INVESTIGATION OF THE EFFECTS OF COPPER SOURCE, COPPER AND ZINC LEVELS, AND DIETARY PROTEIN SOURCE ON CU BIOAVIALABILITY IN RAINBOW TROUT

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ABSTRACT

Limited research has examined the effects that plant-based diets have on copper (Cu) and Zinc (Zn) absorption and utilization in rainbow trout. Few studies have been conducted to determine if interactions exist in the utilization of Cu when increasing levels of supplementary Zn were offered. The objectives of this research were to: first determine what effect protein source (plant vs. animal based), Cu source (complex vs. inorganic) and concentrations of Cu (0, 5, 10, 15, 20 ppm) in the diet had on rate and efficiency of gain and Cu tissue levels in rainbow trout. The second experiment was to determine if interactions occur due to increasing diet content of Zn (0, 30, 300, 1500 ppm) and Cu provided at two levels (0 or 10 ppm) on tissue levels of Cu in rainbow trout. From experiment one, trout fed plant-based diet had higher ($P < 0.05$) ADG and improved ($P < 0.05$) FCR in comparison to fishmeal fed trout. Highest ($P < 0.05$) hepatic Cu concentrations were also observed in trout fed plant-based diets. No differences ($P > 0.05$) were observed in growth or hepatic concentrations due to Cu source. From experiment two, no antagonistic interactions were observed between increasing levels of dietary Zn and Cu. Trout fed the two highest levels of dietary Zn (300 and 1500 ppm) had the greatest ($P < 0.05$) weight gains. Dietary Zn supplementation increased ($P < 0.05$) whole body Cu at 12 wks. With increasing dietary Zn supplementation, resulted in increased ($P < 0.05$) whole body Zn. Cataracts and tail rot were observed at 12 wks in trout fed the Cu and Zn deficient diet. In conclusion, plant-based diets enhanced Cu bioavailability indicated by higher weight gains and hepatic Cu concentrations in experiment one, compared to trout fed fishmeal-based diets. Cu supplementation is required in a plant-based in order to achieve optimal growth in trout. Results of the second study indicate rainbow trout fed plant-based diets require Zn supplementation to obtain sufficient growth. The highest levels of Zn supplementation did not impair Cu uptake in rainbow trout.

INTRODUCTION

Proper supplementations of plant-based diets with essential nutrients can allow for full replacement of fishmeal as a protein source for farmed-fish, including rainbow trout (Gaylord et al., 2010). Part of providing adequate levels of such nutrients requires investigation into what effect plant-based protein sources have on nutrient bioavailability, including micronutrients. Limited research has examined the effects that plant-based diets have on copper (Cu) and Zinc (Zn) absorption and utilization in rainbow trout. Copper is essential for the formation of hemoglobin, bone formation as well as acting a co-factor to important enzymes in the body. Zinc is involved in important processes throughout the body including protein synthesis, reproduction, growth, and G.I tract function. Few studies have been conducted to determine if interactions exist in the utilization of Cu when increasing levels of supplementary Zn were offered. The objectives of this research were to:

1) determine what effect protein source (plant vs. animal based), Cu source (complex vs. inorganic) and concentrations of Cu (0, 5, 10, 15, 20 ppm) in the diet had on rate and efficiency of gain and Cu tissue levels in rainbow trout.

2) determine if interactions occur due to increasing diet content of Zn (0, 30, 300, 1500 ppm) and Cu provided at two levels (0 or 10 ppm) on tissue levels of Cu in rainbow trout.

LITERATURE REVIEW

Aquaculture is currently one of the fastest growing food-producing industries in the world (FAO, 2006). In comparison to other food markets that are increasing at a slower rate, aquaculture continues to expand each year (Palti et al., 2011). It is predicted that within the next 15 years, the demand for aqua food products as a protein source will result in substantial increases in global production of farmed fish (Barrows et al., 2008).

Rainbow Trout Production

Rainbow trout are an important aquaculture species and are the most cultured, cold, freshwater fish in the world. Global production of trout reached 576,289 mt in 2008 (Palti et al., 2011). Rainbow trout, are members of the Salmonoidae family (FAO, 2006), and are a freshwater fish, indigenous to river and lakes in the western United States and Canada from Alaska to Mexico. Significant production of rainbow trout as a farm-raised fish increased during the 1950's due to the development of pelleted diets (FAO, 2006). The primary outlets for rainbow trout are food production and as recreation for angling in lakes and rivers. While trout farming has become well established, there is still need to nutritionally balance diets and to reduce costs and increase production efficiencies (FA0, 2006).

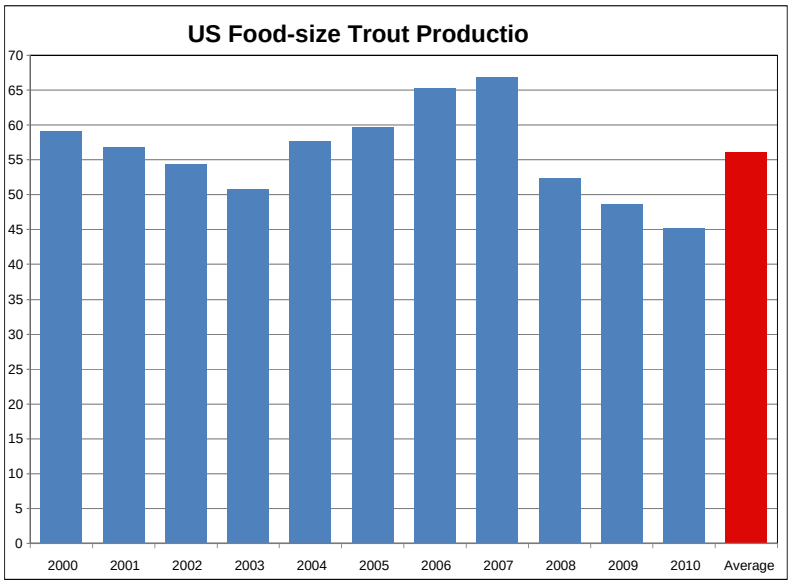

Figure 1. Change in rainbow trout production (millions) (NASS, 2012).

Alternatives to Fishmeal for Rainbow Trout

Because rainbow trout are a carnivorous species, they have historically been fed diets supplemented with fishmeal as a protein source. Fishmeal, as a general term describes several wild-harvested ocean fish species including menhaden and herring. Fishmeal is used extensively in aquaculture feeds because of its high concentration of protein, balanced amino acid profile, high digestibility, and lack of anti-nutrients. It is expected that within the next ten years, the demand for aquaculture food products will exceed the amount of fishmeal that can be produced (Gatlin et al., 2007). While mammalian-based protein sources offer high levels of essential amino acids and other important nutrients, increased concern regarding their use in animal feeds due to concerns about safety and taste preference by the consumer has reduced research in this area (Gaylord et al., 2010).

In order to meet aquaculture feed protein demands, the aquaculture industry has investigated more sustainable alternatives. The primary alternatives that have been investigated are plant-based protein sources. Plant-based protein sources usually consist of a mixture of cereal grains such as barley, wheat or corn gluten meal as well as soybean meal (Gaylord et al., 2010). In order to successfully replace fishmeal, plant protein sources need to meet nutritional requirements and growth performance standards, as well as being economically sound, and environmentally safe (Gatlin et al., 2007; Naylor et al. 2009).

The major limitations of plant-based protein sources for carnivorous fish include low levels of amino acids and fatty acids, and the presence of anti-nutrient factors. Anti-nutrient factors are substances that negatively impact food utilization and digestion. Anti-nutrients that specifically block protein utilization include tannins and lectins. Phytates, gossypol pigments, and oxalates are anti-nutrients that directly block mineral utilization (Francis et al., 2001). One primary example of a plant-based protein that has limited utilization in some species due to anti-nutrients is soybean meal, which contains a high level of protein and an adequate amino acid profile. Due to the amount of anti-nutrient factors contained in soybean meal, it can only be used in a limited amount (< 20%) in trout diets (Sealey et al., 2010). Specific anti-nutrients found in soybean meal include lectins, and phytic acid. However, such anti-nutrients can be removed from soybean meal, resulting in soy protein concentrate, thus reducing the amount of soybean meal present in the diet (Gaylord et al., 2010) or supplementation of substances can be added to soybean meal diets to counteract the effect of the anti-nutrients (Sealey et al.

2010). Sealey et al. (2010) conducted a study to determine if pro-biotics could improve production of rainbow trout fed diets with high levels of soybean meal. Trout in the starter phase could tolerate the higher levels of soybean with pro-biotics supplemented.

Other research suggests that dietary protein source, in part due to the anti-nutrients may also have an effect on the uptake and utilization of other nutrients, specifically, micronutrients such as vitamins. Barrows et al. (2008) found that different vitamin premixes were necessary to optimize growth and feed efficiency in plant-based diets compared to those previously utilized in traditional fishmeal-based diets. Vitamin levels higher than previously recommended levels were necessary to maximize growth potential. Reduced trout survival and nutrient retention were also measured in trout fed diets supplemented with vitamins at NRC levels, indicating such levels are inadequate for current trout diets currently fed (Barrows et al., 2008).

