study in humans has shown positive associations between coronary heart disease, acute myocardial infarctions, and plasma zinc concentrations (21). Tissue and bone zinc concentrations can also provide information about how the deficiency state is affecting homeostatic regulation of zinc. Bone is the chief reservoir of mobilizable zinc (22). "Low priority" tissues such as bone and skin may have zinc mobilized from them for use in "high priority" tissues such as the muscle and brain (23). Plasma zinc falls when homeostatic regulation through tissues such as bone can not be maintained (24).

Tissue and bone chromium concentrations seem to provide indicative information about the nature and extent

of diabetes. In areas where the incidence of maturity-onset diabetes and atherosclerosis are high, tissue concentrations of chromium tend to be lower (25). This may be of importance because some individuals with inadequate chromium intake exhibit diabetes-like symptoms (26). In addition, chromium supplementation may improve glucose control in subjects with marginal chromium status or in insulin resistant subjects (27,28). Therefore, analysis of tissue chromium could provide valuable information as to the nature of diabetes-like conditions.

Objectives

The objectives of this research are to investigate the effects and interactions of three trace minerals (copper, zinc, and chromium) on plasma lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides), plasma glucose, tissue (liver and spleen) and bone minerals and the maximum force required to fracture femurs in male Japanese quail.

Hypotheses

The following hypotheses were developed for this study:

1. There will be no statistically significant independent and/or interactive effect(s) of dietary copper, zinc, and/or chromium on plasma lipids (total cholesterol, LDL-

cholesterol, HDL-cholesterol, and triglycerides) in male Japanese quail.

2. There will be no statistically significant independent and/or interactive effect(s) of dietary copper, zinc, and/or chromium on plasma glucose in male Japanese quail.
3. There will be no statistically significant independent and/or interactive effect(s) of dietary copper, zinc, and/or chromium on liver, spleen, and bone mineral concentrations in male Japanese quail.
4. There will be no statistically significant independent and/or interactive effect(s) of dietary copper, zinc, and/or chromium on the maximum force required to fracture femurs in male Japanese quail.

Limitations

The rat is typically not the animal model of choice when addressing cholesterol issues because rodents genetically have a high level of HDL cholesterol (13). Japanese quail are an appropriate small animal model for plasma lipid research because they are relatively atherosclerotic and they mature rapidly. However, data from animal research cannot be extrapolated directly to humans. The quail model has other limitations. Quail are very susceptible to bacterial overgrowth in early stages of

life. This bacterial overgrowth, if not lethal may interfere with nutrient requirements and complicate the study.

Format of Thesis

Chapters III and IV are written in manuscript form using the guide for authors for the Journal of Nutrition.

CHAPTER II

REVIEW OF LITERATURE

This chapter includes a review of the literature on copper, zinc, and chromium. The effects of these minerals on plasma lipid concentrations and tissue and bone mineral concentrations are reviewed.

Copper

Copper is an essential element for man. The duodenum is the major site for copper absorption (29). The primary storage sites are the liver and spleen (29). The liver, in most animals, has the highest concentration of copper (29, 12). The copper content of the entire human body is about 50 to 120 mg. This amount is usually found bound to proteins (12). Serum copper concentrations have been found to range from 0.50 mg/l to 2.2 mg/l (30). As age increases so do copper concentrations (30).

Copper has numerous functions in the human body. For example, it has a role in antioxidation, skeletal integrity, glucose metabolic regulation, and myelin formation (31,32). The estimated safe and adequate daily dietary intake for copper is 1.5 to 3.0 mg/day for adults (26).

Copper Deficiency and Lipids

Copper is more recently known for it's influence on heart disease. Copper deficiency has been implicated in the development of hypercholesterolemia (33) and may play an important role in cardiovascular system integrity (31). Animal *in vivo* studies have demonstrated that hypercholesterolemia is associated with copper deficiency (15,33,34). This marked elevation in cholesterol is attributed to increases in different lipoproteins and subfractions (33-37). Allen and Klevay (33), although noting an increase in both high density lipoprotein-cholesterol and low density lipoprotein-cholesterol, observed a significant relative decrease in the percent of plasma cholesterol associated with the high density lipoproteins (HDL) in copper-deficient rats. They observed that high density lipoprotein-cholesterol increased 85%; however, when the HDL-bound cholesterol was expressed as a percentage of total plasma cholesterol concentration there was a 9.6% decrease (33). The bulk of the hypercholesterolemia observed came from a 187% increase in plasma low density lipoprotein-cholesterol.

Harvey and Allen (34) also noted a decrease in plasma HDL-cholesterol associated with copper deficiency in rats. The cholesterol in this fraction decreased by 17% and 12% when compared to *ad libitum* and pair-fed controls, respectively. More specifically, the percent cholesterol esters in the HDL fraction was significantly decreased,

while a significant increase in the free cholesterol in HDL was observed. In contrast, very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were characterized by a significant decrease in free cholesterol and a significant elevation in the percent of cholesterol esters. The total cholesterol from VLDL and LDL in the copper deficient animals increased 184% compared to pair fed controls and 203% compared to ad libitum controls. Copper deficiency essentially reversed the normal HDL/(VLDL +LDL) cholesterol ratio of 2:1 in copper adequate rats (34). Harvey and Allen (34) attributed the differences between the observed decrease in HDL cholesterol and the observed increase in HDL cholesterol by Allen and Klevay (33) to the different durations of the studies. However, both studies observed significant increases in triglycerides. A 252% increase in the plasma triglyceride concentration was observed by Allen and Klevay (33) and 188% and 195% increases in triglycerides were observed between copper-deficient rats and the pair-fed and ad libitum controls by Harvey and Allen (34).

Croswell and Lee (36) found increases in all lipoprotein levels in copper-deficient rats with the largest increases occurring in very low density lipoprotein (VLDL) and low density lipoprotein (LDL) fractions. Lefevere and co-workers (37) also noted a large increase in plasma total cholesterol of copper-deficient rats. However, they attributed the hypercholesterolemia in copper

deficiency to selective increases in HDL cholesterol. They did not observe a difference in VLDL cholesterol and LDL cholesterol or triglycerides between control and copper-deficient rats. Additionally, Lee and Koo (35) also observed an increase in total cholesterol with a marked increase in the HDL fraction among copper-deficient rats.

Conflicting findings have been reported (35-37) on the effect of copper deficiency on HDL subclasses. Lee and Koo (35) concluded that the hypercholesterolemia they observed was primarily due to an increase in the HDL subfraction that contained large amounts of apolipoprotein A-I (Apo A-I) but contained no apolipoprotein E (Apo E). Croswell and Lei (36), in contrast, attributed the hypercholesterolemia induced by copper deficiency to increases in cholesterol and apolipoprotein contents of the Apo E-rich HDL; however Apo A-I concentrations were also significantly higher in copper-deficient rats compared to copper-adequate controls (36). Lefevere and co-workers (37) also found significantly higher Apo A-I levels in the HDL fractions of copper-deficient rats than in control animals. Additionally, Apo E-rich fractions also increased but did not reach significance (37).

Induced hypercholesterolemia, by the addition of 0.50% cholesterol and 0.25% cholic acid, in rats has been shown to decrease or disappear with copper supplementation (38). This modification of hypercholesterolemia occurred late in the experiment. When the level of added cholesterol and

cholic acid was reduced to 0.20% and 0.10%, respectively, the addition of dietary copper was sufficient to restore liver copper concentrations from decreased levels.

In humans, similar copper deficiency effects have been observed (39). In one study of a healthy man with induced copper depletion, significant increases in total and low density lipoprotein cholesterol levels were observed (39). Although ceruloplasmin, plasma copper, and superoxide dismutase were sensitive indicators of copper status, lipid changes were more sensitive to copper depletion than hematological measurements, including hemoglobin, hematocrit, mean corpuscular volume, and plasma iron measurements (39).

Other factors appear to affect the severity of lipid changes with copper deficiency (40-42). Fructose as the primary carbohydrate in the diet seems to increase the requirement for copper and augments copper deficiency (41). However, this effect was not observed with sucrose (42). Both aspirin intake (40) and beer consumption (15) mitigated some of the effects of copper deficiency, resulting in lower plasma cholesterol concentrations and more appropriate tissue copper concentrations.

Excessive Copper and Lipids

Although copper deficiency is linked to hypercholesterolemia, elevated serum copper concentrations are a risk factor for ishemic heart disease (30). Men in

Eastern Finland, as a population, have been observed to have elevated serum cholesterol concentrations. This elevation has been attributed to high serum copper concentrations. An abundance of dietary copper in the soil and drinking water has been identified as a possible cause. It has been hypothesized that the increased copper intake acts to interfere with the absorption of selenium which is inversely associated with cardiovascular disease risk (30). Thus, both copper excesses and deficiencies have been correlated with increased risk of heart disease.

Copper and Tissue Minerals

Tissue trace mineral concentrations are often used to assess dietary adequacy and decreased tissue concentrations are often concomitant with copper deficiency and altered lipid concentrations (15,34-40,43). Depressed body weights and increased tissue weights relative to body size also accompany changes in lipid concentrations and illustrate the extent of copper deficiency. More specifically there is usually an impaired weight gain (35,36) and an enlargement of the heart (35-37). Lee and Koo (35) observed significantly lower body weight gains in copper-deficient rats compared with copper-adequate controls after ten weeks. Enlarged liver weights as a percentage of body weight were also observed (34,35). Plasma copper concentrations have been observed to decrease in copper deficiency (35,37), sometimes to half that of control

animals (37). Hepatic copper concentrations also decrease markedly with copper deficiency (34-37). Hepatic zinc concentrations appear to correlate with hepatic copper concentrations (41,43).

Copper supplementation seems also to have an effect on tissue minerals. Tissue concentrations have been observed to increase with copper administration (44). However, factors such as the form of copper and the protein component of the diet are influential. Chicks fed 150, 300, or 450 mg copper/kg diet either as reagent grade acetate or feed grade carbonate or sulfate had proportionally increased liver copper concentrations (44). Dietary copper from the oxide source did not significantly affect liver copper. The copper source did not influence bone copper, however, bone copper concentrations were lower in chicks fed 150 mg of copper/kg diet compared to other dietary levels (44). Differences in tissue accumulation of copper with different protein components have also been observed (45). Tissue copper accumulation was lower in chicks consuming a corn-soybean meal complex diet than in chicks fed a casien-dextrose semi-purified diet (45).

Copper and Bone

Copper deficiency and the resultant lysyl oxidase deficiency primarily affect the development of bone by contributing to the inadequate cross-linking of collagen and elastin (10). The connective tissue abnormalities that

are caused by the lysyl oxidase deficiency lead to osteoporosis (10). The inadequacy of knowledge about copper sources within tissues, the exchange of copper between tissues, and recycling of tissue copper interferes with interpretation of tissue mineral analyses (10). The information available about bone copper concentrations is limited and the topic needs further research.

In Vitro Copper-Mediated LDL Oxidation

In vitro copper mediates oxidation of LDL (46,47). Kalyanaraman et al. hypothesized that formation of a copper-LDL complex is crucial to copper oxidation of LDL (48). Oxidized LDL is more susceptible to degradation and uptake by macrophage foam cells (46,47). Oxidized LDL is taken up by a macrophage scavenger receptor pathway (49). Pure macrophage-derived foam cells from atherosclerotic rabbit aortas (46), mouse resident peritoneal macrophages (47), 5 uM $CuSO_4$ (47), and endothelial cells from human arteries, veins, and microvessels (50), all modify LDL. Modification and oxidation of LDL often induce changes in the LDL particles including increased electrophoretic mobility (50-52) and buoyant density of the LDL particles, as well as altered lipid composition of LDL (50). Thiobarbituric acid reactive substances are also generated (51,53,54). Immunoreactive apolipoprotein B (55), or apo B 100 (53,56), undergoes fragmentation (52,53,55,56) and disappears during oxidation of LDL (55). The formation of

hydroperoxides and depletion of endogenous alpha-tocopherol also occur with LDL oxidation (56,57). Higher protein/cholesterol ratios in LDL are also seen with LDL oxidation (58).

Lipid hydroxy and hydroperoxy derivatives also increase with copper oxidized LDL (51). These increases occur as linoleate (18:2) and arachidonate (20:4) decrease in oxidized LDL (51). These decreases were observed after a 4-6 hour period. Thomas and Jackson (54) found that trace amounts of lipid hydroperoxides in the lipoprotein were required for the initiation of copper dependent oxidation.

Yia-Herttuala and co-workers (59) observed that lesion LDL was similar to oxidized LDL indicating that atherosclerotic lesions may contain oxidized LDL. They found increased electrophoretic mobility, a higher density, a higher free cholesterol content, and the presence of apolipoprotein B fragments in the lesion LDL. The lesion LDL was degraded more rapidly than plasma LDL. A subfraction of plasma LDL, equal to 1% of total plasma LDL, has similar properties to oxidized LDL in that it has an increased negative charge, a higher protein/cholesterol ratio, and a higher density than LDL (58). This may be associated with oxidation of LDL and atherosclerotic changes.

Superoxide dismutase prevents transition metal-induced lipid peroxidation changes (50,53). Hindsberg and

colleagues (50) also noted variation in the degree to which LDL from various donors could be modified. Diet and lipophylic antioxidants have been hypothesized as contributing to this variation (50). Free radical scavengers, such as butylated hydroxytoluene and butylated hydroxyanisole, also inhibited copper-mediated modification of LDL in monkey smooth muscle cells (60). Flavonoids conserved the endogenous alpha-tocopherol content of LDL, postponed lipid peroxidation, and inhibited $CuSO_4$ mediated oxidation of LDL (57). The 4-6 hour lag period noted with LDL modification (47,57) might be due to the alpha-tocopherol content of LDL. The alpha-tocopherol is depleted during this time (57). Alpha-tocopherol can only inhibit oxidated modification of LDL for 5 hours (61). Ascorbate also has an inhibitory effect but for a longer duration of 24 hours (61). The decreased electrophoretic mobility and thiobarbituric acid reacting substances, as well as the decreased uptake by macrophage scavenger receptors show the extent of the inhibitory effects on cell free oxidation. Saito and colleagues (62) showed that rabbits fed purified eicosapentaenoic acid yielded LDL that was somewhat resistant to oxidative modification by copper in comparison to control animals.

HDL interferes with LDL modification (50,63). Although HDL inhibits macrophage degradation of oxidized LDL, it did not affect the thiobarbituric acid reacting

substances nor did it affect the electrophoretic mobility of oxidized LDL (63).

Zinc

Zinc is essential for optimal health as it is associated with biological functions of growth, wound healing, and the regulation of zinc containing enzymes (64). With the functional emphasis of zinc centering around tissue synthesis and repair, it is alarming that the two groups at risk for low zinc intake are older women and two year old children (8). Zinc status is dependent upon three factors; homeostatic regulation (64), bioavailability (26), and interactions with other dietary factors (26,64). An individual's zinc requirement depends on his/her zinc status and body pool of usable zinc (64). This body pool is small and undergoes rapid turnover (26,64), as can be seen by the rapid onset of deficiency symptoms as well as the quick restoration to a zinc-adequate state (64). Zinc is more efficiently used by people with compromised zinc status than by persons with an adequate body pool of zinc (26).

Bioavailability of zinc varies widely among food items (26). Animal products account for 70% of zinc consumption in the United States (26). Meat, liver, seafood, eggs, and dairy products are good sources of zinc (26). Whole grain sources of zinc are less available. Total calorie intake, protein, fiber, phytates, calcium, phosphorus, and iron all

may interfere with zinc absorption and utilization (26,64). Typical diets of North American adults seem to be adequate with consumption patterns of zinc ranging from 10-15 mg/day (26). Balance studies show a need for at least 12 mg/day of zinc and the RDA for zinc has been set at 12 mg/day for women and 15 mg/day for men (26). Although zinc is absorbed in both the small and large intestine, absorption occurs primarily in the duodenum (64). Competition for absorption between copper and zinc can occur in the intestinal lumen (65). If the luminal concentration of one of these minerals is high, inhibition of the other mineral's absorption ensues (65).

Loss of appetite, growth retardation, skin lesions, immunological abnormalities, and reproductive impairment are common signs and symptoms of zinc deficiency in humans (26,64). In Japanese quail, inadequate feathering and skeletal abnormalities are observed with zinc deficiency (64).

Zinc Deficiency and Lipids

Hypocholesterolemia has been observed with zinc deficiency in both humans and animals (66), primarily due to decreases in HDL cholesterol (66,67). This finding is important because of the inverse association between HDL cholesterol and coronary heart disease (66). Thus, marginal zinc intake could serve as a risk factor in terms of cardiovascular health (66). Schneeman and colleagues

(67) observed a decrease in HDL-cholesterol concentrations in zinc-deficient rats, as well as a decrease in VLDL-triglycerides. However, they attributed these changes to reduced food intake, common in zinc deficiency. Koo and Williams (66), in contrast, observed significant decreases in total serum cholesterol, HDL-cholesterol and triglycerides in zinc-deficient rats compared to pair-fed controls. A significant decrease in serum zinc concentration was also observed; however, serum copper was not affected (66). Schneeman and co-workers (67) found no significant difference in HDL parameters between zinc-deficient animals and restricted-intake controls. In a more recent study, Koo and Lee (68) found that zinc deficiency in rats significantly lowered the concentration of actual circulating HDL particles; concentrations of total protein, triglycerides, phospholipids, and cholesterol were not affected. Koo and Lee (68) hypothesized the younger age of the animals or the extremely low zinc level (0.4 mg/day) in the zinc-deficient diet could explain the conflicting results between their studies. Age-related changes have been observed in plasma HDL (68).