Similarly, macro-minerals and inositol supplementation were found to be necessary to optimize performance of rainbow trout fed plant-based diets (Barrows et al., 2009). Fishmeal based diets often contain nutrients such as macro-minerals and inositol that are lacking in plant-based diets. Barrows et al., (2009) investigated the need for marco-minerals and inositol to be provided in plant-based diets. Over a 105 d growth period, juvenile trout (avg wt. 4.8 g) were fed either a control diet, which was fishmeal-based, or one of three plant-based diets with both macro-minerals and inositol present, only inositol present, or neither macro-minerals nor inositol. The macro-minerals supplemented included sodium chloride, potassium chloride and magnesium oxide. Growth parameters, feeding behavior, and histology were measured over the course of

the study. While there were no significant mortalities, liver pathologies were reported in trout fed the plant-based diet lacking macro-minerals. Improved weight gain was observed in trout fed the plant-based diet supplemented with macro-minerals and inositol (Barrows et al., 2009).

The vitamin requirements for rainbow trout were established nearly 30 yr ago (Barrows et al., 2008). Within the last ten years alone, changes in diet processing and formulations have dramatically altered the type of diets being fed to trout thus also potentially necessitating re-evaluation of trace minerals, needs which can vary greatly due to these changes.

Importance of Trace Minerals in Animal Diets

Trace minerals are nutrients essential for normal health in animals. Trace minerals play an extensive and critical role in key processes throughout the body. Such processes include the formation of bone and connective tissue, as well as providing stability. Other roles include aiding in acid-base balance, nerve impulse transmission, membrane permeability, cell replication, gene transcription, and acting as co-factors to important enzymes and hormones in the body (Suttle, 2010). Minerals cannot be produced by the animal, and must therefore be ingested or administered in some form (McDowell, 1992).

It is also important to note that minerals are interrelated, and often work together to make a process possible. One primary example is with Cu and Zn and the enzyme superoxide dismutase. Superoxide dismutase, (SOD) is an antioxidant enzyme which

helps get rid of superoxide radicals, acting in the first line of defense against free radical damage by protecting cellular membranes (Tomlinson et al., 2008).

Trace minerals like Cu and Zn must be supplemented at adequate levels and in a useable form in animal diets. Consideration as to the degree of bioavailability of trace minerals is also important in determining proper supplementation levels (McDowell, 1992). Proper supplementation is needed in order to reduce over-supplementation, which could lead to toxicity, or excess excretion (NRC, 1993).

Copper Nutrition

Copper is a trace mineral whose major role is that of a catalyst allowing the body to utilize Fe for formation of hemoglobin. Copper is necessary for bone growth, reproduction, immune response, cross-linking of connective tissue, and pigmentation. Copper also acts as cofactor to several important metalloenzymes including cytochrome oxidase, which aids in cellular respiration and lysl oxidase that aids in collagen formation. Anemia, diarrhea, bone disorders, poor growth and impaired immune response are all manifestations of a Cu deficiency (McDowell et al., 1992). In mammals, Cu is absorbed from the small intestine, but rate of Cu uptake is dependent upon the physiological demands of the animal.

Increased Cu absorption occurs in animals during growth, lactation or during a Cu deficiency (McDowell et al., 1992). Studies conducted by Skoryna (1971), demonstrated this behavior. Rats fed either Cu deficient or Cu adequate diets were given the same dose of radioactive Cu. Rats fed the Cu deficient diet had much higher tissue retention rates

and there were three patterns of Cu absorption observed. Rats fed the copper deficient diet retained the highest amount of supplemented Cu in the body.

Dietary Cu absorption from the small intestine occurs through two routes: simple diffusion and active transport. Metallothionein (MT) found within the epithelial cells of the intestine plays a key role in regulating Cu uptake (McDowell, 1992). Once Cu has been absorbed, it is transported bound to albumin and amino acids throughout the body, primarily to the liver. Copper in the liver has three main target locations; bound to the metalloprotien ceruloplasmin (Figure 2) and then transported to target organs throughout the body (McDowell, 1992), and primarily stored in the liver and the gallbladder (Suttle et al., 2010).

Dietary uptake of Cu is related to the level of Cu bioavailability within the body. Bioavailability is defined as the degree to which essential nutrients are absorbed into a useable form to the body (Ammerman et al., 1995). One method commonly used to measure Cu bioavailability is to feed below and above the Cu requirement. These increased levels of Cu allow for accumulation of Cu in specific tissues and organs. (Clearwater et al., 2002) The liver is one of the best indicators of body Cu status, as the primary site of Cu concentration and metabolism. Because Cu is also bound to cerulosplasmin (Ammerman et al., 1995), a primary indicator of Cu depletion would be decreased concentrations of Cu in the liver, followed by decreased levels of ceruloplasmin in the plasma (Suttle et al., 2010).

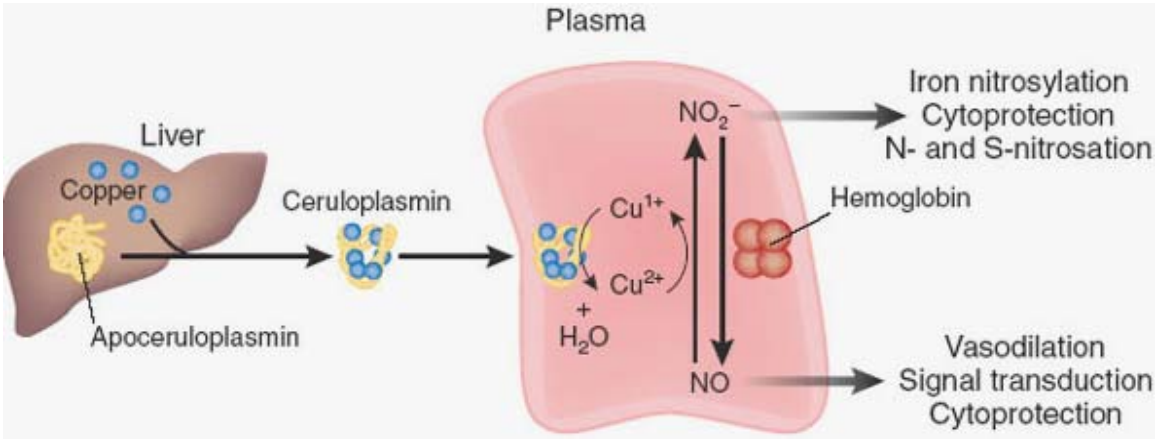

Figure 2. Transport of absorbed Copper from the liver to the plasma (Samuel, 2006).

Zinc Nutrition

Zinc acts as cofactor to over 1,000 enzymes and proteins, which are involved in protein synthesis, growth, the nervous system, GI tract function, and in reproduction. Zinc plays a key role in regulating gene expression; Zn plays a role not only as cofactor, but has a direct effect on hormones like insulin, and growth factor, IGF-1, as well as with important vitamins like D and A. Specifically with vitamin A, Zn aids in converting retinol to retinal. Deficiencies in zinc can be manifested in poor growth development, due to the impairment of the IGF-1 factor. Primary signs of Zn deficiency include cataracts and a decrease in protein synthesis. Zinc is involved in aiding with taste sensation, which may explain why animals fed a Zn-deficient diet could have reduced feed intakes. The relationship between Zn and appetite, when a decrease in dietary Zn levels occurs, was followed by an increase in the synthesis and expression of cholecystokin, which is an

appetite regulating hormone. Similarly, leptin, the hormone that causes the sensation of satiety also increases in the body when fed low levels of dietary Zn (Suttle et al., 2010).

Metallothionein (MT), a metal-binding protein produced by the liver, transfers Zn once it has passed through to the intestinal mucosal cell. It plays a key role in controlling the amount of Zn that enters the body. Both level of Zn in the diet as well as in the plasma will directly impact the amount of MT that is produced. The age and Zn status of the animal also will have an impact on how much zinc is absorbed. According to McDowell (1992) reported that Zn-deficient animals absorb 80% greater amounts of Zn provided, in contrast to animals that absorbed less than 10%, when high amounts of Zn were supplemented.

A majority of Zn released in the plasma is bound to albumin. Once in this form it is readily available for uptake by the body. Zinc transported from the plasma arriving at the liver is separated by MT, and from there directed to different locations in the body. Zinc is most readily accessible in the pancreas, kidneys, liver and spleen. In contrast, once taken up by the brain and especially by the bone, access to Zn in these tissues is limited (McDowell, 1992).

Though found throughout the body, little of the Zn stored in the body is in a readily useable form. When a Zn deficiency does occur there is little impact on the levels of Zn found in the bone, brain, liver and kidney. Excretion occurs primarily through the feces and a portion in urine. If the animal is in physiological demand for Zn (i.e. growing or being fed low Zn diet), the levels of excreted Zn are reduced (McDowell et al., 1992).

When under a state of Zn deprivation, movement of Zn from primary locations of storage such as the bone and muscle is unlikely.

Copper and Zinc Nutrition of Rainbow Trout

The dietary requirement of Cu for rainbow trout has been reported to be 3 μg /Cu g dry mass food (Ogino et al., 1980). Common indices of Cu status in rainbow trout include whole-body Cu retention, which includes skin, gut, and kidneys (Clearwater et al., 2002). Fish growth can also be used as an indicator since both Cu deficiency and toxicity result in retarded growth (Kamunde et al., 2002).

Copper absorption in rainbow trout occurs primarily in the pyloric caecae and intestine similar to mammalian species. Once Cu has passed the basolateral membrane, Cu is absorbed first by the liver and gallbladder, followed by the gill muscle and kidney. The mucus found on the intestinal lining protects the internal organs from uptake of high concentrations of Cu.

The exact process of Cu absorption in trout is still not well understood. However, Clearwater et al., (2002) reported one study where trout were fed high levels of dietary Cu for a week and Cu accumulation was highest in the liver and gallbladder, with lower levels in the muscle and kidney. In a second study where radioactive Cu was administered to rainbow trout, within the first 24 hrs Cu accumulation was observed in the intestine and liver. By 72 hr, Cu was spread throughout the internal organs and the primary route of excretion for rainbow trout is through bile (Clearwater et al. 2002).