Koo and Lee (68) also observed changes in the HDL apolipoprotein profile with marginal zinc deficiency. They reported an increase in Apo A-I and decreases in the relative proportions of Apo E and Apo C. Lefevere et al. (69) did not observe a decrease in plasma HDL-cholesterol

concentrations or in HDL-apolipoprotein concentrations in zinc-deficient rats. However, HDL tended to have increased free cholesterol and decreased triglycerides (69). In addition, a significant decrease in HDL Apo C concentrations was observed. In a subsequent study, Koo and Lee (70) observed a decrease in circulating HDL particles containing no Apo E in zinc-deficient rats. Apo A-I and Apo C concentrations were also decreased in the plasma (70).

In addition to the alterations in the lipoprotein and apolipoprotein profiles, impaired absorption of dietary lipids with zinc deficiency (71) has also been observed in rats, as well as delayed plasma clearance of chylomicron cholesterol (72,73). Koo and colleagues (72) attributed this delayed clearance to morphological changes in the chylomicron and altered chylomicron apolipoprotein composition rather than impaired chylomicron synthesis. The increased chylomicron size may delay hepatic removal and decrease hepatic synthesis and release of cholesterol (72). Apo B content of lymphatic chylomicrons was significantly lower in zinc-deficient rats compared to controls (71).

Excessive Zinc and Lipids

Human dietary zinc intakes between 100 and 300 mg zinc/day are considered pharmacological doses and may result in a wide range of symptoms and disorders. Copper

deficiency characterized by anemia, hypocupremia, leukopenia, and neutropenia, in addition to an impaired immune function, and alterations in serum cholesterol and lipoprotein profiles are complications associated with excessive zinc intake (16).

Extreme zinc intakes in rats are associated with an increase in serum cholesterol (16). In humans, increased LDL concentrations have been observed, while HDL concentrations decrease with excessive zinc (16). Chandra (74) observed a significant decrease in HDL concentrations of eleven healthy men supplemented with 150 mg zinc/day and a slight elevation of LDL which overall resulted in a marked rise in the LDL/HDL ratio. Samman and Roberts (75), in contrast, did not observe any significant effects in males on lipoprotein profiles or copper indices with excessive zinc supplementation. However, they did observe a significant 9% decrease in LDL cholesterol in females with daily supplementation of 150 mg zinc for 12 weeks. HDL-cholesterol was redistributed with a slight increase in HDL-2 and decrease in HDL-3 subfractions (75). HDL-2 is positively associated with a lower risk of coronary heart disease (75). Ceruloplasmin and erythrocyte superoxide dismutase activity were decreased in the women participants, possibly illustrating a compromised copper status (75). However, total plasma mineral concentrations in these studies did not change for either males or females. The lack of response in men could be due to a

gender difference. However, Samman and Roberts (75) support the view that the differences between gender could be dose related, since the women, although weighing less, received the same dose of 150 mg zinc/day.

Freeland-Graves et al. (76) observed a transient decrease of HDL cholesterol in women supplemented with 100 mg zinc/day. HDL-cholesterol decreased by 8.4% during the 4th week of the experiment and returned to normal values by the 6th week of the experiment (76). Plasma zinc values peaked during the 4th week of the experiment and subsequently declined toward initial values. Freeland-Graves and colleagues postulated that certain plasma zinc values need to be maintained in order to observe HDL alterations (76).

Moderately excessive intakes of zinc also have been observed to affect cholesterol concentrations (77,78). Black et al. (77) investigated the effects of zinc supplements at 50 and 75 mg/day for twelve weeks in humans. They observed a significant decrease in HDL-cholesterol concentrations, at weeks six and twelve in males supplemented with 75 mg zinc/day as zinc gluconate compared to placebo subjects. HDL concentrations at weeks 6, 8, and 12 were also significantly lower than baseline values. Those males receiving supplements of 50 mg zinc/day had significantly lower HDL-cholesterol concentrations at week twelve compared to baseline values or twelve week HDL-cholesterol concentrations of the placebo group (77).

However, Freeland-Graves et al. (76) observed no plasma cholesterol alterations with moderate supplementation (15 or 50 mg zinc/day) in men. Crouse and colleagues (79) monitored plasma cholesterol concentrations of sedentary and endurance-trained men during eight weeks of low dose zinc supplementation (50 mg zinc/day). They did not observe any changes in total cholesterol, HDL-cholesterol, LDL-cholesterol or triglyceride concentrations (79). Brewer and co-workers (80) looked at the long-term effects of zinc supplementation (2 years) in Wilson's disease patients. Zinc therapy combats the excessive copper levels of Wilson's disease by induction of intestinal cell metallothionein, blocking intestinal copper absorption (80). In this study they reported that zinc therapy reduced total cholesterol by about 10% in males and females and reduced HDL-cholesterol by about 20% in males (80). Cho et al. (78) noted a significant decrease in serum HDL-cholesterol in rats upon short term (5 days) supplementation with 40 mg zinc/kg. Zinc-supplemented animals had decreased cholesterol-induced liver peroxidation and damage.

In normal subjects and subjects with compromised health, the zinc-induced derangement of copper metabolism has been viewed as an influential factor in lipid and lipoprotein regulation (5,16). Human cholesteryl ester transfer protein (CETP) may be another vehicle by which zinc influences cholesterol metabolism (81). This protein

facilitates the exchange of neutral lipids between lipoproteins (81). Additional zinc in the drinking water of CETP transgenic mice resulted in an increase of the CETP mRNA in the small intestine and liver, indicating that zinc can induce the expression of that transgene. Plasma CETP levels rose by 140% when 10 mM of $ZnSO_4$ was supplemented in the drinking water for 3 days and by 200% when 25 mM $ZnSO_4$ was administered in the drinking water for 7 days. This zinc-induced expression of the CETP transgene resulted in a significant decrease in total plasma cholesterol, specifically due to decreases in plasma HDL-cholesterol (81).

Zinc and Plasma and Tissue Minerals

Zinc deficiency contributes to restricted food intake in animals (67). Significant decreases in body weights have been observed in zinc-deficient animals compared to pair fed-controls (67-72). In addition, liver and plasma zinc concentrations are significantly decreased in zinc deficiency (67-69,72). Plasma zinc concentrations have been observed to increase significantly with zinc supplementation (74-76). Black et al. (77) observed transient increases in serum zinc concentrations in adult males supplemented with 50 mg zinc/day. Supplementation of 75 mg zinc/day resulted in increased serum zinc throughout the 12-week study (77). Crouse and colleagues (79) observed a 15% increase in plasma zinc concentrations among

zinc supplemented males and a 0.4% zinc increase in the placebo group; however, this increase in plasma zinc did not reach statistical significance. Cho and co-workers (78) observed increases in both serum and liver zinc concentrations in zinc-supplemented rats.

In a retrospective study of patients with acute myocardial infarction (AMI) and coronary heart disease (CHD), a significant increase in plasma zinc concentrations was observed (21). Elevated plasma zinc was believed to interfere with the lecithin:cholesterol acyltransferase (LCAT) reaction in these AMI and CHD patients. Significantly decreased LCAT activity may have importance as a risk factor for CHD (21).

Zinc and Bone Minerals

Bone mineral content as well as tissue mineral concentrations serve as indicators or markers, providing information on the extent or nature of a deficiency or disease state.

Zinc intake was observed to be significantly correlated with changes in mineral content of the radius in post-menopausal non-supplemented women between the ages of 35-65 years of age (11). Excessive dietary zinc retarded bone resorption and differentiation in pigs fed high-zinc, low-copper diets (82). Zinc-deficient guinea pigs had significantly lower plasma and bone zinc concentrations than zinc-adequate controls (22). In this study, bone

served as the chief reservoir of mobilizable zinc; significant decreases in zinc concentrations of soft tissues such as muscle, brain, liver, or skin were not observed (22). In a zinc-deficient rabbit study, however, bone zinc concentrations decreased by 45% while liver zinc decreased by 20% and skin zinc concentration decreased by 35% (23). Bone and skin may be "low priority" tissues that mobilize zinc for the "high priority" tissues such as the muscle and brain (23).

Both in laboratory animals and in humans it appears that zinc concentrations are compromised in the bone, liver, testes, and plasma to maintain normal zinc concentrations in other tissues such as muscle (24). Plasma zinc falls when homeostatic regulation through tissues such as bone can not be maintained (24). The reduction in growth, possibly through decreased food intake, allows animals to maintain a normal whole body zinc concentration and may be a survival mechanism (24). Weight gain in zinc-deficient rats stimulates zinc depletion due to decreased protein catabolism and increased anabolism (83). Park et al. (83) found that zinc-depleted force-fed rats could not survive beyond 8-10 days. The increased zinc requirement during anabolism and the animals' inability to regulate growth and skeletal mass via reduced food intake brought about the rapid results. Bone zinc seems to correlate with zinc intake while lower liver zinc correlated with undernutrition (84).

Chromium

Chromium, also, is an essential element for animals and humans (25). Chromium is a transition element that occurs in eight oxidation states (25). The most common oxidation states are 0, +2, +3, and +6 (25). The trivalent state is the most stable (85), and is required for maintenance of normal glucose metabolism (26). Chromium must be in a biologically active form in order to perform its physiological functions (25). The biologically active form of chromium has been isolated from brewer's yeast and the yeast possibly contains nicotinic acid, glycine, glutamic acid, and cysteine, in addition to chromium (25). The precise arrangements and composition of the structure remain undefined and controversial (25). These amino acids and nicotinic acid have demonstrated biological activity when synthetically arranged in chromium complexes (25). Glutathione, chromium, and nicotinic acid complexes also have demonstrated biologically active properties (25).

Although intestinal absorption of chromium is low (85), it appears that it is primarily absorbed in the jejunum (25). Chelation and mineral interactions influence chromium's absorption (25,85). Oxalate increases chromium absorption, while phytate, to a lesser extent, decreases absorption through chelation (25,85). Chromium and iron may share a transport mechanism; intestinal concentrations of iron either promote or interfere with chromium absorption. Iron-deficient animals absorb more chromium in

comparison with iron-supplemented animals. When oral iron was administered to the iron-deficient animals, chromium absorption was inhibited (25). Zinc in a similar manner influences chromium absorption; oral zinc decreased chromium absorption while chromium decreased zinc absorption (25). Once absorbed, trivalent chromium appears to be transported to the tissues primarily by transferrin (25).

Chromium consumption in typical western diets ranges between 25 ug chromium/day to 200 ug chromium/day, with most intakes below 100 ug/day (26). These levels could be a cause for concern because only 1% of oral chromium is absorbed (25). An estimated safe and adequate range has been established between 50 and 200 ug of chromium/day (26). At intakes near the lower end of the range; 50 ug of chromium/day, no signs or symptoms of chromium deficiency develop (26). Chromium intake reaching the upper end of the range; 200 ug chromium/day, over a period of time also has shown no adverse signs. In a varied diet providing an adequate intake of other micronutrients, chromium absorption averages 0.5% (26). In general, the more unprocessed and fibrous the food is, the higher it will be in chromium content (7). Mushrooms, brewer's yeast, prunes, asparagus, rhubarb, black pepper, wine, and beer are foods high in chromium (7). The bioavailability of chromium in calf liver, American cheese, and wheat germ also make these products good sources of chromium (26).

Chromium and Glucose

Chromium's physiological role in animals and humans centers around regulation of glucose metabolism through insulin potentiation (7,25,85). Glucose breakdown can be increased by the addition of the biologically active chromium; in contrast, more insulin is needed at suboptimal chromium levels (7). Diabetes-like symptoms may occur in some individuals with inadequate chromium intake (26).

Chromium supplementation studies are conflicting regarding chromium's role in glucose metabolism. The biologically active form of chromium in brewer's yeast along with the nicotinic acid, glutamic acid, glycine, and a sulfur containing amino acid is known as the glucose tolerance factor (85). It is unclear how much of chromium's influence on insulin and glucose regulation is due to the chromium itself or due to chromium complexes such as the glucose tolerance factor (20,86). In addition, many studies have reported that chromium supplementation benefits only those with marginal chromium status or those who are insulin resistant (27, 28).

Chromium and Lipids

Marginal chromium status also has been implicated as a risk factor for atherosclerosis and altered lipid metabolism (7). Elevated insulin concentrations, seen with suboptimal chromium status have been associated with the incidence of coronary heart disease (7). However,

comparison of the inhibitory effects of low chromium and high chromium derived fractions on liver cholesterol synthesis in vitro concluded that chromium compounds do not appear to account for the inhibitory effects of brewer's yeast (87).

Offenbacher and Pi-Sunyer (20) investigated the effects of 9 grams of chromium-rich brewer's yeast on serum glucose, insulin, cholesterol, total lipids, and triglycerides in both diabetic and nondiabetic elderly subjects. Elderly subjects supplemented with 9 grams of chromium-poor torula yeast served as controls. Glucose tolerance improved significantly in the experimental group. In addition to a decreased insulin output, serum total cholesterol, and total lipids were significantly lower in the brewer's yeast-supplemented group compared to the controls. However, a significant decrease in serum total cholesterol of nondiabetic participants in the chromium-poor torula yeast-supplemented group also was observed (20). This suggests that there may be another factor in yeast contributing to the cholesterol lowering effect (20). In a subsequent study, Offenbacher et al. (88) examined the effects of a 10 week supplementation study with 5 grams of brewer's yeast or 200 ug of trivalent chromium as chromium chloride in free-living elderly subjects. Glucose tolerance, plasma insulin, cholesterol, and triglycerides did not change significantly in either of the two experimental groups, compared to the placebo group (88).

Plasma insulin output did decrease slightly in both the brewer's yeast and the chromium chloride groups after a glucose load. The brewer's yeast group exhibited decreased insulin output 45 and 90 minutes after the glucose load, while insulin output of the chromium chloride group decreased 45 minutes post-glucose load (88). However, these decreases in glucose load did not reach significance. Plasma chromium concentrations increased significantly in the chromium chloride group only (88).

There are some experimental details between the studies which may explain the descrepancies. One possible explanation is that the supplement in the later study provided too little chromium to evoke a response (88). In addition, the first study recruited participants from a retirement home as opposed to free-living elderly (20). The institutionalized nature of the first group could have contributed to a suboptimal intake of chromium. The free-living volunteers in the later study were very well nourished, consuming over 100% of the RDA for eight indicator nutrients (88). The retirement home elderly who exhibited a significant decrease in total serum cholesterol and lipids were initially hypercholesterolemic (20). A combination of the these factors could explain the lack of response from the chromium supplements in chromium adequate elderly.

Vinson and Bose (89) more extensively addressed the influence of chromium supplementation on glucose control

and plasma lipid responses in the following subgroups; normal subjects, hyperglycemic subjects, insulin-dependent diabetics, and noninsulin-dependent-diabetics for six months. Participants consumed 100 mg of yeast daily which contained 218 ug of chromium. A transient improvement was observed in the percent glycosylated hemoglobin at months two and four in the insulin dependent diabetic group (89); however, at six months the percent glycosylated hemoglobin was close to pre-dose values in this group. The hyperglycemic participants averaged a significant 57% improvement in blood glucose control at the end of six months. The observed improvements in glucose regulation were attributed to the actual chromium in the original yeast rather than to other components in the yeast. Although transient improvements in serum cholesterol and triglyceride concentrations were observed, no group sustained statistically significant improvements in serum lipid concentrations after six months of chromium supplementation (89). However, participants were not subdivided into hypercholesterolemic risk groups (89). HDL concentrations were significantly elevated in the hyperglycemic group at six months and HDL concentrations were also temporarily increased in normal participants after two months. The hyperglycemic group was the only group after four months that observed a significant decrease in serum LDL concentrations as well as a significant decrease in the cholesterol to HDL ratio. This

group decreased its risk for coronary heart disease by 50% by the end of the study (89).

Anderson et al. (86) observed significantly lower fasting glucose concentrations in male and female subjects after two months of supplementation with 200 ug of chromium as chromic chloride. Again, these values returned to pre-supplementation concentrations after three months of supplementation. However, the effects of chromium supplementation on serum glucose varied based on subject's 90-minute post-glucose challenge glucose concentrations (86). Subjects whose 90-minute post glucose values were greater than or equal to 100 mg/dL had a significant decrease in serum glucose. On the other hand, subjects whose 90-minute glucose concentrations were less than or equal to fasting levels had a significant increase in serum glucose concentrations. Subjects whose 90-minute blood glucose were greater than fasting but less than 100 mg/dL did not have any significant changes in serum glucose (86). Chromium supplementation did not alter any lipid variables (86).

Uusitupa et al. (27) did not observe any significant improvements in glucose tolerance or serum lipid concentrations in noninsulin dependent diabetics after supplementation of 200 ug trivalent chromium for 6 weeks. However, they did observe a significantly decreased 1-hour post-glucose serum insulin level in chromium supplemented diabetics as opposed to insulin levels of placebo-treatment

diabetics (27), thus indicating that less insulin is required at optimal chromium levels. Twenty-four hour urinary chromium excretion increased nine-fold upon supplementation indicating that subjects were in chromium balance (27). Riales and Albrink (28) observed a significant increase in HDL cholesterol concentration and a decrease in the body weights of human males consuming 200 ug of trivalent chromium in 5 ml of water, five days a week for twelve weeks. A decrease in insulin relative to plasma glucose levels also was observed, exhibiting increased insulin sensitivity (28).

Several studies investigating the effect of chromium on the formation of atherosclerotic lesions have used rabbits as the animal model (19,90,91). Abraham et al. (90) observed a 50% reduction in the aortic intimal plaque and aortic total cholesterol content in Loewnstein male rabbits upon intraperitoneal injection of 20 ug of potassium chromate in 0.2 ml distilled water (90). Serum total cholesterol, LDL-cholesterol, and triglyceride concentrations were lower and HDL fractions were higher in chromium-treated rabbits compared to control rabbits, but the differences were not significant (90). Flexon and colleagues (91) also observed a non-significant decrease in serum cholesterol among New Zealand White rabbits injected intraperitoneally with 20 ug of potassium chromate in 0.2 ml of distilled water, five days a week for ninety days. However, no significant changes in total cholesterol

content per gram of aorta or in the percent intima covered with plaque were observed (91). Possible explanations for the differences observed between these studies are the different rabbit breeds used, differences in initial weights resulting in a different dose relative to body weight and different methods for determining aortic plaque (91). Abraham et al. (19) observed a marked reduction in the percent of aortic intimal surface covered by plaque and in the cholesterol content of chromium-treated Loewenstein male rabbits. The trivalent form of chromium as 20 ug chromium chloride elicited a better response than rabbits treated with the hexavalent chromium in doses of 10 or 20 ug potassium chromate (19).

Donaldson et al. (92) observed a transient increase in postprandial plasma glucose concentrations in male rats fed a low-chromium (60-100 ug chromium/g diet), high-sucrose, high-cholesterol diet at four and eight months when compared to control rats fed a chromium-supplemented, high-sucrose, high-cholesterol diet. No other significant changes were observed throughout the 18 month study. Donaldson and colleagues (92) suggested that other factors aside from dietary chromium could play a part in the manifestation of glucose intolerance in this animal model. Large inter- and intra- animal variability in glucose disappearance rates in young adult rats upon preliminary i.v. glucose tolerance tests are examples of such factors (92).

Other factors may influence the effect of chromium supplementation on lipid concentrations. Stoecker and Oladut (93) observed a synergistic effect between chromium and ascorbate deficiency. Male Hartley guinea pigs, deficient in both ascorbate and chromium, had higher plasma glucose concentrations ninety minutes post-glucose load than animals receiving adequate ascorbate. They also observed a significant relationship between chromium depletion and blood cholesterol levels ($p<0.0003$) (93). On the other hand, Cupo and Donaldson (94) found no effect of chromium on glucose metabolism or lipid synthesis in chicks supplemented with 20 ppm chromium and/or 20 ppm vanadium. In addition, chromium did not significantly interact with vanadium.

Chromium and Tissue and Bone Minerals

Newer analytical methods and improved awareness of trace mineral contamination have contributed to increased accuracy of reported chromium concentrations (25). In humans, chromium concentrations decrease markedly within the first few months of life in lung, heart, and spleen tissues (25). Liver and kidney retain neonatal concentrations until 10 years of age. In areas where maturity-onset diabetes and atherosclerosis are high, tissue concentrations of chromium tend to be lower (25). In addition, Hunt et al. (95) observed significantly lower

hair chromium concentrations in diabetic humans when compared to non-diabetics.

Chromium supplementation in humans has been observed to significantly increase serum chromium concentrations (96). Kidney and bone chromium concentrations increased in chromium-supplemented lean and obese mice; however, chromium tissue accumulation was greater in the bone and spleen tissues of obese mice than in lean mice (97). Liver chromium concentration was not affected by chromium supplementation.

Donaldson et al. (92) observed lower liver and kidney chromium concentrations in rats fed low-chromium diets than in rats fed chromium-supplemented diets. However, only differences in kidney concentrations between rats fed low-chromium or chromium-adequate diets reached statistical significance (92).

CHAPTER III

INDEPENDENT AND/OR INTERACTIVE EFFECT(S) OF COPPER, ZINC, AND CHROMIUM LEVELS ON PLASMA LIPIDS AND GLUCOSE IN MALE JAPANESE QUAIL

Nihiser, R.G., Hermann, J.R., Stoecker, B.J., Arquitt, A.B., and Chang, H.H.

Department of Nutritional Sciences, Oklahoma State University, Stillwater, OK 74078.

Shortened Title:

TRACE MINERAL EFFECTS ON LIPIDS AND GLUCOSE

ABSTRACT

The effect(s) and interaction(s) of copper, zinc, and chromium on plasma lipids and glucose concentrations were evaluated with 72 male Japanese quail, Coturnix coturnix japonica, using a three-factor central composite response surface design. Quail were randomly assigned to one of fifteen experimental diets containing variable concentrations of copper (1.6 to 8.3 ug/g diet), zinc (11 to 51 ug/g diet), and chromium (71 to 1124 ng/g diet). After six weeks, blood samples were collected by cardiac

puncture for plasma lipid and glucose analyses. No significant differences were observed for plasma total cholesterol, LDL-cholesterol, triglycerides, or glucose concentrations for the mineral ranges studied. However, chromium and copper had a significant interactive effect on increasing HDL-cholesterol ($p<0.02$).

INDEXING KEY WORDS: Japanese quail, lipids, chromium, copper, zinc

Cardiovascular disease is the leading cause of death in the United States (1), and hypercholesterolemia is considered to be a major contributing risk factor in the development of cardiovascular disease (2). Additionally, hypercholesterolemia is one of the top three modifiable factors related to cardiovascular disease (3). Substantial nutritional research has focused on dietary modifications to improve plasma lipid profiles and thus lower cardiovascular disease risk (4).

Diets low in saturated fat and cholesterol, and increased exercise are beneficial in improving plasma lipids (5,6). However, other dietary factors including adequate trace minerals may also improve plasma lipids. This is of great importance because typical American diets often provide inadequate amounts of trace minerals (7). Low intakes of several minerals, including copper, zinc, and chromium have been implicated with abnormal lipid profiles (8-13). Thus, these minerals may have the

potential for improving plasma lipids and thus lowering cardiovascular disease risk.

No conclusive benefits of copper, zinc, or chromium for hypercholesterolemia have been established. However, it is possible that the therapeutic potential of copper, zinc, and chromium in relationship to hypercholesterolemia may lay in the interrelationship of these or other trace minerals. In this study, response surface regression analysis was used to investigate the effect(s) and/or interaction(s) of copper, zinc, and chromium on plasma lipids and fasting glucose concentrations in male Japanese quail.

MATERIALS AND METHODS

Animals

Japanese quail were the experimental animal model selected for this study. Rodent models are not the most suitable animal model for lipid studies because the rat has unusually high levels of high density lipoproteins (12). The protocol for this study was reviewed and approved by the Oklahoma State University Institutional Animial Care and Use Committee.

Two hundred hatchling Japanese quail (Coturnix coturnix japonica) (B and D Game Farms, Harra, OK) were held on a holding diet for four weeks until gender could be determined by feathering, at which time seventy-two male

Japanese quail were weighed and randomly assigned to the experimental diets for six weeks.

The animals were housed in an isolated laboratory, in accordance with USDA and NIH guidelines for laboratory animals. The temperature and light-controlled laboratory was maintained by personnel from Laboratory Animal Resources in the College of Veterinary Medicine at Oklahoma State University. During the initial four week holding period animals were group housed in polystyrene cages with supplemental heat provided by heat lamps, with decreasing heat intensity. During the first week, 100 watt bulbs provided the heat; 75 watt bulbs supplied the heat in the second week and during the third and fourth weeks, 50 watt bulbs were used. During the experimental period animals were individually housed in polystyrene cages. Ceramic feed cups and water containers were used to reduce possibilities for mineral contamination.

Japanese quail are a promising small animal model for lipid research (14-19). Japanese quail mature rapidly and are economical for animal studies with large numbers.

Diets

The experimental diets, vitamin and mineral mixes were formulated to contain all nutrients recommended by the National Research Council for growing Japanese quail (20) except for copper, zinc, and chromium as dictated by the experimental design (Table 1). Semi-purified diets,

vitamin and mineral mixes were prepared in our laboratory after assaying all components for trace mineral content.

TABLE 1

Diet composition

Ingredient	g/kg diet
Casein	250.0
Arginine	5.0
D-L Methionine	3.0
Choline Bitartrate	5.9
Corn oil	40.0
Exthoxyquin	0.1
Celufil	40.0
Vitamin mix[1]	10.0
Mineral mix[2]	60.0
Dextrose	586.0

[1]Vitamin mix formulated to meet the National Research Council recommendations for growing Japanese quail.

[2]Mineral mix formulated to meet the National Research Council mineral recommendations for growing Japanese quail except for chromium, copper and zinc.

The experimental mineral mixes for this study were based on a three factor central composite rotatable response surface design (21) (Table 2). Response surface analysis is a statistical technique which evaluates the interrelationship of two or three independent variables with a minimal sample size (21).

TABLE 2

Zinc, copper, and chromium concentrations[1] of experimental diets using a three-factor response surface design

Diet	RS Code[2] (Zn,Cu,Cr)	Zinc ppm	Copper ppm	Chromium ppb	n
Holding	(-1,-1,-1.68)	19.3	3.1	68	
1	(1, 1, 1)	40.2	6.3	821	4
2	(-1, 1, 1)	19.6	5.1	856	4
3	(1,-1, 1)	46.1	3.3	811	4
4	(1, 1,-1)	45.6	8.1	228	4
5	(-1,-1, 1)	19.4	3.0	729	4
6	(-1, 1,-1)	20.9	8.0	261	4
7	(1,-1,-1)	41.3	2.8	268	4
8	(-1,-1,-1)	20.3	2.7	270	4
9	(1.68,0,0)	51.0	5.0	532	4
10	(-1.68,0,0)	11.2	4.7	489	4
11	(0,1.68,0)	30.5	8.3	485	4
12	(0,-1.68,0)	31.4	1.6	488	4
13	(0,0, 1.68)	30.5	4.5	1124	4
14	(0,0,-1.68)	29.2	5.5	71	4
15	(0,0,0)	28.7	5.7	465	16

[1]As determined by atomic absorption spectrophotometry.
[2]Response surface code.

The ranges of copper, zinc, and chromium studied were; copper 1.6 ug/g diet to 8.3 ug/g diet, zinc 11 ug/g diet to 51 ug/g diet, and chromium 71 ng/g diet to 1124 ng/g diet. The initial four week holding diet consisted of a

moderately low mineral combination to prevent tissue mineral accumulation (Table 2).

The mineral mixes were prepared from reagent grade or ultrapure chemicals, after assaying each mineral lot number, because different lots have been found to vary widely. The experimental diets were analyzed for total mineral content before feeding. The experimental diets and deionized water were available ad libitum except as specified by the experimental design.

Plasma Lipid and Glucose Analyses

After six weeks on the experimental diets the quail were fasted for 12 hours prior to blood collection. Methoxyflurane was used for anesthesia. The birds were weighed and blood was collected by cardiac puncture into a heparinized, trace-mineral-free syringe and centrifuged to obtain plasma. One milliliter of plasma was chilled immediately and used for plasma total cholesterol and HDL-cholesterol analyses using an enzymatic method (22). Plasma for triglycerides was frozen and analyzed using a microenzymatic procedure (23). Plasma LDL-cholesterol was determined by calculation using the equation LDL=TC-(HDL+TG/5) (24). Plasma for glucose was frozen and analyzed using an enzymatic colorimetric procedure utilizing glucose oxidase (25,26).

Data Analyses

Data were analyzed by the Statistical Analysis System (SAS) using the SAS response surface regression analysis (RSREG). This procedure allows the use of two or more independent variables to determine maximum and minimum responses of the dependent variables. In this study, response surface regression analyses were performed to find relative minimum response levels for plasma total cholesterol, LDL-cholesterol, triglycerides, and glucose, and a relative maximum response level for HDL-cholesterol at various levels of copper, zinc, and chromium. Response surface regression analyses produced regression equations for each of the dependent variables: total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, and glucose. Because of the complexity of the response surface analyses, two-dimensional response surface contour plots were produced for each of the dependent variables using RSREG. To graph the dependent variable response surface contours, the three variable regression equations were reduced to the most useful two variables by holding the third variable in the equation at the optimal value as determined by RSREG. These response surface contours can be used to determine dependent variable responses at various combinations of the independent variables. In interpreting the response surface contours, the lightest shading represents decreased dependent variable responses

while the darkest shading is indicative of higher dependent variable responses. The level of significance for this study was set at $p \leq 0.05$. Probabilities are cited from the total regression model, unless otherwise specified.

RESULTS

No significant differences were observed in quail initial weights, final weights or weight gain between treatments. Initial body weights averaged 79 g, final body weights averaged 149 g, and weight gain averaged 70 g.

No significant differences were observed for plasma total cholesterol, LDL-cholesterol, triglycerides, or glucose for the mineral ranges studied (Figures 1-4). However, chromium and copper had a significant linear interactive effect ($p<0.02$) on HDL-cholesterol (Figure 5), with HDL-cholesterol increasing as chromium and copper increased in the diet.

DISCUSSION

Although significant differences for total-cholesterol and LDL-cholesterol were not observed for the ranges studied, the data indicates a trend towards decreasing total cholesterol, LDL-cholesterol, triglycerides, and glucose at combined higher chromium and copper levels than were evaluated. The data also indicate a trend for HDL-cholesterol continuing to increase at combined higher chromium and copper levels than were evaluated.

Most of the research addressing copper concentrations and lipid responses concentrates on copper depletion and deficiency (27-29). Copper deficiency has been observed to result in increased plasma total cholesterol, triglycerides, and LDL-cholesterol concentrations in rats (27-29). However, HDL-cholesterol concentrations only moderately increased with copper depletion and deficiency in rats (27-29). Figures 2 and 3 indicate that at combined higher levels of copper and chromium in the diet, the LDL-cholesterol and triglyceride responses are beginning to decline. Railes and Albrink (30) observed that chromium supplementation increased HDL-cholesterol and decreased fasting blood glucose through increased insulin sensitivity in adult men. In the present study, HDL-cholesterol concentrations were significantly increased in quail fed diets with combined higher copper and chromium concentrations (Figure 5). However, at the lower copper, higher chromium diet combination HDL-cholesterol concentrations were reduced. Interactions between the minerals at minimal copper levels could negate the increase in HDL-cholesterol concentrations as seen in animals fed chromium-supplemented diets (30,31). Plasma glucose responded similarly in the Japanese quail with minimal glucose responses at the combined higher chromium, lower copper combination. Many researchers have observed improved glucose tolerance responses or improved plasma glucose values upon chromium supplementation (25,30,31).

Other researchers (32,33) have observed no significant changes in plasma glucose and lipid responses in subjects supplemented with chromium. Some researchers believe that only subjects with marginal chromium status respond to chromium supplementation (30). Additional research is needed on lipid and glucose responses in relation to trace mineral interactions. However, a hypothesis can be drawn from the present data that combined higher levels of chromium and copper will continue to improve lipid profiles, and that there may be an higher combination of chromium and copper which will provide optimal lipid profiles.

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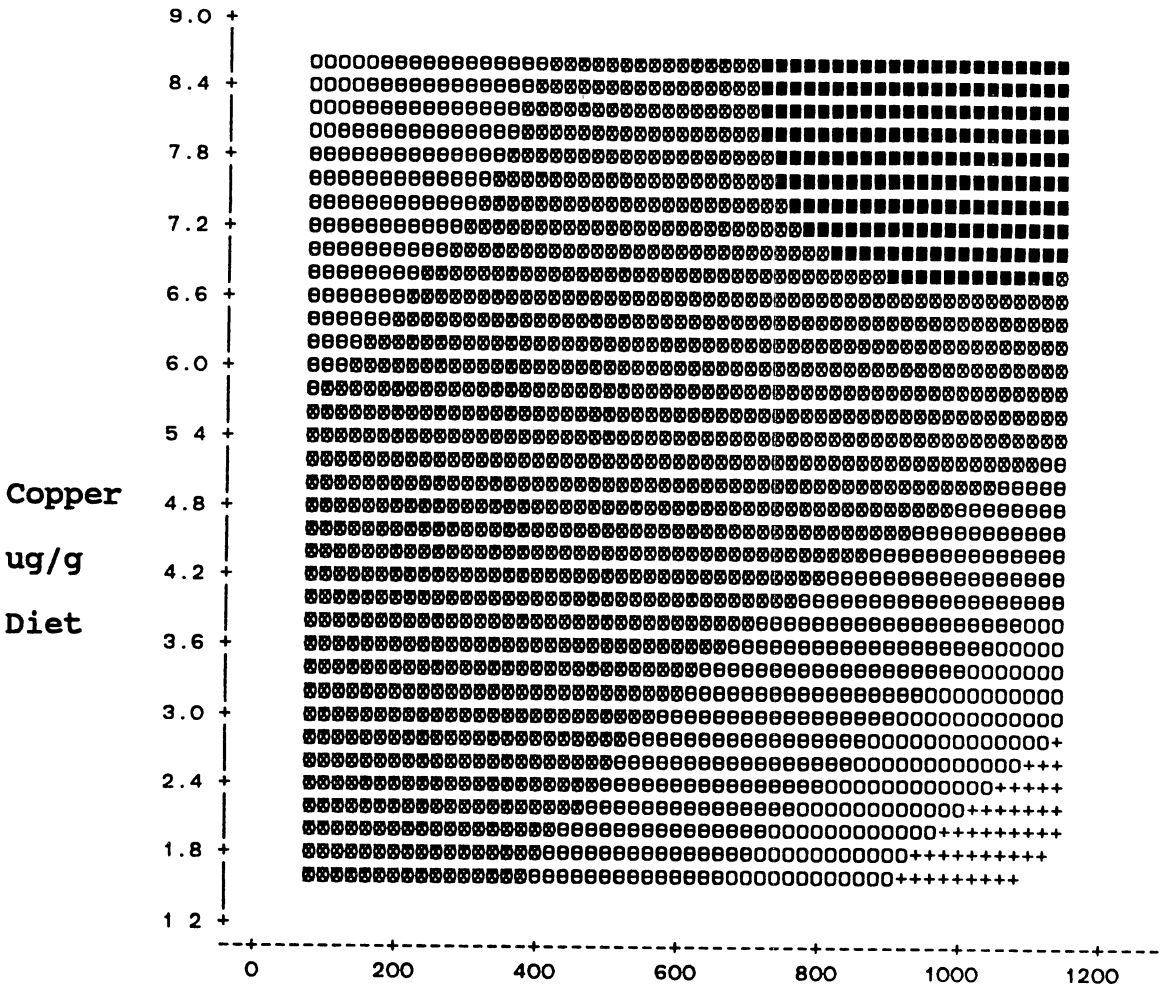

FIGURE 1 Digital contour plot of plasma total cholesterol (mg/dl) as a function of copper and chromium in the diet with zinc in the diet set at 27.7 ug/g.