The form, in which Cu is bound in the diet, can also make a significant difference in how Cu is absorbed. Although, research as to whether the organic or inorganic form of Cu has a greater degree of bioavailability is still actively debated. Studies in rats (Guo et al., 2001) and heifers, (Rabiansky et al., 1999) found increased tissue Cu accumulation in Cu-lysine fed animals when fed equal amounts of Cu-Lysine and $CuSO_4$. In contrast, studies like those of Ward et al. (1993) with steers and a loading and depuration study by Kjoss et al. (2006) with rainbow trout, found that $CuSO_4$ and Cu-Lysine were equivalent in Cu bioavailability.

In the Kjoss study, two experiments were conducted to examine what effect different forms of Cu and Zn would have on Cu and Zn uptake. In experiment one, juvenile trout weighing 200 mg were evenly distributed between one of nine tanks and acclimated to tanks for one wk. During this time trout were fed a diet lacking supplemental Cu or Zn. Following acclimation, trout were fed one of the nine diets including a control, one of four Cu sources (CuO, $CuSO_4$, Cu-Lysine, or Cu-Proteinate) diets supplemented at 400 ppm, or one of four Zn sources (ZnO, $ZnSO_4$, Zn-Proteinate, or Zn-methionine) diets supplemented at 1000 ppm, over a two week loading phase. Depuration immediately followed, with all trout returning to the control diet for two wk. Tissue sampling, which consisted of liver, gut and whole body, occurred every three days, starting on day zero. Experiment one was followed by a second experiment, where only Cu bioavailability was examined. Trout weighing 1.2 g were acclimated for two weeks prior to the start of the study. Trout were fed the same control and Cu compound supplemented diets. The loading phase lasted four wk, with a two-wk depuration.

There were no differences in growth rates among the respective treatments. The form of Cu supplemented did affect Cu uptake. Trout fed CuO had low levels of Cu accumulation that were similar to those of trout fed the control diet, suggesting that CuO was not readily available. In contrast, Cu-Lysine, Cu-Proteinate and $CuSO_4$ all had similar and high tissue levels of Cu, suggesting no difference in Cu uptake between the inorganic and organic forms of Cu. Depuration of Cu of the gut occurred quickly, suggesting that Cu does not remain in the intestine very long if the form of Cu is readily available (Kjoss et al., 2006).

Different results were observed in trout fed the various forms of Zn. The form of Zn supplemented did not make a difference in Zn uptake. Zn accumulation was similar among all forms of Zn provided and Kjoss et al., (2006) suggested that Zn is under tight homeostatic control.

Zinc Nutrition of Rainbow Trout

The Zn requirement for rainbow trout is recommended to be between 15-30 ppm (Ogino et al., 1978). Zinc is found in the bone, body tissues, of rainbow trout with the highest concentration being in the eyes. Signs of Zn deficiency in rainbow trout include cataracts, erosion of fins and scales mortality, and reduced growth. There is still uncertainty as to what level of dietary Zn is toxic for rainbow trout. Clearwater (2002) reported that trout exhibited no impairment to growth or health due to high concentrations of dietary Zn (approximately 1,000 ppm diet). In a study conducted by Wekell et al. (1983), two sources of Zn ($ZnSO_4$ and Zn-Proteinate) were supplemented (4

to 1700 ppm) to rainbow trout fingerlings over a 173 d period. Poor growth and increased mortalities were measured in the Zn deficient diet. But once Zn levels reached 90 ppm, no further improvement in growth, reduced mortalities and increased Zn accumulation in the liver blood and gills were measured.

Routes of Copper and Zinc Uptake in Rainbow Trout

According to Clearwater et al. (2002) uptake of minerals from the lumen of the small intestine for rainbow trout was similar to that of mammals, with a few differences. Along with the well-known route of absorption through the small intestine that occurs similarly to mammalian species, rainbow trout are also exposed to water borne levels of Cu and Zn, with uptake via the gills. Kamunde et al., (2002) conducted a study comparing dietary and waterborne Cu uptake after pre-exposure to Cu waterborne levels. Over a 28 d period trout were exposed to either 2 µg/ l Cu (control) or 22 µg /l waterborne Cu. This was then followed by administration of radioactive Cu by both dietary or waterborne means and being monitored over a 48 h period. Results showed that dietary Cu exposure resulted in higher concentrations of Cu in the body of compared to waterborne Cu exposure. These findings are similar to research by Kamunde et al., (2002) that dietary Cu accounts for more than 90% of Cu concentrations in the body of rainbow trout. Trout pre-exposed to the waterborne Cu followed by exposure to radioactive Cu in the waterborne form had reduced Cu uptake via the gills, with no negative impact on intestinal dietary Cu uptake. From these findings it was concluded

that while excessive levels of waterborne Cu would have an impact on Cu concentrations in the body, more emphasis should be given to dietary Cu concentrations.

According to Clearwater et al. (2002) dietary Zn accounts for a greater amount of Zn uptake than does waterborne Zn. Several studies were conducted examining the ratio of daily dose of dietary vs. waterborne Zn. Findings from these studies, indicate that when the diet: waterborne ratio was greater than 1, dietary Zn greatly increased Zn uptake compared to waterborne Zn (Clearwater et al., 2002). It was suggested that the route that will have the greatest impact on Zn uptake depends on the concentration of Zn available from each route; waterborne or diet-borne. Trout exposed to reduced waterborne Zn concentrations (< 20 µg Zn 1) and moderate diet-borne concentrations (90-200 mg Zn kg dry diet) met Zn requirements primarily from the diet-borne concentrations in the feed. In contrast, when diet-borne concentrations were dramatically lowered (1 mg Zn kg dry diet), waterborne Zn was the only route of Zn uptake for the trout (Clearwater et al., 2002). While trout will absorb Zn provided in the water, this alone cannot compensate for a Zn deficient diet (Clearwater et al., 2002).

Interactions between Zinc and Copper

Zinc absorption is affected by the presence of other components in the diet. Levels of other nutrients in the diet including Ca and phytate, P, Cr, Cd, and Cu all can impair Zn uptake (McDowell et al., 2003). Similarly, Cu absorption is impacted by four main factors including the amount of dietary Cu, the chemical form of Cu provided, age

of the animal, and the dietary level of other trace elements in the diet including Zn, Fe, Cd, among others (Underwood, 1977).

Previous research has addressed interactions between Cu and Zn Skoryna (1971) and Ammerman (2003). Skoryna (1971) observed in rats that an antagonism existed between Cu and Zn. Typical toxicity signs found due to excess dietary Zn levels were anemia, reduced growth, and lowered concentrations of Cu and Fe in the tissues of the rats. Additionally, levels as low as 50 ppm Zn supplemented in a Cu-deficient diet result in an antagonistic response on tissue Cu levels in chicks. Skoryna's (1971) conclusions from these studies were that there was an antagonistic relationship between Cu and Zn at the point of absorption in the small intestine. Further support of an antagonistic relationship between Cu and Zn, is the review by Ammerman et al (2003), who described several studies in a wide range of species including rats, chicks, pigs and sheep. In that review paper, detrimental effects on Cu status were seen due to excess dietary Zn levels. It was concluded from these studies that higher levels of dietary Zn had a greater impact on Cu status than vice versa. The primary location of impact proposed was at the point of absorption in the small intestine and the proposed mode of action was that increased dietary Zn levels triggered synthesis of intestinal MT. Because MT preferentially binds with Cu and the Cu once bound to MT cannot be absorbed, the Cu remains unavailable and is sloughed off with mucosal cells.

Beyond the point of absorption, uncertainty still remains as to what other Cu related processes might be affected by high levels of Zn. However, Ammerman (1995)

reported reduced activity of cytochrome oxidase and superoxidase due to high levels of Zn. Indicating the potential for interactions other than competition for absorption.

Although less studied, interactions between Cu and Zn in rainbow trout have been previously investigated. Clearwater (2002) examined the effect of different factors (CA2+, and cysteine) including Zn on Cu uptake, when Cu was provided at a low dose of radioactive Cu. While Zn did not impede Cu uptake, it was suggested that neither Cu nor Zn was supplemented at a high enough concentration for an interaction to occur. Knox et al, (1984) examined the effects of dietary Zn on Cu metabolism in rainbow trout. Trout weighing 8 g were fed a purified diet supplemented with either 2.5 ppm or 500 ppm Cu and 34 to 1000 ppm dietary Zn for 20 weeks. Trout performed similar in growth and feed efficiency across all treatments. Liver Cu concentrations were reduced with the higher levels of dietary Zn and this response was due to the action of MT. Knox et al., 1984).

Gatlin et al., (1988) followed up the Knox (1984) study by determining what levels of Zn were necessary in a practical diet. Fingerling channel catfish were fed either 1 or 20 ppm Cu and either 100 or 200 ppm Zn over a 12-wk period. There was no effect on growth performance of feed efficiency. It was suggested that Zn could be supplemented in a practical diet at higher levels without any impact on Cu bioavailability.

MATERIAL AND METHODS

Study One

Objective

The objectives of experiment one were to determine the main effects of dietary protein source (fishmeal or plant-based), Cu source (CuSO4 or CuLys) and Cu level (0-20 ppm) on Cu bioavailability in rainbow trout.