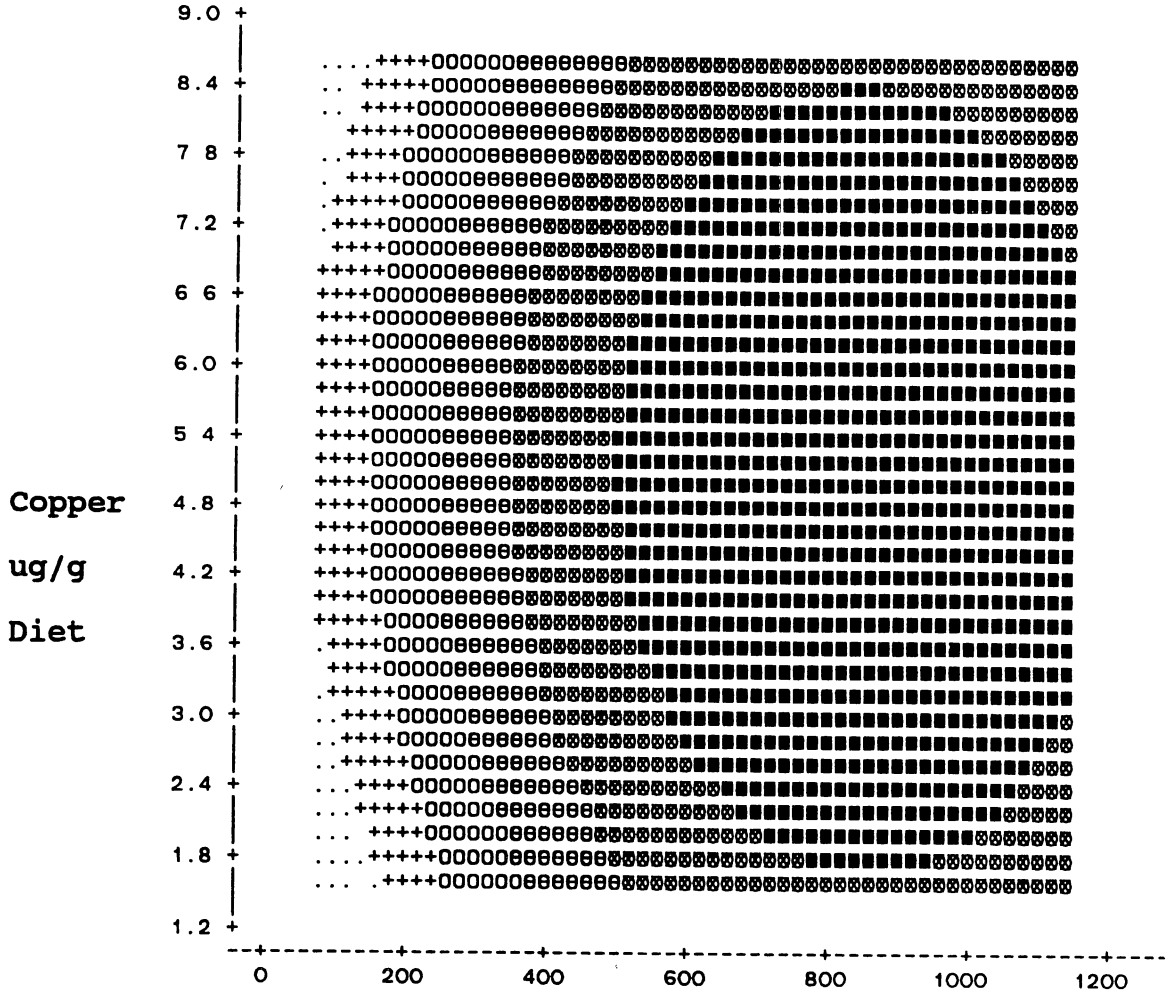

FIGURE 2 Digital contour plot of plasma LDL-cholesterol (mg/dl) as a function of copper and chromium in the diet with zinc in the diet set at 24.9 ug/g.

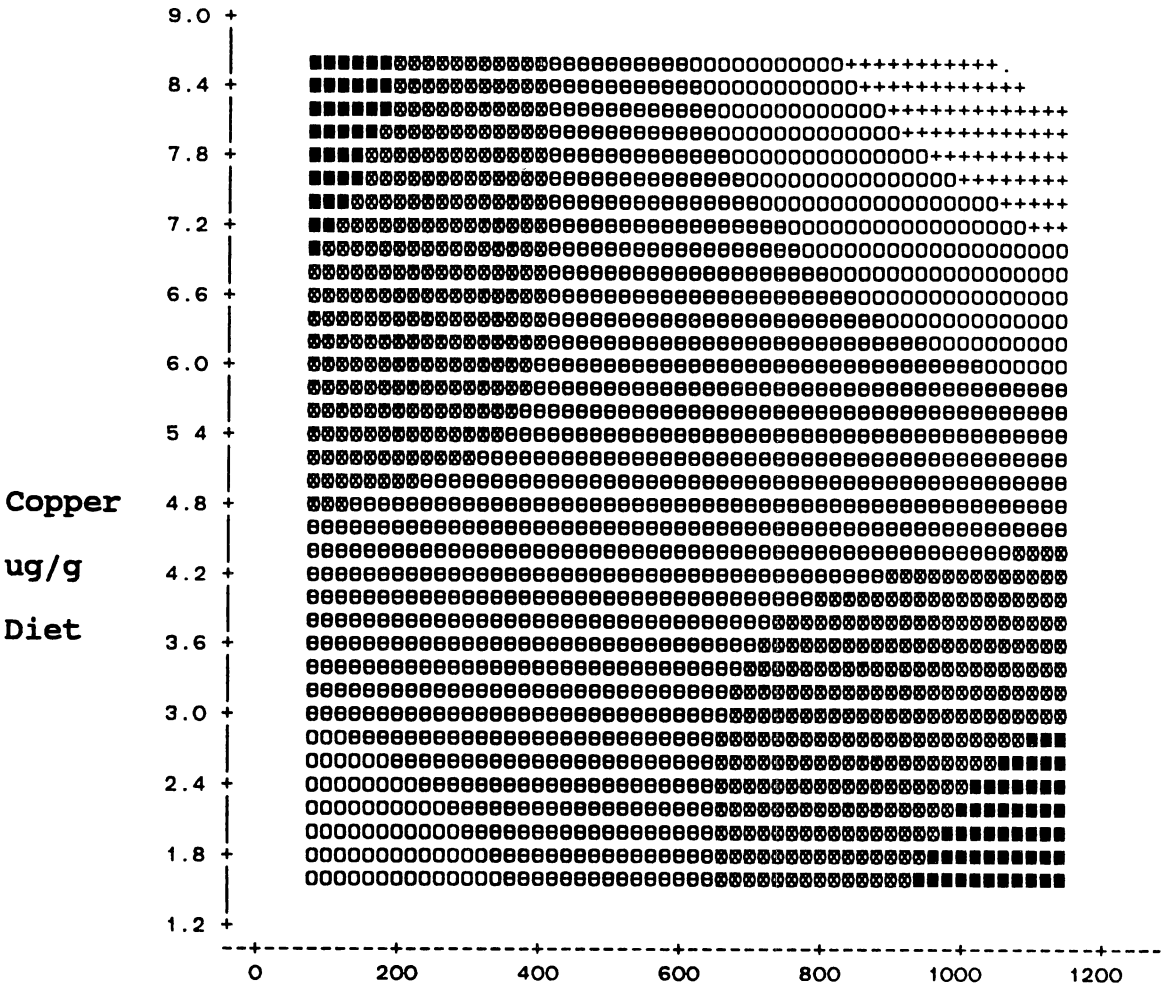

FIGURE 3 Digital contour plot of plasma triglycerides (mg/dl) as a function of copper and chromium in the diet with zinc in the diet set at 24.8 ug/g.

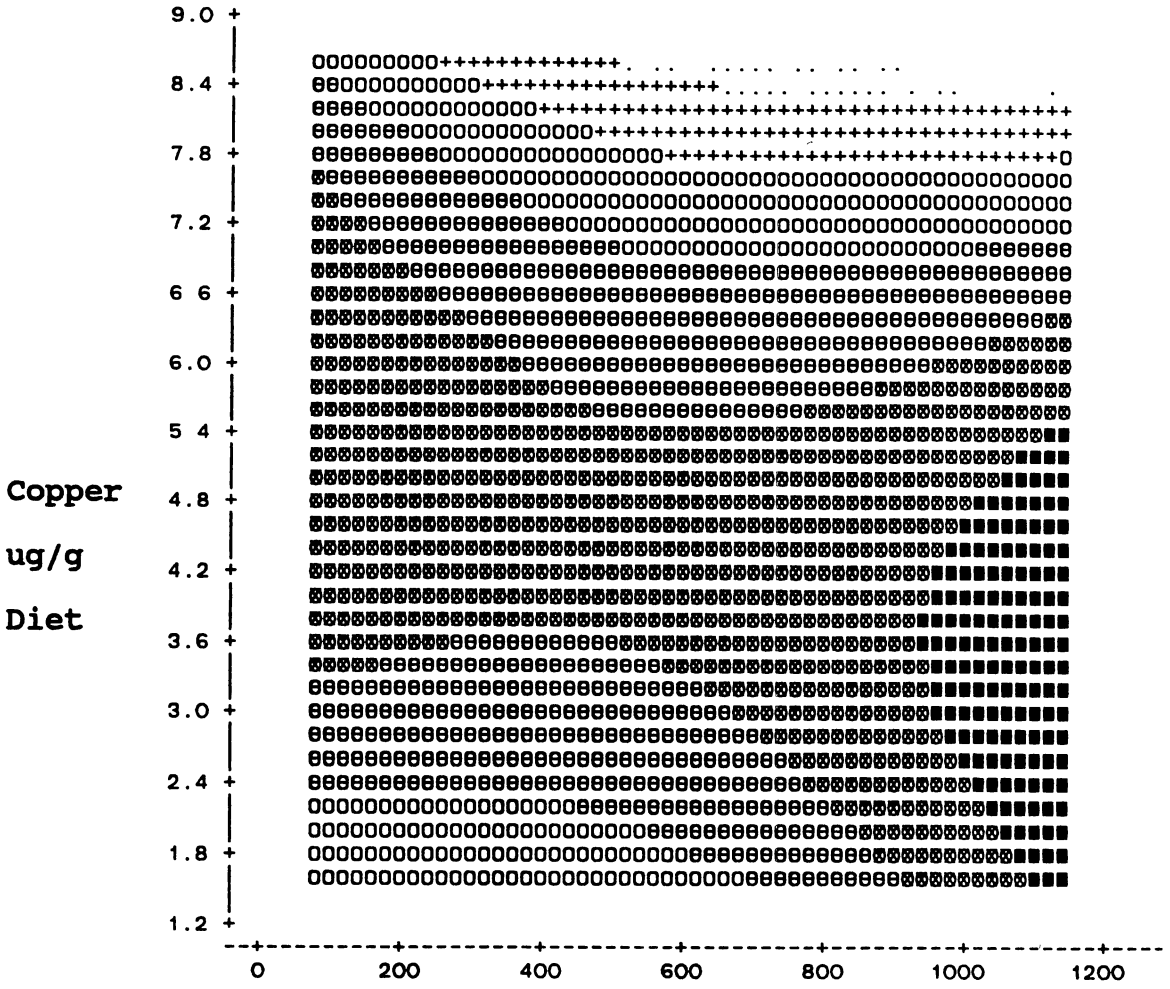

FIGURE 4 Digital contour plot of plasma glucose (mg/dl) as a function of copper and chromium in the diet with zinc in the diet set at 30.8 ug/g.

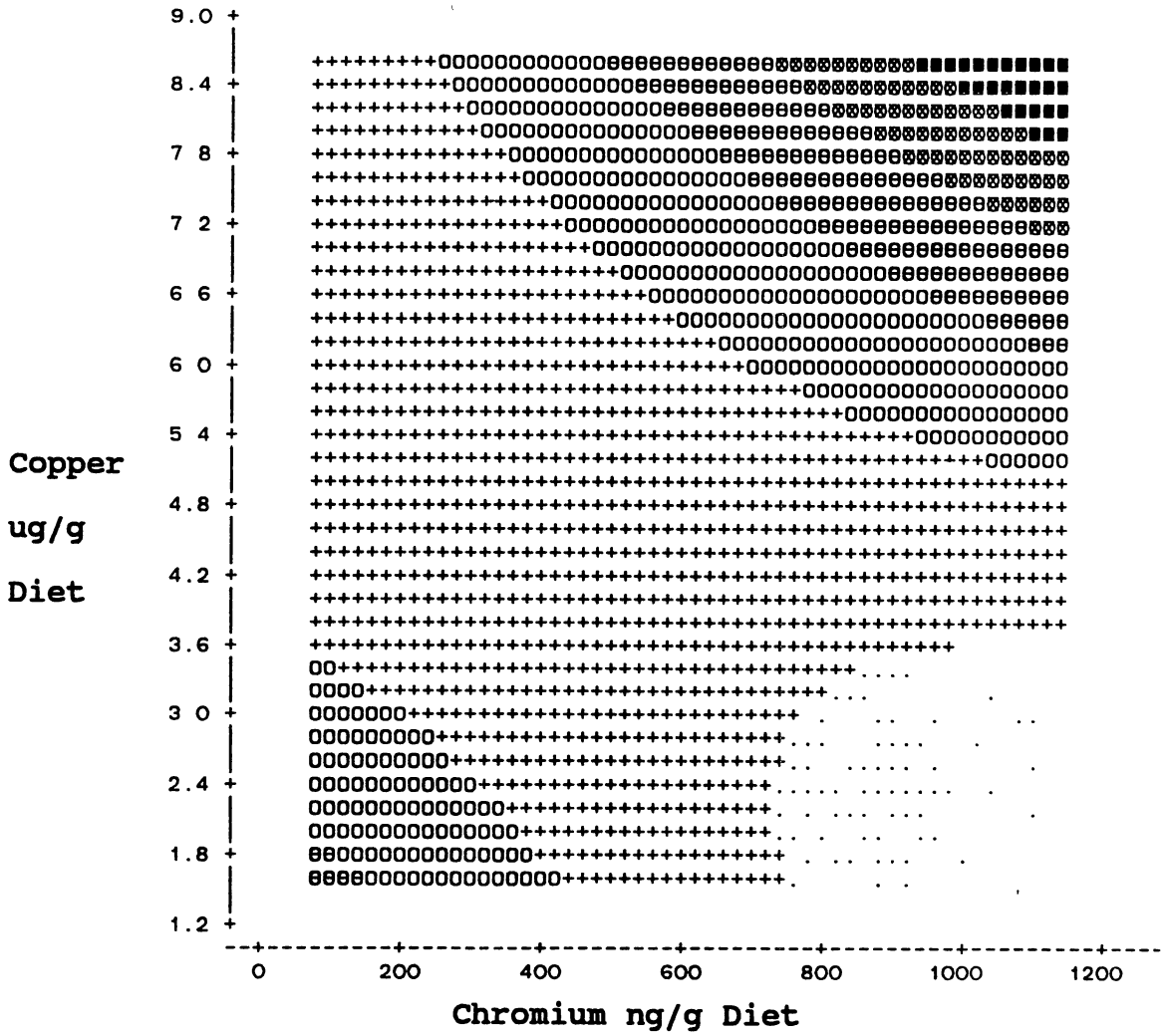

FIGURE 5 Digital contour plot of plasma HDL-cholesterol (mg/dl) as a function of copper and chromium in the diet with zinc in the diet set at 51.5 ug/g.

CHAPTER IV

INDEPENDENT AND/OR INTERACTIVE EFFECT(S) OF COPPER, ZINC, AND CHROMIUM LEVELS ON BONE AND TISSUE MINERAL CONCENTRATIONS IN MALE JAPANESE QUAIL

Nihiser, R.G., Hermann, J.R., Adeleye, B.O.,
Stoecker, B.J., Arquitt, A.B.,
and Brusewitz, G.H.

Department of Nutritional Sciences, Oklahoma State University, Stillwater, Oklahoma 74078.