Fish Handling and Use

A commercially available strain of juvenile rainbow trout (Trout Lodge, Seattle WA, USA) was used for experiment one. All procedures for the care and handling of the rainbow trout that used for experiment one were conducted in accordance with the Bozeman Fish Technology Center, Institutional Animal Care and Use Committee.
The study was conducted over a 14-wk period from October 2010 to January 2011 at the USFWS, Fish Technology Center in Bozeman, Montana. Thirty- two, 168 L tanks were stocked with 18 fish (average initial weight 28 g, +/- 1.2 g per tank. Tank was considered the experimental unit for all response variables. Two tanks were randomly assigned to each dietary treatment. In addition all tanks received 9.5 L of water/ min to maintain fresh water flow via a re-circulating pump system with particulate and biological filtration to maintain optimum water quality. Water temperature was held constant at 15°C and photoperiod was maintained at a 13:11 diurnal cycle.

Diets

From the two base formulas (Table 1), supplemental Cu was added to create sixteen experimental diets. All diets were formulated to contain 40% crude protein and 20% crude lipid (Table 1). The protein sources for the plant-based diet series included soy protein concentrate, corn protein concentrate, and soybean meal (Table 1). Diet one was the plant-based control diet containing no supplemental Cu. Of the remaining plant-based diets, CuSO4 was supplemented at levels at 5, 10, 15 and 20 ppm. Four additional diets of the plant-based contained Cu-lysine (CuPLEX 10% Cu) at the same four levels. The remaining diets 10-16, were the fishmeal-based series. For this series there was also a control fishmeal-based diet, without supplemental Cu. The six remaining fish meal-based diets contained either CuSO4 or Cu-lysine, each at levels of 5, 10, and 20 ppm.

Each diet was manufactured as a 3 mm sized pellet. Diets were processed using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 s exposure to 127°C in the extruder barrel. The die plate was water cooled to an average temperature of 60°C. Pressure at the die head varied from 200 to 400 psi. Pellets were dried in a pulse bed drier (Buhler AG, Uzwil, Switzerland) for 25 min at 102°C with a 10 min cooling period to a final moisture level of less than 10%. A topcoat of fish oil was added to the cooled feed using a vacuum-assisted top-coater (A.J. Mixing, Ontario, Canada). Diets were stored in plastic lined paper bags at room temperature until fed. All diets were fed within six months of manufacture (Barrows et al., 2008).

Feeding

Trout were fed twice daily to visual satiation, 6 days a week. Before initiation of experimental diet feeding, fish in each tank were randomly fed one of the two basal diets without Cu supplementation to "deplete" body stores. After depletion, fish within each basal diet group were randomly allocated to their respective diet for an additional 12 wks.

Proximate Analysis

Dry matter analysis of ingredients and diets was performed according to standard methods (AOAC 1995) on a Leco thermogravi- metric analyzer (TGA701, LECO Corporation, St. Joseph, MI, USA). Protein (N X 6.25) was determined by the Dumas method (AOAC 1995) on a Leco nitrogen determinator (TruSpec N, LECO Corporation). Total energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, IL, USA.

Table 1. Ingredient composition of plant and fishmeal-based diets (g/100g).

Ingredients (%DM)	Plant-based	Fishmeal-based
Soy protein concentration	24.64	-
Fish oil, Menhaden	15.59	16.75
Corn protein	17.54	5.5
Wheat Flour	16.27	22.72
Soybean Meal	13.3	12
Menhaden Meal	-	33.7
Blood Meal	-	7.43
Mono-Dical Phosphate	2.65	-
L-Lysine	1.99	-
Vitamin Premix ARS 702	1	1
Choline-CL	0.6	0.6
Potassium Chloride	0.56	-
Taurine	0.5	-

Table 1 Continued

Dl-Methionine	0.5	-
Sodium Chloride	0.28	-
Threonine	0.23	-
Stay-C	0.2	0.2
Trace mineral premix[a]	0.1	0.1
Analyzed composition of basal diets		
Copper (mg/kg)	4.16	1.21
Formulated Composition		
Crude Protein (%)	40.1	40.1
Crude Fat (%)	20	20
Crude Fiber (%)	0.93	1.08

[a] Trace mineral premix; contributed in mg/kg of diet: zinc- 40; manganese – 17; and iodine – 6.

Tissue Sampling

During the 12-week study, fish samples were obtained at the start, six weeks, and at the end of the study. At six and twelve wks, three-five fish from each tank were sampled to determine liver and whole body Cu levels. Fish obtained from each of the 32 tanks were euthanized and liver samples and whole body composition were obtained. Body indices including hepatosomatic index, and muscle ratio were also measured. At the end of the 12-week period, all remaining fish from each tank were combined as a pooled sample to determine mineral retention. Mineral retention was determined by use of the Induced Couple Plasma-Optical Emission Spectroscopy, following nitric acid digestion (Anderson, 1996).

Statistical Analysis

The design of this study was a 2 X 2 X 5 factorial with two protein treatments, two Cu sources and five dietary concentrations of Cu. Analysis of variance (ANOVA) was used to identify the main effects of plant-based vs. fishmeal diets, Cu source ($CuSO_4$ vs. Cu-Lys), interactions of protein source by Cu source, Cu source by Cu level, were also tested. Regression was used to examine the effects of linear and quadratic effects of increasing Cu levels (0, 5, 10, 15, 20 ppm). Differences were considered significant at $P < 0.05$.

Study Two

Objective

The objectives of experiment two were to determine if any interactions existed between Cu and Zn, when two levels of Cu (0 or 10 ppm) and four levels of Zn (0,30,300,1500 ppm) were provided in a plant-based diet.

Fish Handling and Use

Over an 18-week period a second study was conducted at the USFWS Fish Technology Center in Bozeman, Montana. A commercially available strain of juvenile rainbow trout (Trout Lodge, Seattle WA, USA) was used for experiment two. All procedures for the care and handling of the rainbow trout that used for experiment two were conducted in accordance with the Bozeman Fish Technology Center, Institutional Animal Care and Use Committee.

Depuration Period

Prior to the start of the study, juvenile trout (avg wt. 17 g) were fed the same plant-based control diet as described in Study one without supplemental Cu and Zn for six weeks. Analyzed levels of Cu and Zn were 9.4 ppm and 37.7 ppm, respectively. The purpose of this period was to reduce Cu and Zn tissue levels.

Feeding Trial

After six wk depuration, 600 fish (avg wt of 40 g) were randomly assigned to a 2 X 4 experimental treatment design with diets containing 0 or 10 ppm added Cu and 0, 30, 300 or 1500 ppm added Zn (eight diets). There were 25 fish/ tank with three replicate tanks per diet. Water was held constant at 15 °C. Lighting followed a 13:11 diurnal cycle. Four gallons/ minute running water was provided for each tank, in a re-circulating water system with particulate and biological filtration. Three wks into the study, trout numbers were readjusted to 20 fish per tank, due to significant non-treatment related mortalities.

Feeding and Weighing

Trout were fed to visual satiation twice daily, six days a week. Feed bins were weighed weekly in order to determine average feed intakes. Weight gain was determined every three wk.

Diets

Diets were formulated to contain 40% CP from soybean meal and corn protein concentrate and 20% crude lipid (Table 2). Each diet was manufactured as a 3 mm sized

pellet. Diets were processed using a twin-screw cooking extruder (DNDL-44, Buhler AG, Uzwil, Switzerland) with an 18 s exposure to 127°C in the extruder barrel. The die plate was water cooled to an average temperature of 60°C. Pressure at the die head varied from 200 to 400 psi. Pellets were dried in a pulse bed drier (Buhler AG, Uzwil, Switzerland) for 25 min at 102°C with a 10 min cooling period to a final moisture level of less than 10%. A topcoat of fish oil was added to the cooled feed using a vacuum-assisted top-coater (A.J. Mixing, Ontario, Canada). Diets were stored in plastic lined paper bags at room temperature until fed. All diets were fed within six months of manufacture (Barrows et al., 2008).

Proximate Analysis

Dry matter analysis of ingredients and diets was performed according to standard methods (AOAC 1995) on a Leco thermogravi- metric analyzer (TGA701, LECO Corporation, St. Joseph, MI, USA). Protein (N 3 6.25) was determined by the Dumas method (AOAC 1995) on a Leco nitrogen determinator (TruSpec N, LECO Corporation). Total energy was determined by isoperibol bomb calorimetry (Parr 6300, Parr Instrument Company Inc., Moline, IL, USA.

Table 2. Ingredient composition of plant-based diets (g/100g).

Ingredients (%DM)	Plant-based
Soy Protein Concentration	24.64
Fish Oil, Menhaden	15.59
Corn Protein Concentration	17.54
Wheat Flour	16.27
Soybean Meal, Solvent Extracted	13.3
Mono-Dical Phosphate	2.65
L-Lysine	1.99
Vitamin Premix ARS 702	1
Choline-CL	0.6
Potassium Chloride	0.56
Taurine	0.5
Dl-Methionine	0.5
Sodium Chloride	0.28
Threonine	0.23
Stay-C	0.2
Trace Mineral Premix[a]	1.0
Formulated Composition	
Crude Protein (%)	40.1
Crude Fat (%)	20
Crude Fiber (%)	0.93

[a] Trace mineral premix; contributed in mg/kg of diet: zinc - 40, manganese - 17, and iodine – 6.

Tissue Sampling

In order to determine Cu and Zn retention, whole body samples were taken pre and post depletion, at 6 weeks and finally at 12 weeks. At six wks, three to five fish from each tank were removed and euthanized for whole body samples. Body indices including hepatosomatic index, visceral index, and muscle ratio were also measured during sampling. At 12 weeks, ten fish from each tank were euthanized, for whole body

samples. Tissue samples were then analyzed for minerals use of the Induced Couple Plasma-Optical Emission Spectroscopy, following nitric acid digestion (Anderson, 1996).