Shortened Title:

TRACE MINERAL EFFECTS ON BONE

ABSTRACT

The independent and/or interactive effect(s) of copper, zinc, and chromium levels on bone mineral concentrations, the maximum force required to fracture femurs, and tissue mineral concentrations were evaluated in 72 male Japanese quail, Coturnix coturnix japonica, using a three-factor central composite response surface design. Quail were randomly assigned to one of fifteen experimental diets containing variable concentrations of copper (1.6 to 8.3

ug/g diet), zinc (11 to 51 ug/g diet), and chromium (71 to 1124 ng/g diet). After six weeks on the experimental diets, the femurs, liver, and spleen were removed, weighed and frozen for mineral analyses and analyses of the maximum force required to fracture femurs. Dietary copper had a significant main effect ($p<0.05$) on minimizing bone copper concentration and dietary zinc had a significant linear main effect ($p<0.01$) on minimizing bone magnesium concentration. Copper and zinc had a significant interactive effect on maximizing the force required to fracture femurs ($p<0.05$). Copper had a significant quadratic effect on maximizing liver zinc and magnesium concentrations ($p<0.03$ and $p<0.01$, respectively). Copper, zinc, and chromium all had significant effects on maximizing liver calcium ($p<0.05$). Zinc had a significant effect on minimizing liver chromium ($p<0.02$), and copper had a significant effect on maximizing liver iron ($p<0.05$) concentrations.

INDEXING KEY WORDS: Japanese quail, chromium, copper, zinc, bone,

Osteoporosis has become the most important bone disease in Western countries. Osteoporosis afflicts 15 to 20 million Americans, resulting each year in an estimated 1.3 million fractures in individuals 45 years of age and older. One-third of women 65 years and older have vertebral fractures, and by age 90, one-third of women will

have hip fractures, leading to death in 12 to 20 percent of all cases and to long-term nursing home care for many survivors (1).

Much of the research relative to nutrition has focused on low intakes of dietary calcium, vitamin D, fluoride, and boron as dietary factors affecting bone mineral concentrations (2,3). However, other dietary factors including trace minerals such as copper, zinc, and chromium, are related to bone loss and have an impact on bone mineral content (4-12). Zinc is an important factor in bone mineralization (1), and copper has been shown to inhibit bone resorption (1). This is of great importance because typical American diets often provide inadequate amounts of trace minerals (13).

Thus, these minerals may have the potential for improving bone mineral concentrations and lowering risk of osteoporosis. In addition, the interaction of these trace minerals on bone mineral concentrations and the maximum force required to break femurs has not been investigated. In this study, response surface regression analysis was used to investigate the independent and/or interactive effect(s) of copper, zinc, and chromium levels on bone mineral concentrations, the maximum force required to break femurs, and liver and spleen mineral concentrations in male Japanese quail.

MATERIALS AND METHODS

Animals

Japanese quail (Coturnix coturnix japonica) were the experimental animal model selected for this study. Japanese quail have been established as an appropriate animal model for evaluating various osteoporotic factors (14, 15, 16). The protocol for this study was reviewed and approved by the Oklahoma State University Institutional Animal Care and Use Committee.

Two hundred hatchling Japanese quail (B and D Game Farm, Harra, OK) were held on a holding diet for four weeks until gender could be determined by feathering, at which time seventy-two male Japanese quail were weighed and randomly assigned to the experimental diets for six weeks.

The animals were housed in an isolated laboratory, in accordance with USDA and NIH guidelines for laboratory animals. The temperature and light-controlled laboratory was maintained by personnel from Laboratory Animal Resources in the College of Veterinary Medicine at Oklahoma State University. During the initial four week holding period animals were group housed in polystyrene cages with supplemental heat provided by heat lamps, with decreasing heat intensity. During the first week, 100 watt bulbs provided the heat; 75 watt bulbs supplied the heat in the second week and during the third and fourth weeks, 50 watt bulbs were used. During the experimental period animals

were individually housed in polystyrene cages. Ceramic feed cups and water containers were used to reduce possibilities for mineral contamination.

Diets

The experimental diets, vitamin and mineral mixes were formulated to contain all nutrients recommended by the National Research Council for growing Japanese quail (17) except for copper, zinc, and chromium as dictated by the experimental design (Table 1). Semi-purified diets, vitamin and mineral mixes were prepared in our laboratory after assaying all components for trace mineral content.

The experimental mineral mixes for this study were based on a three factor central composite rotatable response surface design (18) (Table 2). Response surface analysis is a statistical technique which evaluates the interrelationship of two or three independent variables with a minimal sample size (18). The ranges of copper, zinc, and chromium studied were; copper 1.6 ug/g diet to 8.3 ug/g diet, zinc 11 ug/g diet to 51 ug/g diet, and chromium 71 ng/g diet to 1124 ng/g diet. The initial four week holding diet consisted of a moderately low mineral combination to prevent bone and tissue mineral accumulation (Table 2).

TABLE 1

Diet composition

Ingredient	g/kg diet
Casein	250.0
Arginine	5.0
D-L Methionine	3.0
Choline Bitartrate	5.9
Corn oil	40.0
Exthoxyquin	0.1
Celufil	40.0
Vitamin mix[1]	10.0
Mineral mix[2]	60.0
Dextrose	586.0

[1]Vitamin mix formulated to meet the National Research Council recommendations for growing Japanese quail.
[2]Mineral mix formulated to meet the National Research Council mineral recommendations for growing Japanese quail except for chromium, copper and zinc.

The mineral mixes were prepared from reagent grade or ultrapure chemicals, after assaying each lot number of minerals, as different lots have been found to vary widely. The experimental diets were analyzed for total mineral content before feeding. The experimental diets and deionized water were available ad libitum except as specified by the experimental design.

TABLE 2

Zinc, copper, and chromium concentrations[1] of experimental diets using a three-factor response surface design

Diet	RS Code[2] (Zn,Cu,Cr)	Zinc ppm	Copper ppm	Chromium ppb	n
Holding	(-1,-1,-1.68)	19.3	3.1	68	
1	(1, 1, 1)	40.2	6.3	821	4
2	(-1, 1, 1)	19.6	5.1	856	4
3	(1,-1, 1)	46.1	3.3	811	4
4	(1, 1,-1)	45.6	8.1	228	4
5	(-1,-1, 1)	19.4	3.0	729	4
6	(-1, 1,-1)	20.9	8.0	261	4
7	(1,-1,-1)	41.3	2.8	268	4
8	(-1,-1,-1)	20.3	2.7	270	4
9	(1.68,0,0)	51.0	5.0	532	4
10	(-1.68,0,0)	11.2	4.7	489	4
11	(0,1.68,0)	30.5	8.3	485	4
12	(0,-1.68,0)	31.4	1.6	488	4
13	(0,0, 1.68)	30.5	4.5	1124	4
14	(0,0,-1.68)	29.2	5.5	71	4
15	(0,0,0)	28.7	5.7	465	16

[1]As determined by atomic absorption spectrophotometry.
[2]Response surface code.

Necropsy

After six weeks on the experimental diets the quail were fasted for 12 hours prior to necropsy. Methoxyflurane was used for anesthesia. The birds were weighed and the liver and spleen were removed carefully to avoid chromium contamination, weighed, and frozen separately for tissue

mineral analyses. Both femurs were removed, cleaned with a glass knife and separately weighed into acid-washed borosilicate glass tubes under a clean air hood to avoid mineral contamination and frozen for bone mineral analyses and maximum force required to break femurs.

Tissue and Bone Mineral Analyses

For tissue mineral analyses, tissues were sampled (250 - 500 mg) using a glass knife under a clean air hood. Samples were weighed into acid-washed borosilicate glass tubes and dried for 24 h at 100°C. Samples were then ashed in a muffle furnace with no exposed metal heating elements (Lindberg, Watertown, WI) using a modified wet-dry ashing procedure (12,19). After ashing, samples were diluted with 1N HCl (G. Fredrick Smith, Columbus, OH) and analyzed at 324.8 nm for copper, 213 nm for zinc, and 357.9 nm for chromium using a Perkin Elmer Model 5000 atomic absorption spectrophotometer with flame and graphite furnace and Zeeman background correction (Perkin Elmer Corp., Norwalk, CT.). Tissue chromium and bone copper were analyzed on the graphite furnace and all other mineral analyses were performed on the flame furnace. Drying and ashing procedures for bone analyses were similar to tissue analyses, however, repetition of the steps was required.

Maximum Force To Break Femur Analyses

The force required to break femurs was measured on a three point bending fixture with an Instron Universal Testing Machine at a loading rate of 1 mm/minute.

Data Analyses

Data were analyzed by the Statistical Analysis System (SAS) using the SAS response surface regression analysis (RSREG). This procedure allows the use of two or more independent variables to determine maximum and minimum responses of the dependent variables. In this study, response surface regression analyses were performed to find response levels for the maximum force required to fracture femurs, and bone and tissue minerals at various levels of copper, zinc, and chromium. Response surface regression analyses produced regression equations for each dependent variable. Because of the complexity of the response surface analyses, two-dimensional response surface contour plots were produced for each of the dependent variables using RSREG. To graph the dependent variable response surface contours, the three variable regression equations were reduced to the most useful two variables by holding the third variable in the equation at the optimal value as determined by RSREG. These response surface contours can be used to determine dependent variable responses at various combinations of the independent variables. In

interpreting the response surface contours, the lightest shading represents decreased dependent variable responses while the darkest shading is indicative of higher dependent variable responses. The level of significance for this study was set at $p \leq 0.05$. Probabilities are cited from the total regression model unless otherwise specified.

RESULTS

Dietary copper had a significant main effect ($p<0.05$) on minimizing bone copper concentration (Figure 1). Although not significant, dietary copper and zinc had an quadratic effect ($p=0.056$) on minimizing bone calcium concentration (Figure 2). Dietary zinc had a significant linear main effect ($p<0.01$) on minimizing bone magnesium concentration (Figure 3). Copper and zinc had a significant interactive effect ($p<0.05$) on maximizing the force required to break femurs (Figure 4). The region where combined copper and zinc increased the force required to break femurs was above the regions where other bone mineral concentrations were minimized. Though not significant, chromium also had a positive effect on the maximum force required to break femurs ($p=0.058$). No significant effect was observed for bone zinc or iron concentrations; however, there was a strong trend for minimization of bone zinc within the mineral ranges investigated in this study (Figure 5).

Copper had a significant quadratic effect on maximizing liver zinc (Figure 6) and magnesium (Figure 7) concentrations, ($p<0.03$ and $p<0.01$), respectively. Chromium, copper and zinc all had a significant effect ($p<0.05$), and copper and chromium had a significant interactive effect ($p<0.001$) on maximizing liver calcium (Figure 8). Zinc had a significant effect on minimizing liver chromium ($p<0.02$) (Figure 9), and copper had a significant effect on maximizing liver iron ($p<0.05$) (Figure 10) concentrations. No significant effect was observed for liver copper concentration; however, there was a trend for maximizing liver copper within the mineral ranges investigated in this study (Figure 11).

Additionally, copper had a significant quadratic main effect on minimizing spleen copper ($p<0.05$) (Figure 12). No significant effect was observed for spleen chromium and zinc concentrations; however, there was a trend for maximizing spleen chromium and minimizing spleen zinc within the mineral ranges investigated in this study (Figure 13 and 14, respectively).

DISCUSSION

Several studies have investigated the effect of excessive or inadequate trace mineral intakes on bone structure and composition. Ledoux and colleagues (10) observed that an inadequate copper intake resulted in decreased bone copper concentrations in chicks. In our

study, we observed that as copper increased in the diet there was a quadratic response on bone copper concentration (Figure 1). This indicates that at suboptimal levels, the bone may serve as a copper reservoir and as intakes increase beyond suboptimal levels, copper deposition begins to occur. Several studies have reported that deficient dietary zinc intake resulted in decreased bone zinc concentrations (5-9). In our study we did not observe a significant effect of dietary zinc on bone zinc. However, there appeared to be a trend for minimizing bone zinc at lower dietary levels (Figure 5). Like copper, dietary zinc also affected other bone minerals. Dietary zinc had a significant linear effect on minimizing bone magnesium (Figure 3). Additionally, copper and zinc had an interactive effect on bone calcium and on the force required to fracture femurs (Figures 2 and 4). Thus, our research indicates that dietary copper and zinc not only affect bone copper and zinc minerals but also independently and interactively affect other bone mineral concentrations.

Several researchers have reported that liver copper concentrations decrease with copper deficiency (20-23). In the present study, although not significant, there was a quadratic response with liver copper concentrations as dietary copper increased. Additionally, liver zinc concentrations have been correlated with liver copper (24). We also observed a significant quadratic effect of dietary copper on maximizing liver zinc, as well as on liver

magnesium, and iron. In addition, copper and chromium had a significant interactive effect on maximizing liver calcium. Thus, it appears that dietary copper may have a significant effect on maximizing many other trace elements.

Numerous researchers have reported that liver zinc concentrations decrease with deficient zinc intake (25-28) and that liver zinc concentrations increase with zinc supplementation (29). Although not significant, we also observed an increase in liver zinc with increasing dietary zinc. In addition, we observed a significant effect of dietary zinc on minimizing liver chromium. It has been reported that zinc influences chromium absorption; more specifically that zinc decreased chromium absorption while chromium decreased zinc absorption in zinc-deficient rats (30). The present results do not demonstrate that dietary chromium has a significant effect on liver chromium. Additionally, Seaborn and Stoecker (12) have reported that liver chromium concentration in mice was not affected by dietary chromium.

Thus, it appears that the dietary intake of trace minerals not only affects bone and tissue mineral concentrations of those minerals being varied, but also bone and tissue concentrations of other minerals. In addition, the interactive effects of trace minerals also influence various bone and tissue mineral concentrations.

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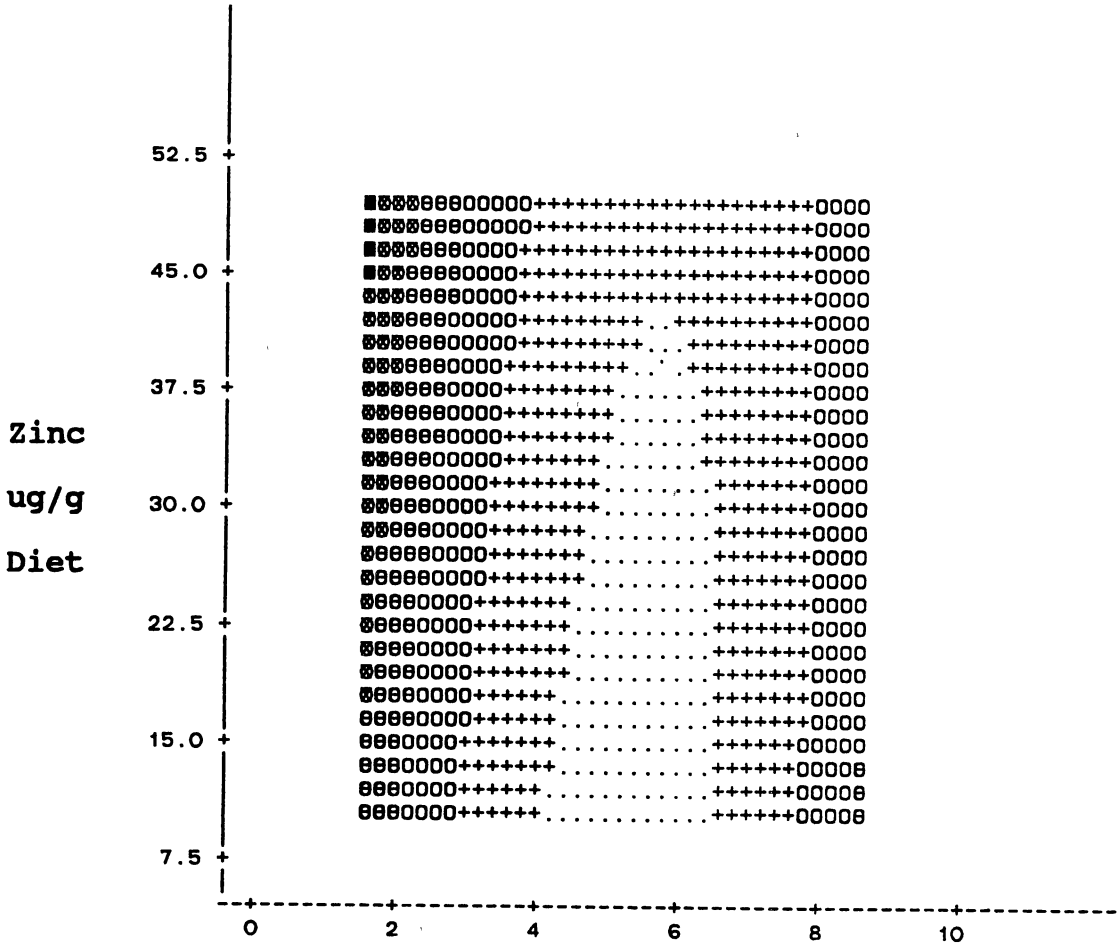
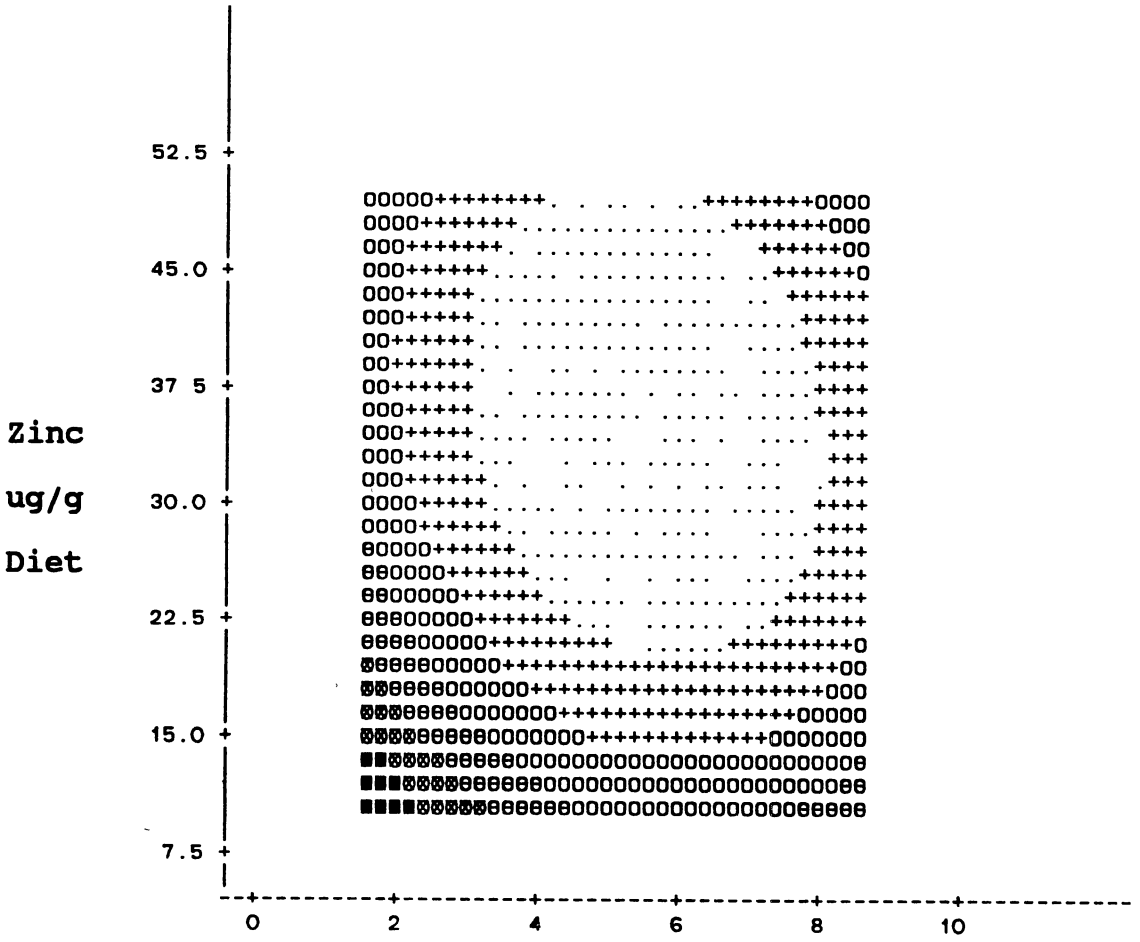