Statistical Analysis

Through the use of SAS GLM PROC, factorial analysis of variance (ANOVA) and least square means were used to examine the main effects and potential interactions of the two levels of Cu and the four levels of Zn and any Cu by Zn interaction. Regression was used to examine the increasing Zn levels (0, 30, 300, 1500 ppm). Differences were considered significant at $P < 0.05$.

RESULTS

Study One

Dietary Analyses

Analyzed dietary proximate composition reflected formulation targets (Table 3). Analyzed dietary Cu and Zn levels were proportionate to the targeted supplementation levels (Table 4).

Table 3. Analyzed composition (SD)[1] of diets fed to rainbow trout for 12 weeks[2].

Protein Source	Diet Cu Source	Cu Level	Gross Energy Kcal/g	Moisture %	Lipid %	Crude Protein %
PB	None	0	5421.5 (9.96)	2.4 (0.03)	15.4 (0.30)	41.0 (.40)
PB	$CuSO_4$	5	5383.1 (0.48)	3.1 (0.33)	17.2 (0.02)	42.1 (.42)
PB	$CuSO_4$	10	5300.5 (3.22)	2.6 (0.08)	14.3 (0.13)	42.1 (.18)
PB	$CuSO_4$	15	5366.7 (3.35)	2.2 (0.19)	15.6 (0.35)	43.3 (.24)
PB	$CuSO_4$	20	5356.4 (9.11)	2.4 (0.09)	15.2 (0.27)	37.0 (.23)
PB	CuLys	5	5357.3 (4.71)	1.9 (0.27)	14.4 (0.30)	37.6 (.14)
PB	CuLys	10	5454.3 (5.22)	1.9 (0.12)	16.4 (0.06)	35.6 (.08)
PB	CuLys	15	5363.4 (1.60)	2.3 (0.21)	14.9 (0.32)	35.7 (.16)
PB	CuLys	20	5357.6 (4.77)	2.5 (0.01)	15.3 (0.10)	37.6 (.07)
FM	None	0	5447.0 (7.01)	1.4 (0.31)	18.8 (0.46)	37.2 (.04)
FM	$CuSO_4$	5	5506.5 (26.18)	2.5 (0.05)	20.0 (0.44)	38.7 (.09)
FM	$CuSO_4$	10	5526.9 (39.49)	1.9 (0.04)	20.0 (0.36)	38.9 (.21)

Table 3 Continued

FM	$CuSO_4$	20	5465.0 (33.79)	1.5 (0.00)	18.9 (0.40)	36.1 (.18)
FM	CuLys	5	5369.9 (6.83)	2.4 (0.12)	19.2 (0.24)	37.7 (.01)
FM	CuLys	10	5386.4 (12.83)	3.1 (0.02)	19.5 (0.23)	48.9 (.03)
FM	CuLys	20	5416.8 (45.20)	2.2 (0.22)	20.0 (0.12)	43.7 (.21)

[1]Standard deviation of [2]Means of two analysis/diet.

Table 4. Analyzed composition of minerals in diets fed to rainbow trout in experiment one.

Protein Source	Cu Type	Cu Level	Cu	Mn	Ca	Fe	Mg	S	Zn
PB	None	0	4.2	48.3	8579.3	340.4	3926.9	6899.0	89.1
PB	$CuSO_4$	5	7.9	42.7	7638.6	294.8	3417.4	6099.1	73.3
PB	$CuSO_4$	10	15.7	49.4	8249.4	321.1	3791.6	6756.6	83.8
PB	$CuSO_4$	15	18.9	43.4	7635.9	301.8	3584.0	6344.3	73.1
PB	$CuSO_4$	20	26.9	42.7	7973.6	319.0	3721.3	6529.1	79.7
PB	CuLys	5	10.9	48.7	9128.3	345.7	4235.0	7110.4	89.2
PB	CuLys	10	17.9	49.7	8593.7	337.7	4036.5	6863.9	85.9
PB	CuLys	15	22.7	50.4	8420.9	331.9	3988.5	6906.9	87.0
PB	CuLys	20	24.5	40.9	7329.7	279.4	3401.4	5987.4	70.9
FM	None	0	1.2	44.0	29321.2	438.8	2448.2	4067.2	127.2
FM	$CuSO_4$	5	6.0	35.8	29412.3	417.9	2306.7	3756.5	120.4
FM	$CuSO_4$	10	11.3	33.3	27326.9	404.6	2163.9	3512.1	107.1
FM	$CuSO_4$	20	24.0	41.4	29599.3	426.2	2312.7	3707.5	121.7
FM	CuLys	5	8.7	32.0	26427.7	392.2	2064.8	3343.5	113.4
FM	CuLys	10	13.9	38.4	30744.4	684.9	2386.3	3781.4	125.7
FM	CuLys	20	24.3	31.8	26486.7	385.9	2062.8	3317.6	107.1

Growth & Feeding Behavior

Protein source and Cu level altered ($P < 0.05$) ADG and feed conversion ratio (FCR). Fish fed plant-based diets had higher ($P < 0.05$) ADG (Figure 3), and better ($P < 0.05$) FCR (Figure 5) at 12 wk than fish fed the fishmeal-based diets. Protein source significantly ($P < 0.05$) altered ADG and FCR by 3 wks (Figures 4, 6). Trout fed plant-

based diets, without Cu supplementation had lower ($P < 0.05$) growth rates (Figure 4, 6) and higher ($P < 0.05$) FCR compared to fish fed Cu-supplemented plant-based diets.

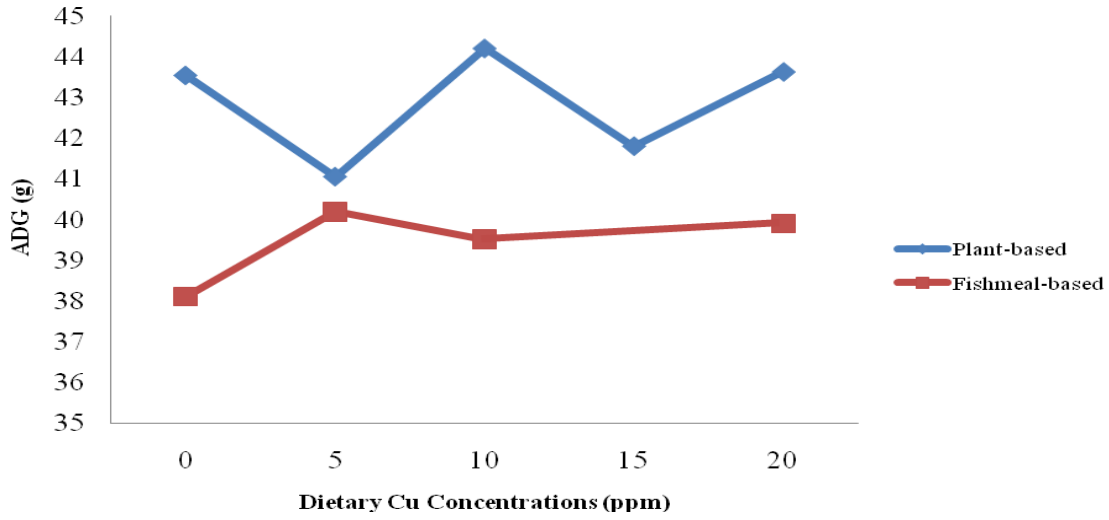

Figure 3. The effects of protein source (plant-based vs fishmeal-based) and level of dietary copper on daily gain by rainbow trout after 12 wk. Significant ($P < 0.05$) effect due to protein source.

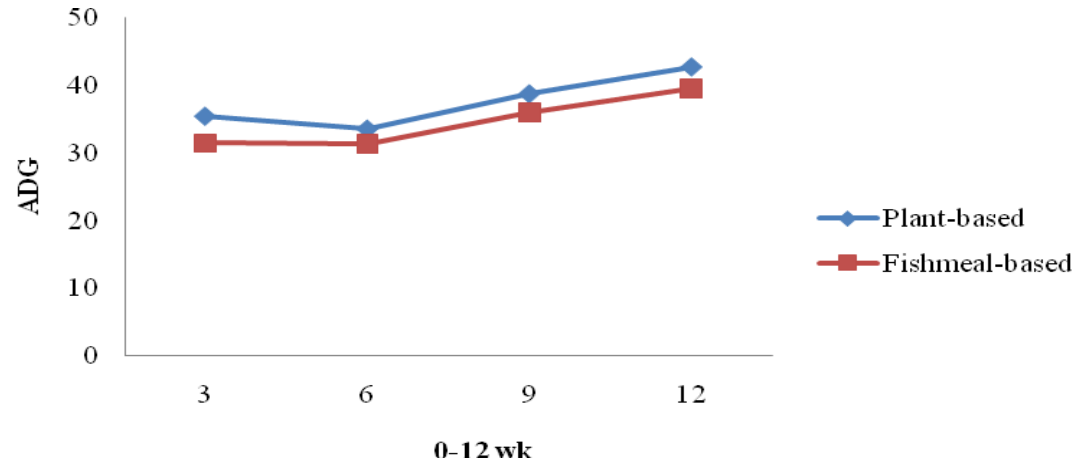

Figure 4. The effect of protein source (Plant vs. Fishmeal-based) on ADG over the 12 wk study. Trout fed plant-based diets had higher ($P < 0.05$) ADG in comparison to trout fed fishmeal-based diet.

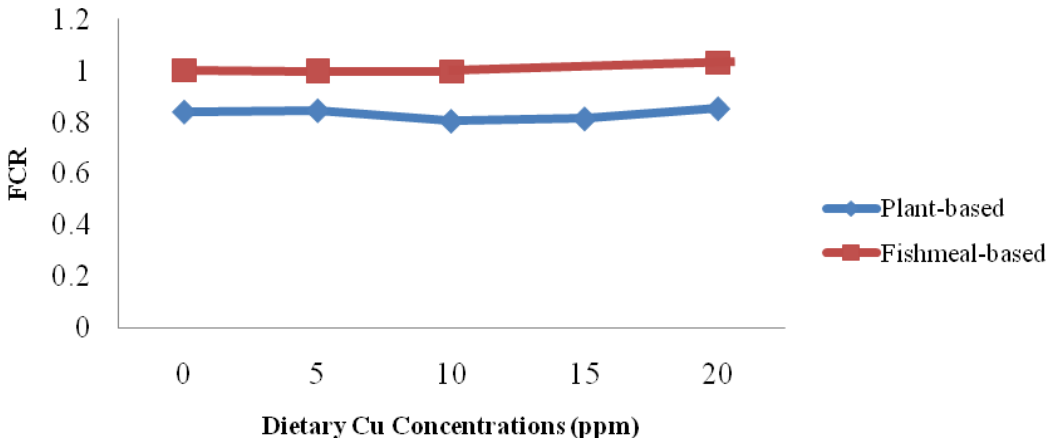

Figure 5. The effects of protein source (plant-based vs. fishmeal-based) and level of dietary copper on feed conversion (FCR) by rainbow trout after 12 wk. Significant ($P < 0.05$) effect due to protein source.

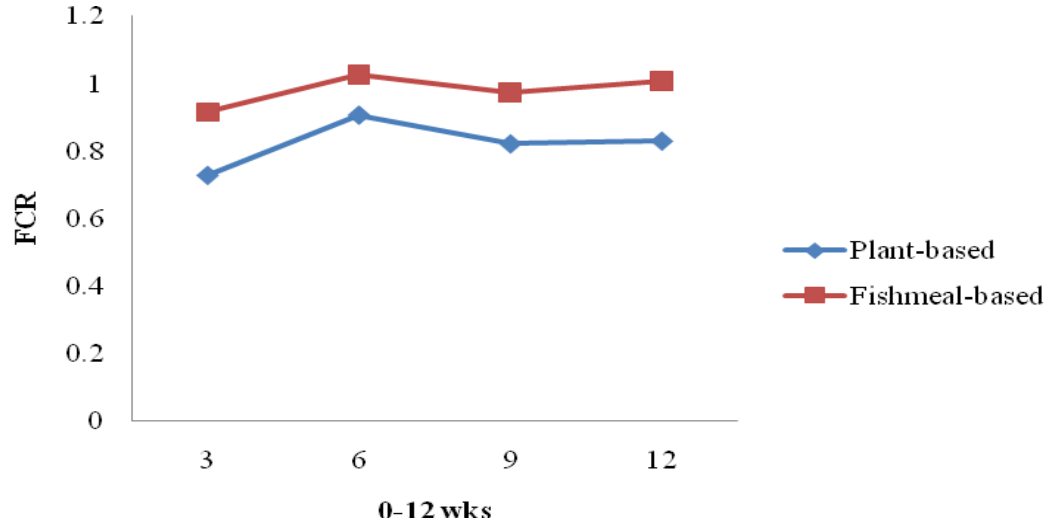

Figure 6. There was a significant effect of protein source over the 12 wk study. Plant-based fed trout had improved ($P< 0.05$) FCR in comparison to trout fed fishmeal-based diets

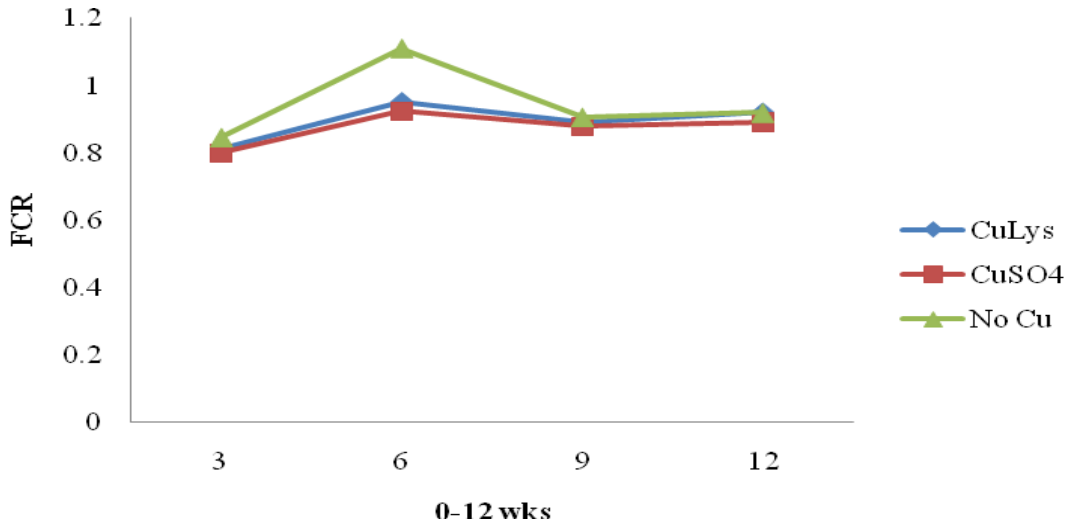

Figure 7. Effect of Cu source over the 12 wk study. Significance was only observed at 6 wks, where trout fed the fishmeal-based diet supplemented with CuSO4 had improved FCR.

An interaction between protein source and Cu source was observed for FCR (Figure 7). Fish fed fishmeal-based diets supplemented with CuSO4, had higher growth rates ($P < 0.05$) and lower FCRs, compared to fish fed fishmeal-based diets supplemented with Cu-Lysine at 6 wks; however, by 12 wks no significant ($P > 0.05$) effects due to Cu source were measured .

Body Indices

Significant effects due to Cu level ($P=0.0027$) and protein source ($P <0.0001$) were observe for muscle ratio at 12 weeks, resulting in a three-way interaction ($P=0.0122$). Muscle ratio was highest in trout fed plant-based diets, when dietary Cu was supplemented at 15 and 20 ppm, intermediate at 0 and 5 ppm, and lowest at 10 ppm.

Significant effects were measured for 6wk HSI. A three way interaction ($P=0.0186$) was detected in trout fed fishmeal-based diets, having the highest HSI when

CuLys was supplemented at > 10 ppm and lowest HSI when no Cu was supplemented. Twelve-wk HSI was effected (P=0.0012) by protein source, with higher HSI indices in trout fed fishmeal-based diets (See Table 5).

Table 5. Body indices[1,2] of rainbow trout at 12 wks in experiment one.

			Hepatosomatic Index[3]	Fillet Ratio[4]
	Diet			
Protein Source	Cu Source	Cu Level		
PB	None	0	1.10	55.13
PB	$CuSO_4$	5	1.13	55.48
PB	$CuSO_4$	10	1.11	55.93
PB	$CuSO_4$	15	1.12	55.90
PB	$CuSO_4$	20	1.12	56.12
PB	CuLys	5	1.10	55.77
PB	CuLys	10	1.18	47.98
PB	CuLys	15	1.17	55.52
PB	CuLys	20	1.02	58.72
FM	None	0	1.29	51.86
FM	$CuSO_4$	5	1.40	52.22
FM	$CuSO_4$	10	1.27	52.96
FM	$CuSO_4$	20	1.36	52.09
FM	CuLys	5	1.31	49.92
FM	CuLys	10	1.14	51.23
FM	CuLys	20	1.18	52.63
		Pooled SE	0.0567	0.762461
		ANOVA, Pr >	0.1311	0.0002
		Protein Source	0.0012	<.0001
		Cu level	0.4955	0.0027
		Cu Source	0.4305	0.1136
		Protein Source*Cu Level* Cu Source	0.6372	0.0032

[1] 3 to 5 fish taken from each tank for sampling.[2]Tank means pooled together within each treatment. [3]Hepatosomatic Index (HSI) = Liver Weight (g)/ whole body weight (g) *100. [4]Fillet ratio (FR) =Fillet weight*2 (g)/ whole body weight (g) *100. No two-way interactions between any of the main effects were observed (P>0.05).

Proximate Analysis

Protein source had a significant effect on whole body moisture and lipid levels (Table 4). Moisture content was higher (P=0.0445) in trout fed plant-based diets in comparison to trout fed fishmeal diets. Whole body lipid levels were higher (P < 0.0001) in trout fed fishmeal based diets (See Figure 8). Whole body energy levels at 12 wks were higher (P=0.0036) in trout fed fishmeal-based diets. Protein whole body composition was higher (P <0.0001) in trout fed plant-based diets.

Nutrient Retention

Protein retention efficiency (PRE) and energy retention efficiency (ERE) were or were not significantly altered by diet at 12 wks (Table 4). PRE was highest (P < 0.0001) in plant-based fed trout (See Figure 9). A similar pattern was also observed in whole body energy retention (ERE), where higher energy was retained in plant-based fed trout.

An effect due to dietary Cu content was measured (P=0.0038), with PRE highest when Cu was supplemented at 15 ppm. A Cu source by Cu level interaction (P=0.0031) was also observed for PRE. Trout fed diets with CuLys had the highest PRE when Cu was supplemented at 15 ppm.

A Cu source by protein source interaction (P=0.0024) was also measured for PRE. Trout fed either Culys (P <0.001) or CuSO4 (P=0.0022) had higher PRE levels when fed a plant-based diet in comparison to a fishmeal based diets. Finally, a Cu level by protein source interaction (P=0.0442) was observed. Highest PRE was observed in trout fed a plant-based diet where dietary Cu was supplemented at 15 ppm.