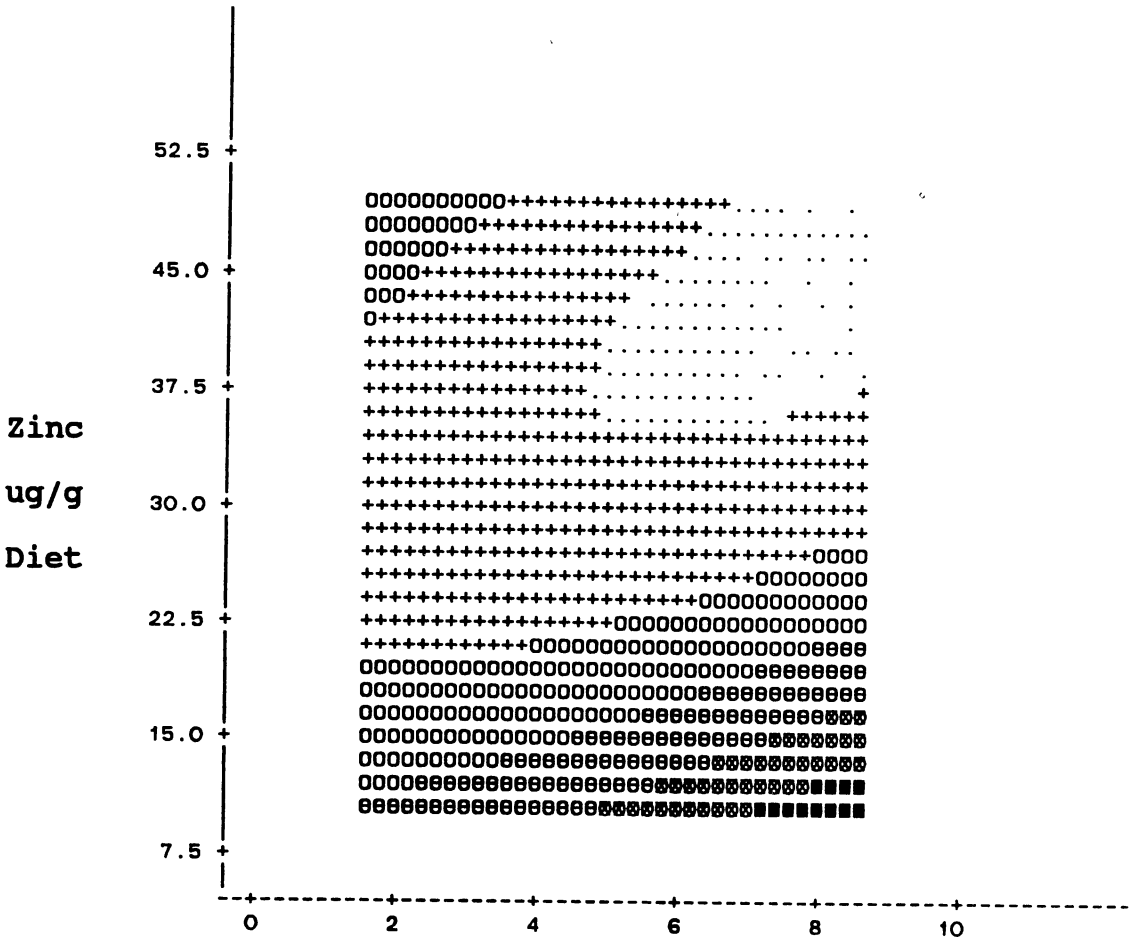
FIGURE 1 Digital contour plot of bone copper (ng/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 241 ng/g.



LEGEND:

.....	110 - 115	ΘΘΘΘΘ	125 - 129
+++++	115 - 120	ΘΘΘΘΘ	129 - 134
OOOOO	120 - 125	ΘΘΘΘΘ	134 - 139

FIGURE 2 Digital contour plot of bone calcium (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 539 ng/g.



LEGEND:

.....	1.5 - 1.8	ΘΘΘΘΘ	2.4 - 2.7
+++++	1.8 - 2.1	◘◘◘◘◘	2.7 - 3.0
OOOOO	2.1 - 2.4	■■■■■	3.0 - 3.3

FIGURE 3 Digital contour plot of bone magnesium (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 530 ng/g.

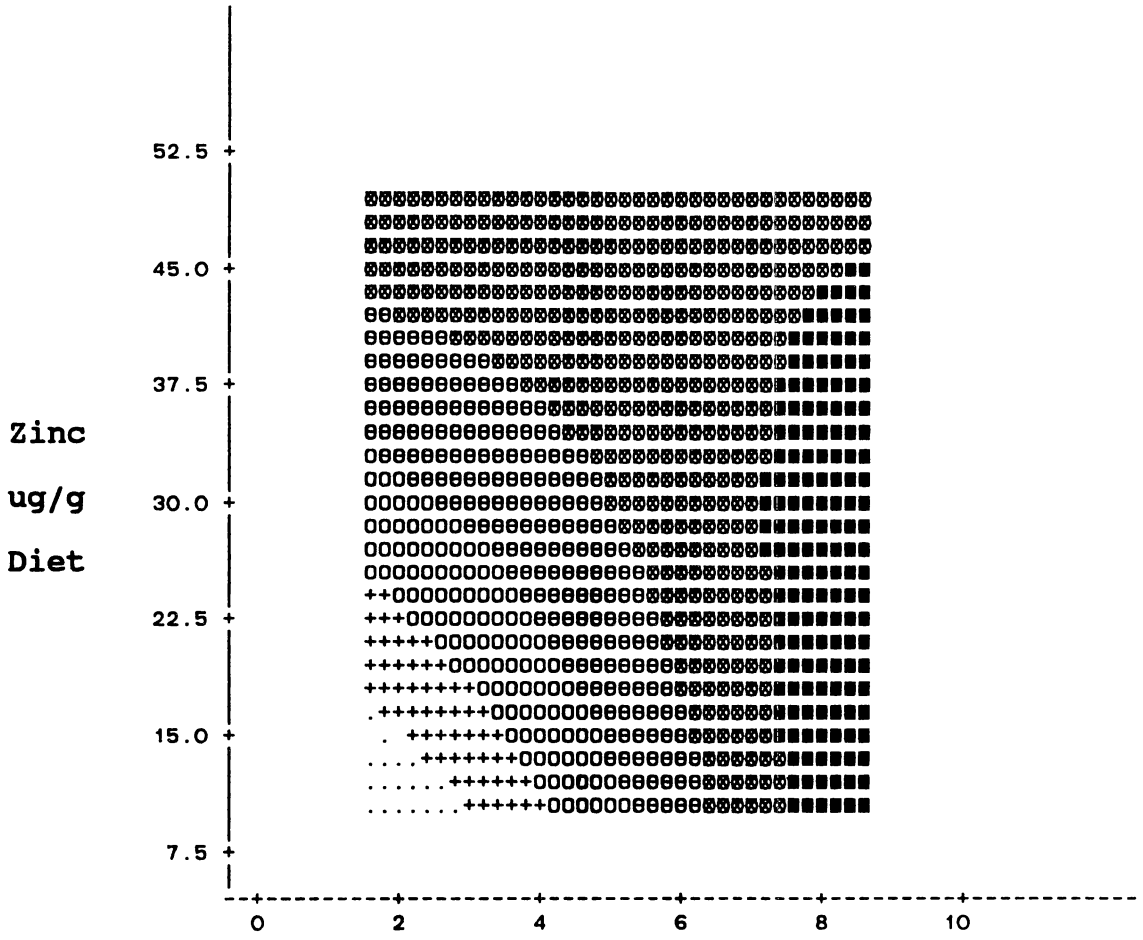


FIGURE 4 Digital contour plot of femur fracture force as a function of zinc and copper in the diet with chromium in the diet set at 129 ng/g.

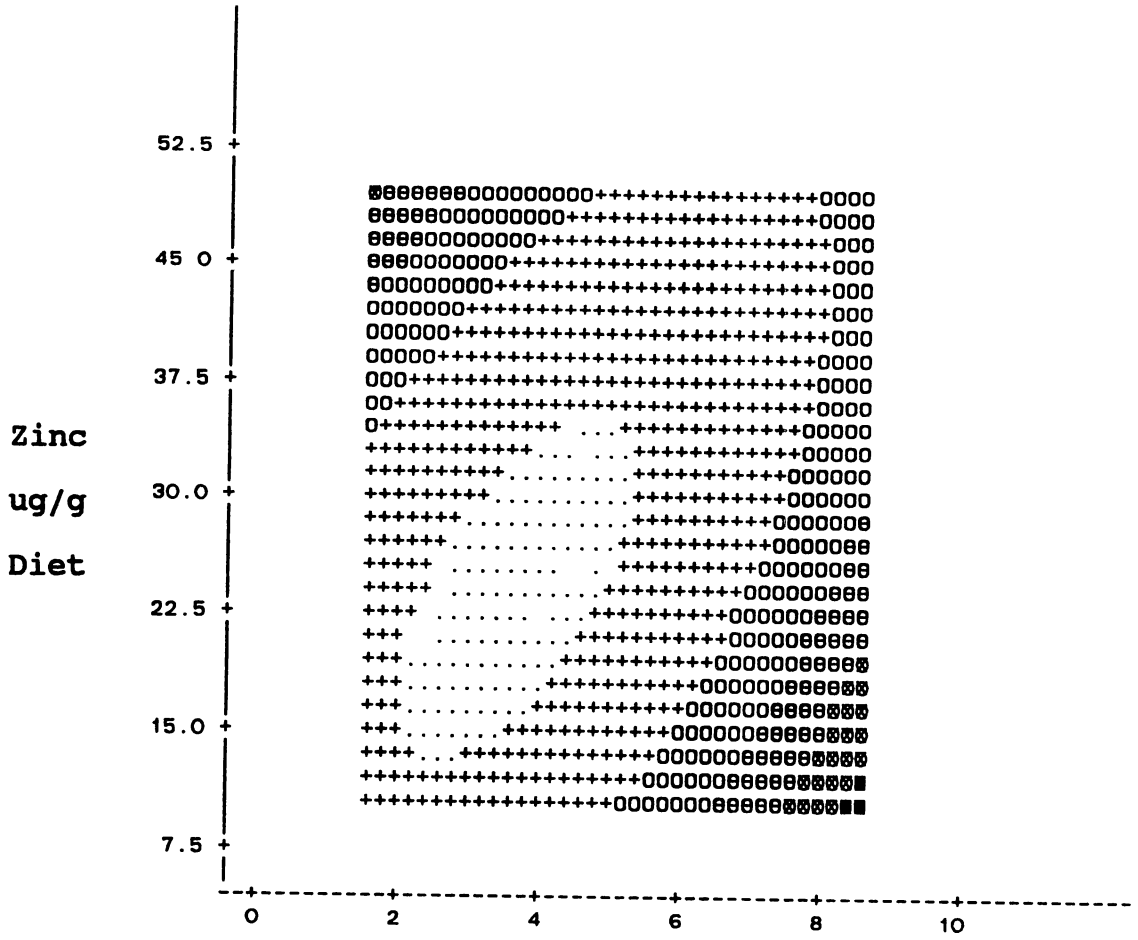


FIGURE 5 Digital contour plot of bone zinc (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 698 ng/g.

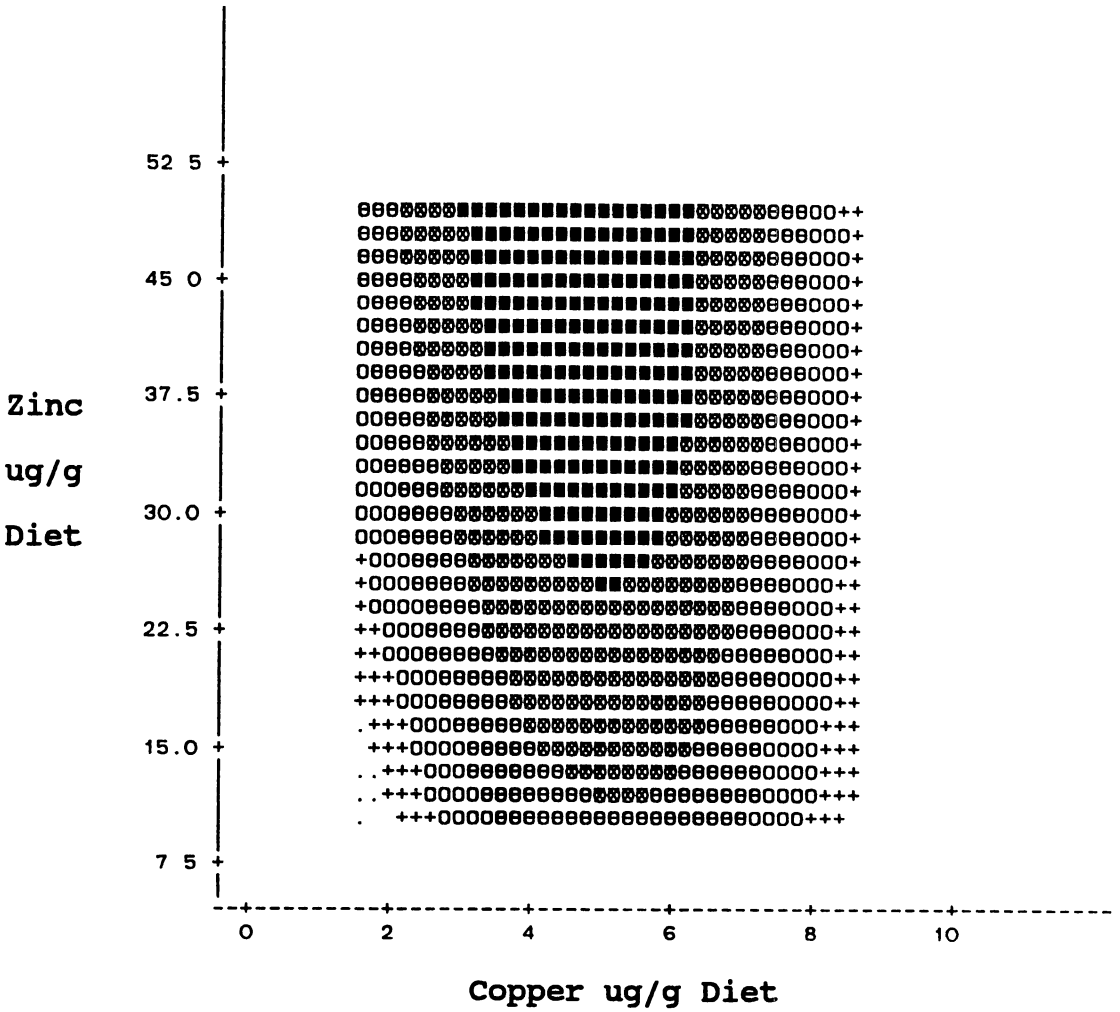


FIGURE 6 Digital contour plot of liver zinc (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 322 ng/g.