A three way interaction (P=.0495) also was found in that trout fed plant-based diets with Cu Lys had the highest PRE at 15 ppm. No differences were measured (P > 0.05) due to any of the three main effects when no Cu was supplemented.

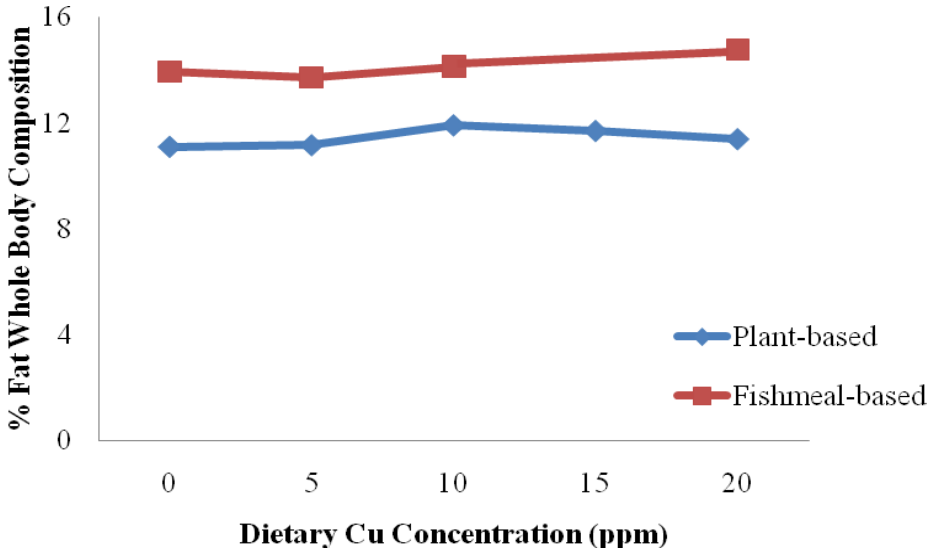

Figure 8. Effect of protein source and Cu level on percent whole body lipid content of rainbow trout at 12 wk.

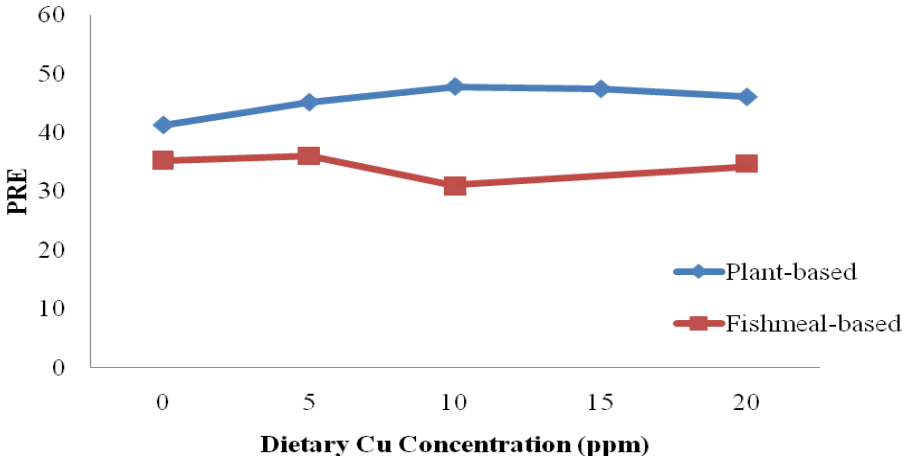

Figure 9. Plant-based fed trout had higher (P<0.05) PRE levels than trout fed fishmeal-based diets.

Mineral Retention

Twelve wk whole body composition levels of Cu, Fe, Mg, S, and Zn were significantly affected by at least one of the three main effects., Cu whole body stores were significantly affected by Cu Source ($P < 0.001$), with lowest Cu whole body levels detected when no Cu was supplemented in the diet. Effects due to Cu level ($P < 0.0001$) were observed with highest Cu whole body stores at 15 and 20 ppm, intermediate at 5 and 10 ppm, and lowest at 0 ppm.

For the observed protein source effect on whole body Cu, highest ($P=0.0037$) whole body Cu levels were measured in trout fed plant-based diets. Fe whole body levels were significantly affected by protein source with trout fed fishmeal-based diets having higher ($P=0.0019$) whole body Fe levels. In contrast, Mg whole body levels were higher ($P=0.0121$) in plant-based fed trout. Both Cu level and protein source altered S whole body levels, with highest ($P=0.0142$) whole body S levels when dietary Cu was supplemented at 15 ppm; highest ($P < 0.0001$) S whole body levels were observed in trout fed plant-based diets. Finally Zn whole body levels were significantly ($P < 0.0001$) higher in trout fed fishmeal-based diets. Zn whole body levels were also observed to be effected by Cu level ($P=0.0135$), with highest Zn whole body levels, when dietary Cu was supplemented at > 5 ppm.

Liver Cu concentrations were found to be effected by both protein source and level of dietary Cu (Figure 10). Liver Cu concentrations were higher ($P < 0.05$) in plant-based diets. Also when no Cu was supplemented in the diet, liver Cu concentrations were lower ($P < 0.05$). Liver Cu concentrations increased with increasing dietary Cu

supplementation in both the plant and fishmeal based diets. As far as other minerals, S was effected by both protein source and Cu source. Liver S concentrations were highest when no Cu was supplemented in the fishmeal-based diets. There were no differences ($P > 0.05$) seen in concentrations of Ca, P, and Zn in the liver.

Dietary protein source and Cu level, but not Cu source, increased ($P < 0.05$) liver Cu stores in the present study. Fish fed Cu-supplemented plant-based diets had higher ($P < 0.01$) liver Cu concentrations than fish fed Cu-supplemented fishmeal-based diets at 6 wks. A quadratic response of increasing levels of Cu was measured at 6 wk ($P < 0.05$) irrespective of protein source or Cu source. Similar results were measured at 12 wks; with Cu- supplemented plant-based fed trout having higher ($P < 0.05$) liver Cu concentration levels (89.4 ppm) than trout fed Cu-supplemented fishmeal-based diets (65.1 ppm, Figure 10). Increased levels of dietary Cu resulted in increased liver Cu levels ($P < 0.05$), with a plateau of approximately 10 ppm in rainbow trout fed plant-based diets. No differences were measured in liver Cu concentrations due to Cu source ($P > 0.05$).

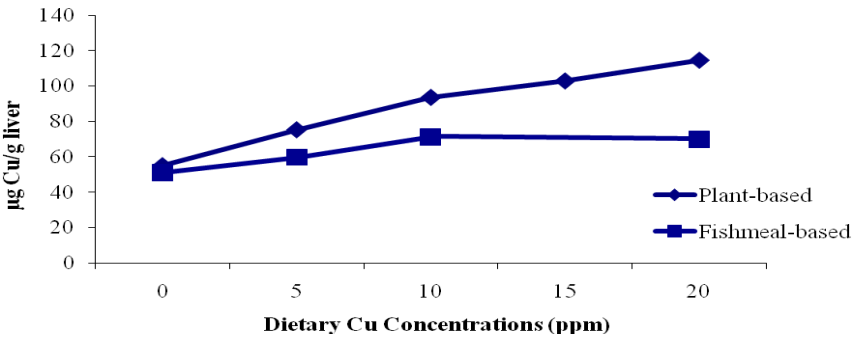

Figure10. The effects of protein source (plant-based vs. fishmeal-based) and level of dietary Cu on liver Cu concentrations in rainbow trout after 12 wk. Significant effect ($P < 0.05$) due to protein source and level of dietary copper.

Study Two

Dietary Analyses

Analyzed dietary proximate composition reflected formulation targets (Table 6). Analyzed dietary Cu and Zn levels were proportionate to the targeted supplementation levels (Table 7).

Table 6. Analyzed composition (SD)[1] of diets fed to rainbow trout for 12 wks[2].

Diet		Crude Protein	Crude Lipid	Moisture	Gross Energy
Cu	Zn	(%)	(%)	(%)	kcal/g
0	0	46.32 (.29)	19.80 (.20)	3.87 (.22)	5633.18 (23)
0	30	43.08 (.08)	15.05 (.25)	2.44 (0.0)	5264.41 (113.44)
0	300	44.65 (.33)	15.46 (.11)	2.60 (.11)	5380.15 (18.18)
0	1500	44.88 (.46)	15.31 (.24)	3.82 (.88)	5414.98 (19.01)
10	0	43.40 (.57)	17.07 (.46)	2.76 (.45)	5518.92 (80.33)
10	30	43.67 (.19)	17.84 (.02)	3.35 (.04)	5422.05 (53.43)
10	300	43.71 (.04)	16.36 (.56)	3.46 (.02)	5413.45 (56.04)
10	1500	42.81 (.17)	15.42 (.09)	3.68 (.09)	5471.59 (20.21)

[1]Standard deviation of [2]Means of two analysis/diet.

Table 7. Analyzed composition (ppm) of minerals in diets fed to rainbow trout in experiment two.