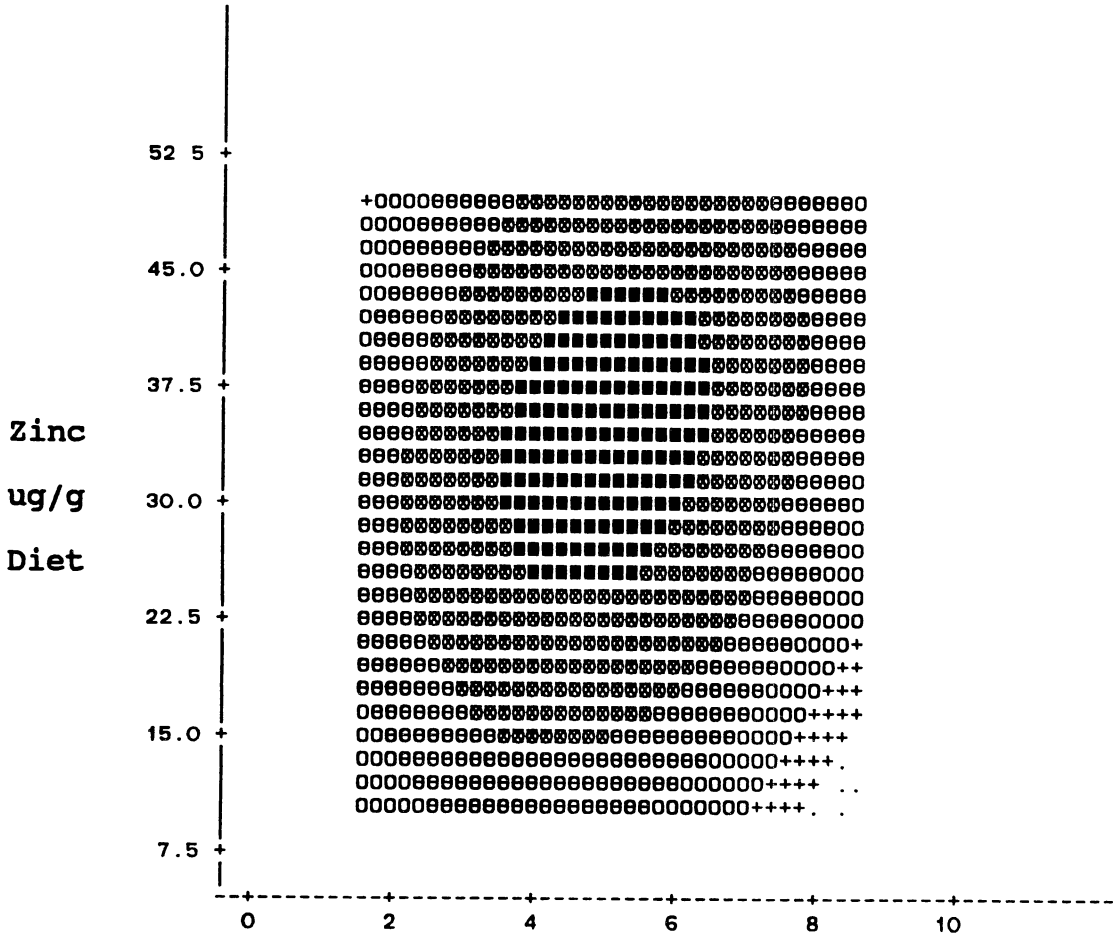


FIGURE 7 Digital contour plot of liver magnesium (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 696 ng/g.

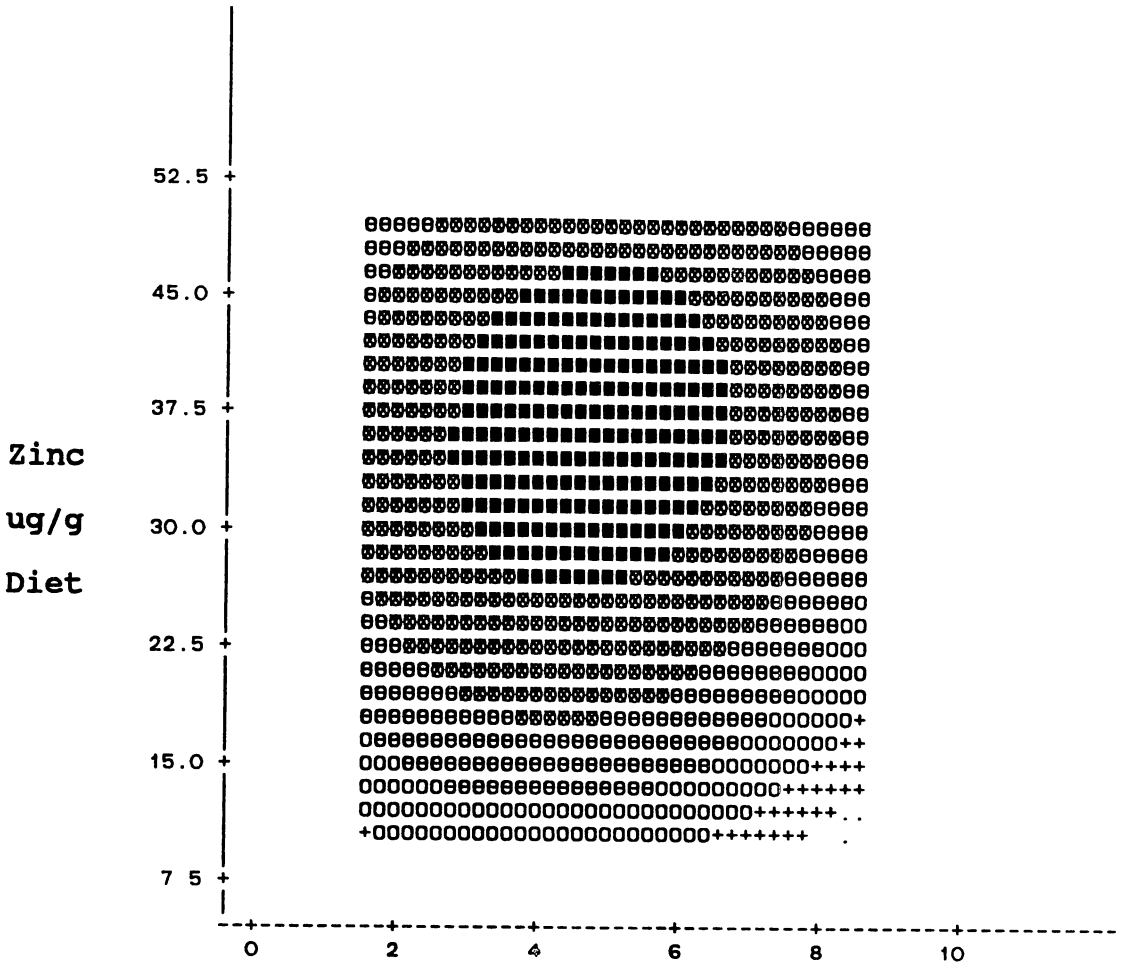


FIGURE 8 Digital contour plot of liver calcium (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 549 ng/g.

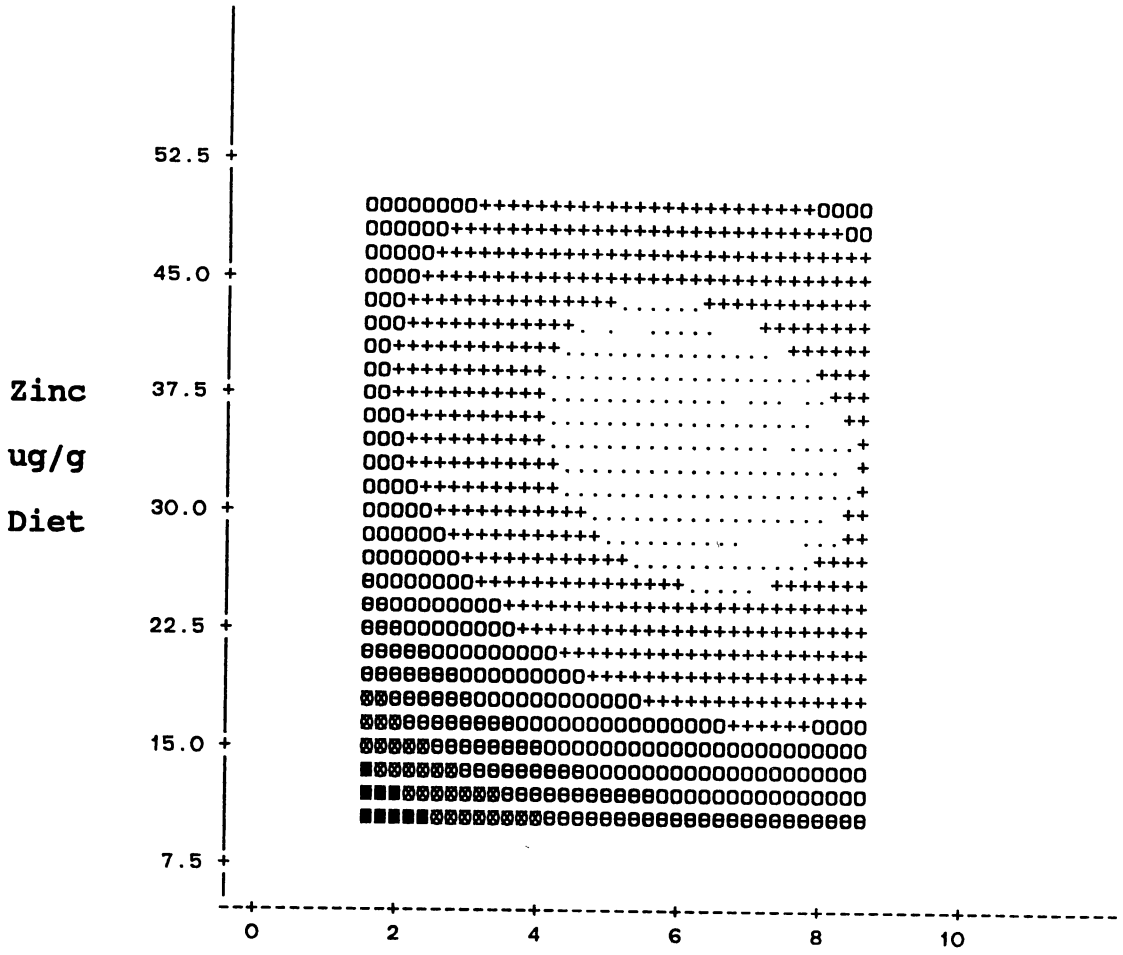


FIGURE 9 Digital contour plot of liver chromium (ng/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 554 ng/g.

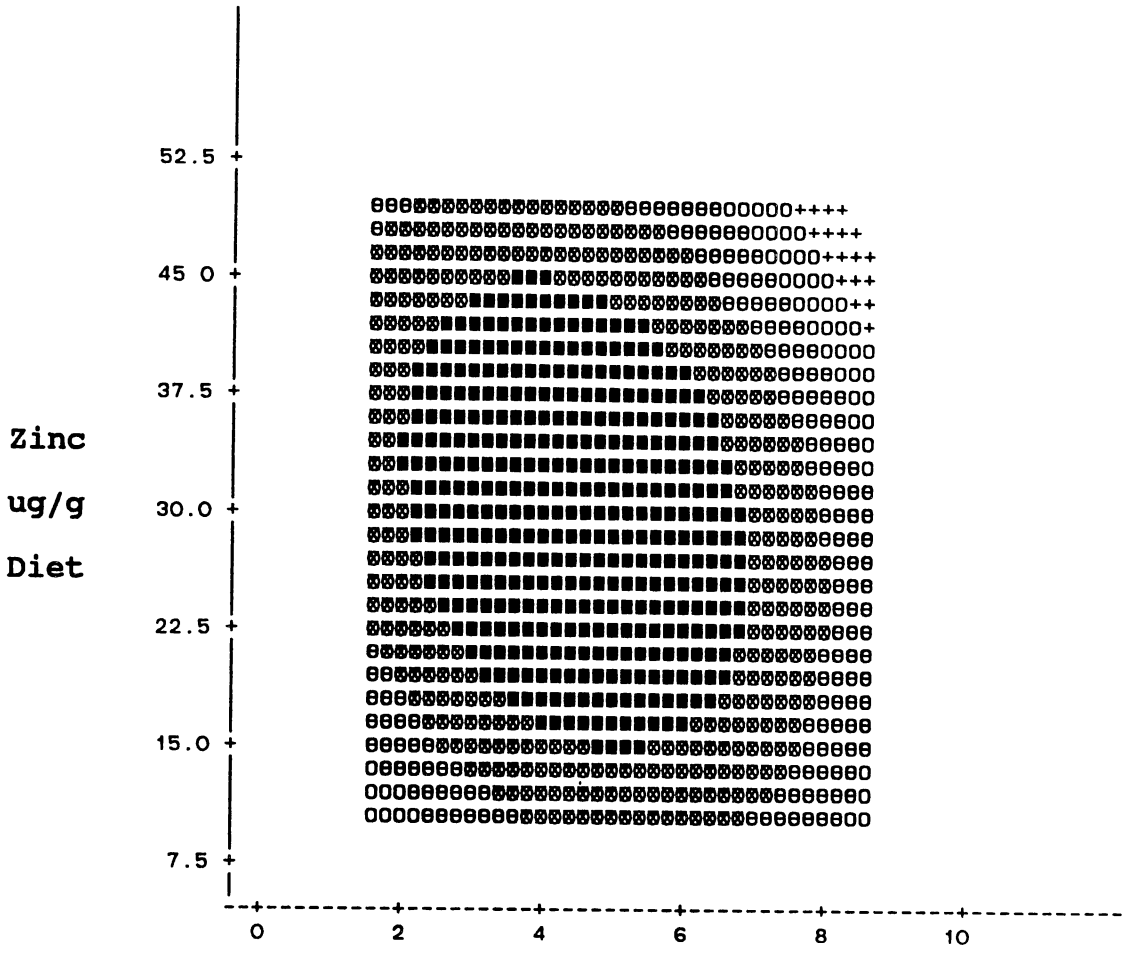


FIGURE 10 Digital contour plot of liver iron (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 526 ng/g.

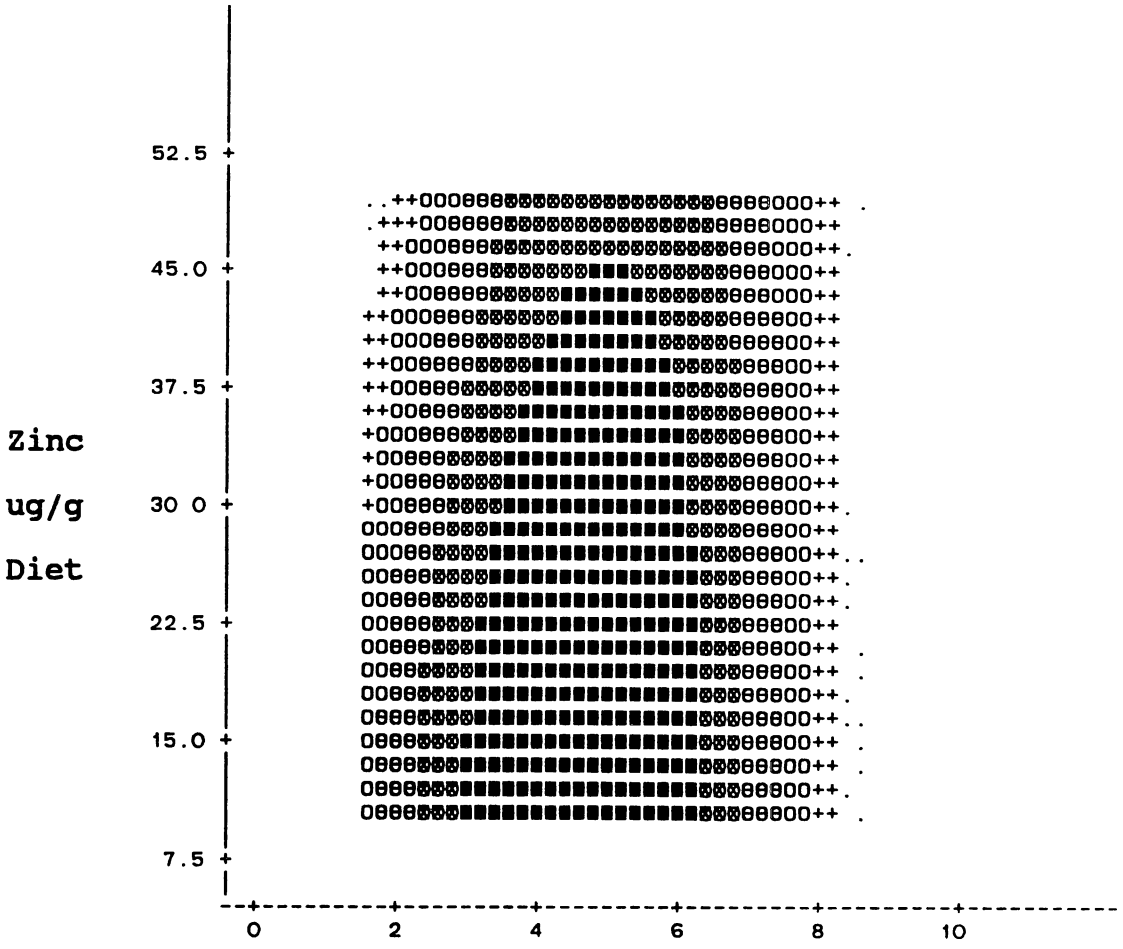


FIGURE 11 Digital contour plot of liver copper (ug/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 381 ng/g.

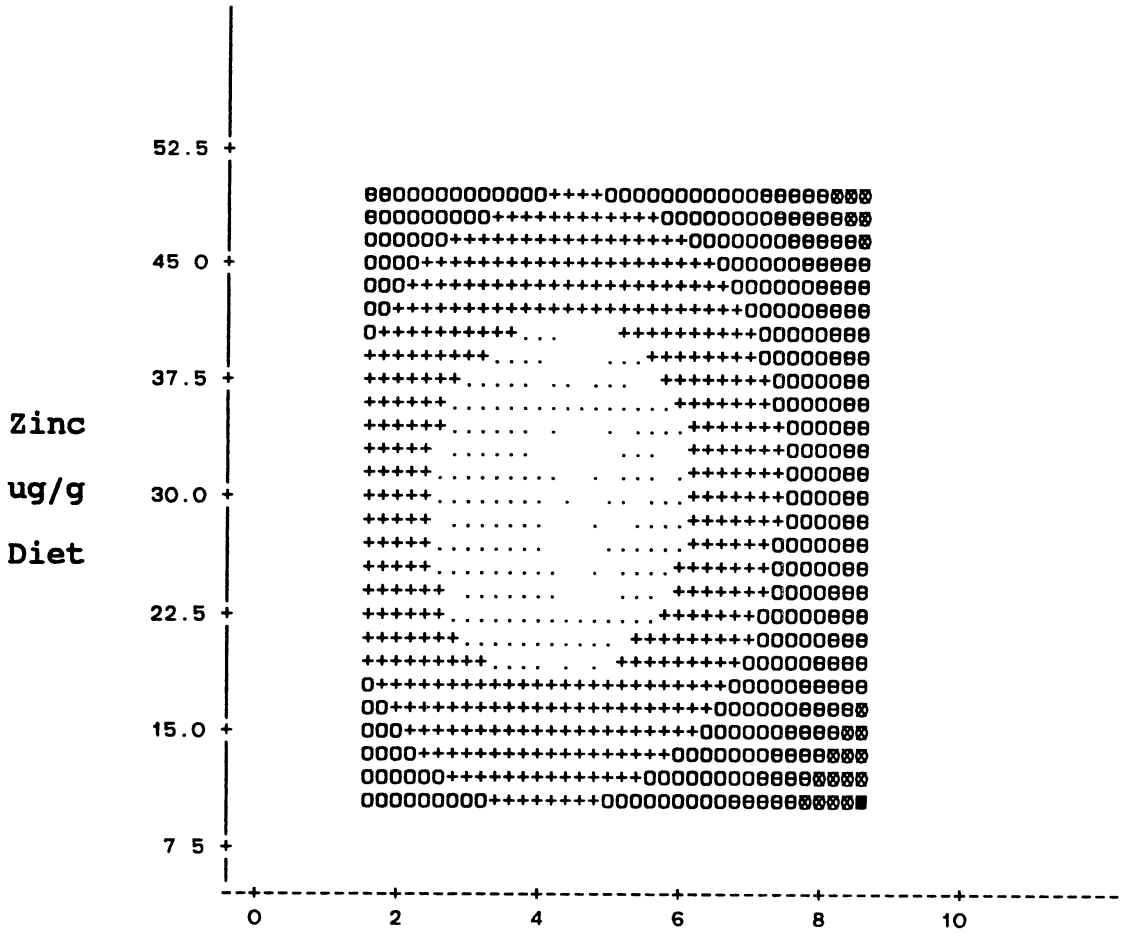


FIGURE 12 Digital contour plot of spleen copper (ug/g dry weight) as a function of zinc and copper in the diet with chromium set in the diet at 565 ng/g.

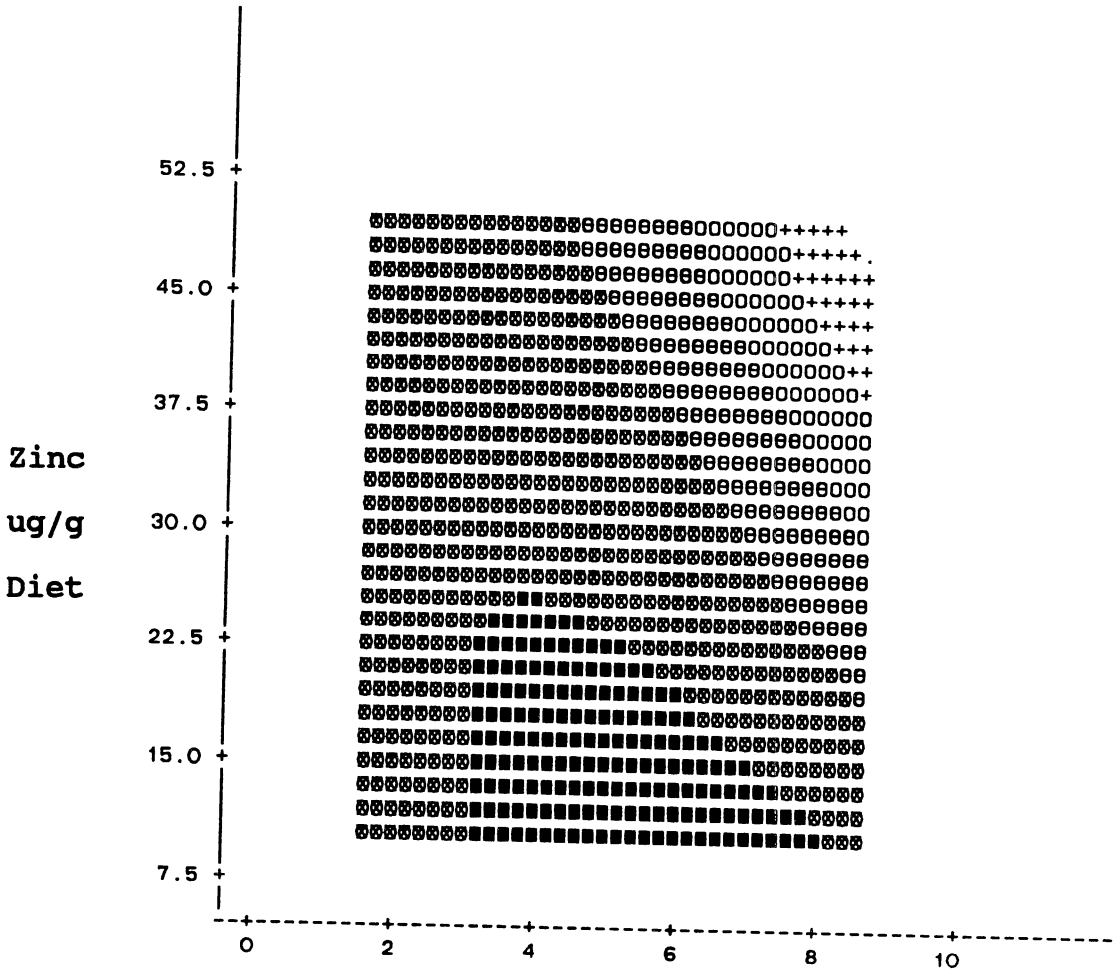


FIGURE 13 Digital contour plot of spleen chromium (ng/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 542 ng/g.

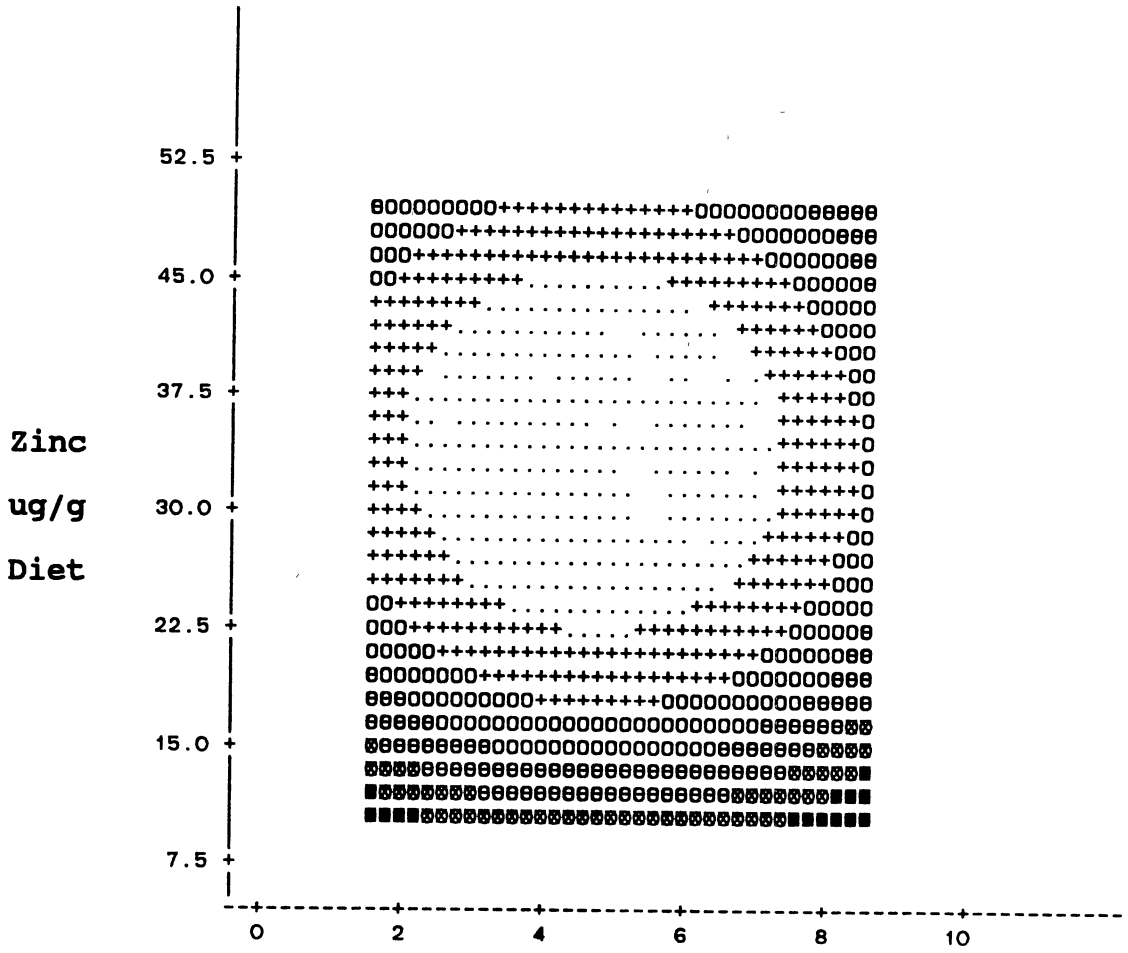


FIGURE 14 Digital contour plot of spleen zinc (ng/g dry weight) as a function of zinc and copper in the diet with chromium in the diet set at 584 ng/g.

CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This research evaluated the independent and/or interactive effect(s) of copper, zinc, and/or chromium on plasma lipids and glucose, in male Japanese quail. In addition, this study evaluated the independent and/or interactive effect(s) of these minerals on tissue and bone mineral concentrations, as well as the maximum force required to fracture femurs in male Japanese quail.

Four hypotheses were listed in the introduction of this thesis. The first hypothesis stated that there would be no statistically significant independent and/or interactive effect(s) of copper, zinc and/or chromium on plasma lipids (total cholesterol, LDL-cholesterol, HDL-cholesterol, and triglycerides) in male Japanese quail. The second hypothesis stated that there would be no statistically significant independent and/or interactive effect(s) of copper, zinc, and/or chromium on plasma glucose in male Japanese quail. No significant differences were observed for plasma total cholesterol, LDL-cholesterol, triglycerides, or glucose between dietary treatments for the mineral ranges studied. However, a

significant linear effect was observed for HDL-cholesterol as a response to copper and chromium ($p<0.02$) after six weeks, with HDL-cholesterol increasing as both copper and chromium increased in the diet. Therefore, based on these results the first null hypothesis is rejected and we fail to reject the second null hypothesis.

Hypothesis three stated that there would be no statistically significant independent and/or interactive effect(s) of copper, zinc, and/or chromium on liver, spleen, and bone mineral concentrations in male Japanese quail. After six weeks, dietary copper had a significant main effect on bone copper ($p<0.05$). Dietary zinc had a significant linear main effect ($p<0.01$) on bone magnesium. Copper had a significant quadratic effect on liver zinc and magnesium concentrations ($p<0.03$), and copper, zinc, and chromium all had significant effects on liver calcium ($p<0.05$), and there was a significant interactive effect between copper and chromium ($p<0.001$) on liver calcium. Zinc also had a significant effect on liver chromium ($p<0.02$), and copper had a significant effect on liver iron ($p<0.05$) concentrations. Additionally, copper had a significant quadratic main effect on spleen copper ($p<0.05$). Therefore, based on these results the third null hypothesis is rejected.

Hypothesis four stated that there would be no statistically significant independent and/or interactive effect(s) of copper, zinc, and/or chromium on the maximum

force required to fracture femurs in male Japanese quail. At six weeks, copper and zinc had a significant interactive effect on the force required to fracture femurs ($p<0.05$). Therefore, based on these results the fourth null hypothesis is rejected.

Conclusions

Copper and chromium were the most significant variables in terms of their effect on blood lipids. Although plasma HDL-cholesterol was the only lipid response that was significant, higher dietary copper and chromium concentrations may further influence plasma LDL-cholesterol, total-cholesterol, triglyceride, and glucose responses. Plasma LDL-cholesterol concentration began to decline at the highest chromium and copper combinations in this study. This may indicate that higher chromium and copper combinations may be beneficial in further reducing LDL-cholesterol concentrations. Plasma triglyceride concentrations were beginning to minimize at the higher concentrations of copper and chromium in this study. Plasma total-cholesterol, maximizing at higher combined concentrations of dietary copper and chromium, may reflect an overlap of the maximum response of plasma HDL-cholesterol. Plasma glucose responses also were beginning to minimize at higher combined concentrations of copper and chromium. An overall conclusion that can be drawn from these trends is that combined higher concentrations of

copper and chromium could elicit optimal lipid responses in Japanese quail.

It is the mineral combinations of dietary zinc and copper, however, that predominantly influenced bone and tissue mineral concentrations as seen by the significant effects on bone copper, magnesium, and calcium as well as the significant effects on liver zinc, magnesium, calcium, and iron, and on spleen copper. There was also a significant interactive effect between dietary zinc and copper on the maximum force required to fracture femurs. Therefore, zinc and copper are important dietary factors affecting bone and tissue mineral concentrations as well as the maximum force required to break femurs. In addition, this research indicates that dietary zinc and copper not only influence the zinc and copper concentrations in the bone and tissues, but these minerals also independently and interactively affect other bone and tissue mineral concentrations, such as magnesium, calcium, and iron.

Recommendations

Trace mineral research and data using Japanese quail as the animal model are limited. Future research verifying the present data and exploring the various responses would be beneficial.

The plasma lipid parameters, although not all significantly, responded to the higher dietary concentrations of copper and chromium. Exploration of the

plasma lipid and glucose responses of Japanese quail at combined higher concentrations of copper and chromium, which surpass the scope of mineral concentrations examined in the present study, is recommended.

In addition, the dietary mineral ranges examined in this study significantly influenced several bone and tissue minerals. Future research looking at the different mineral concentrations and the corresponding influence on bone strength could provide valuable information.

Lastly, exploring the influence of trace minerals, within safe and adequate ranges, on plasma lipids and minerals in humans could prove to be very informative. Not only are trace minerals low in typical American diets, but improving lipid profiles and bone strength are pertinent health issues for many Americans. Therefore, similar studies investigating dietary trace minerals and their effects on lipid profiles and bone metabolic enzymes would be beneficial.

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VITA

Rebecca G. Nihiser

Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS AND INTERACTIONS OF COPPER, ZINC, AND CHROMIUM, ON PLASMA LIPIDS, GLUCOSE, AND TISSUE AND BONE MINERALS IN MALE JAPANESE QUAIL

Major Field: Nutritional Science

Biographical:

Personal Data: Born in Denver, Colorado, September 1, 1969, the daughter of Raymond and Oletha Nihiser.

Education: Graduated from Sallisaw High School, Sallisaw, Oklahoma in May 1987; recieved Bachelor of Science degree in Food, Nutrition and Institution Administration in May 1991; completed requirements for the Master of Science degree at Oklahoma State University in December 1992.

Professsional Experience: Dietary Aide, Stillwater Medical Center, Stillwater, Oklahoma, 1990; Research Assistant, Department of Nutrition, Oklahoma State University, 1990-1992; Provisional Liscensed Dietitian and AP4 Student, Oklahoma Memorial Hospital, Oklahoma City , Oklahoma, 1991-1992.

Professional Organizations: American Dietetic Association, Kappa Omicron Nu, Phi Eta Sigma, Golden Key National Honor Society.