Cu	Zn	Cd	Cu	Mn	Mo	Ca	Fe	S	Zn
0	0	0.16	9.40	49.36	3.21	6634.64	333.86	5060.09	37.71
0	30	0.15	10.34	53.09	3.48	9734.50	366.12	5767.60	91.59
0	300	0.16	9.67	51.32	3.19	8652.95	341.71	5246.11	475.79
0	1500	0.18	10.29	63.69	3.41	7503.48	390.75	6396.91	2289.96
10	0	0.16	20.24	50.88	3.36	9149.43	358.66	5340.49	114.24
10	30	0.15	21.10	52.72	3.37	8968.33	370.47	5445.85	93.38
10	300	0.15	21.00	53.04	3.29	8864.38	359.06	5421.60	508.32
10	1500	0.15	19.86	62.97	3.13	7066.02	368.56	6096.97	2302.71

Growth and Feeding Behavior

Dietary Zn content had significant effects on growth, with minor effects on feeding behavior. Increasing levels of dietary Zn (P=.0079) increased average fish weight. Greatest average fish body wt were measured when trout fed diets were supplemented with ≥300 ppm dietary Zn.

A Cu by Zn interaction (P=0.0017) was also measured for 3 wk average fish wt. At both 0 and 10 ppm Cu, dietary Zn increased average fish weight. Similar trends were also observed for total weight gain at 3 wks, with trout fed 300 and 1500 ppm having the highest (P .0226) weight gains (See Figure 11). A Cu by Zn interaction (P=0.0026) was also measured with 3wk weight gain, where dietary Zn ≥ increased fish weight gain regardless of dietary Cu level.

An interaction (P= 0.0214) between Cu and Zn on FCR was observed at 3 weeks. FCRs was highest when the lowest (30 ppm) and Zn deficient diets, with lower FCRs measured in the fish fed the higher dietary Zn supplemented diets (See Figure 12).

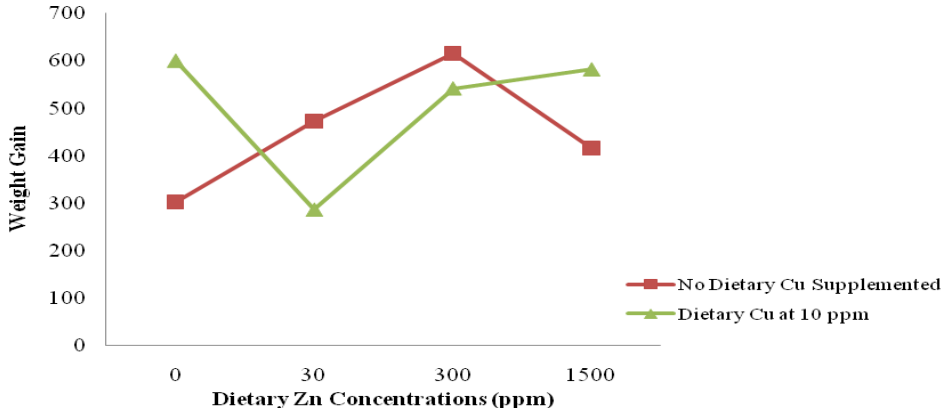

Figure 11. Effects of supplemented levels of dietary Zn on weight gain at 3 wks with or without dietary Cu supplementation with greatest weight gain found in trout fed diets supplemented with 300 and 1500 ppm dietary Zn.

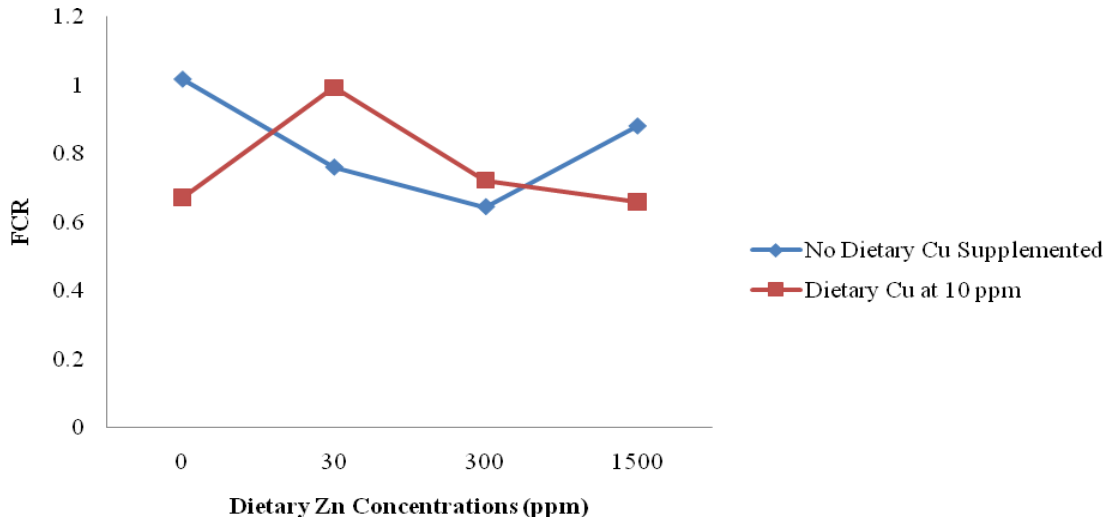

Figure 12. Effects of dietary Zn levels on FCR at 3 wks, with lowest ($P < 0.05$) FCRs in trout fed the higher levels of dietary Zn (300 and 1500 ppm).

Feed intake at 3wks was impacted ($P= .0043$) by dietary Zn concentrations, with feed intake lowest in trout fed the diets with 0 or 30 ppm dietary Zn, with no difference in feed intake among the other levels of dietary Zn supplementation. A Cu by Zn interaction ($P= 0.0029$) was also observed for feed intake at 3 wks, with dietary Zn supplementation (>30 ppm) significantly increased feed intake.

Dietary Zn concentration influenced ($P = 0.0023$) weight gain at 6 wks. Higher weight gains were seen in trout fed 300 and 1500 ppm dietary Zn than trout fed 0 or 30 ppm Zn. An interaction ($P < 0.0001$) between Cu and Zn was found, whereby dietary Zn supplementation $\geq$300 ppm increased weight gain at both levels of dietary Cu supplemented.

At 12 wks, dietary Zn had a significant effect ($P = 0.0015$) on total tank weight gain. Highest weight gain was measured in trout fed Zn supplemented diets at 300 and

1500 ppm, with lowest gains at 0 and 30 ppm Zn (See Figure 13). A Cu by Zn interaction (P= 0.0081) was also observed. Zn significantly (P< 0.0001) increased weight gain when no Cu was supplemented. However, once Cu was added to the diet, Zn supplementation was no longer significant (P= 0.0967).

Although not significant (P= 0.0840), a Cu by Zn interaction was observed in that when no Cu was supplemented in the diet, dietary Zn had a significant (P= 0.0007) effect on cumulative survival. When Cu was supplemented, dietary Zn supplementation did not have a significant (P= 0.0967) effect on cumulative survival.

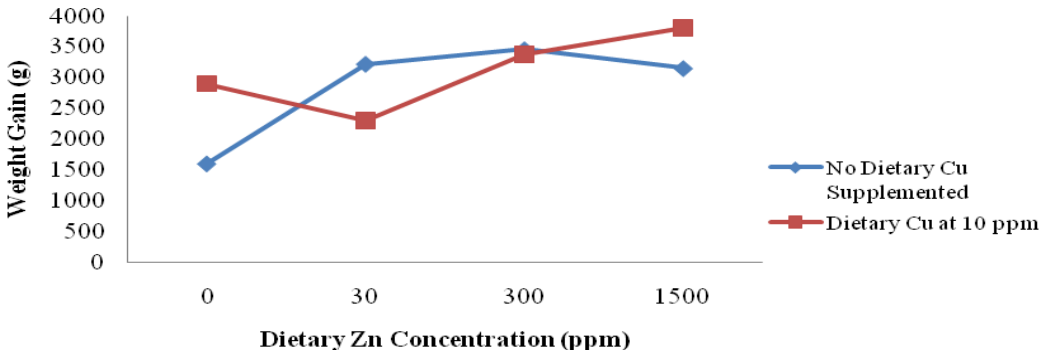

Figure 13. Shown above the highest levels of dietary Zn at 300 and 1500 ppm resulted in the highest (P= 0.0015) trout weight gains at 12 wks. An interaction (P=0 .0081) was also observed with dietary Zn supplementation increasing weight of trout fed the Cu deficient diet.

Body Indices

There were no differences found due to Cu or Zn on 6 and 12 wk VSI, HIS, and MR (See Table 8) with the exception of 12 wk HSI. The 12 wk HSI was lowest (P= 0.0297) when no dietary Zn was supplemented.

Table 8. 12 wk body indices[1,2] of rainbow trout in experiment two.

Diet		Visceral Index[3]	Hepatosomatic Index[4]	Fillet Ratio[5]
Cu	Zn			
0	0	9.17	0.81	24.93
0	30	20.32	1.04	24.72
0	300	11.92	1.01	25.82
0	1500	12.71	1.07	23.68
10	0	10.37	0.89	24.11
10	30	10.97	0.91	23.05
10	300	11.67	1.01	25.23
10	1500	12.62	1.00	22.55
Pooled SE		1.99	0.0639	1.69
ANOVA, Pr > F		0.33	0.1107	0.39
Cu Level		0.24	0.1986	0.16
Zn Level		0.22	0.0297	0.41
Cu * Zn		0.51	0.8060	0.47

[1] 3 to 5 fish taken from each tank for sampling.
[2]Tank means pooled together within each treatment.[3]Visercal somatic index (VSI) = Gut weight (g)/ whole body weight (g)*100.[4]Hepatosomatic Index (HSI) = Liver Weight (g)/ whole body weight (g) *100. [5]Fillet ratio (FR) =Fillet weight*2 (g)/ whole body weight(g) *100.

PRE and ERE

Dietary Cu and Zn both had significant effects on protein and energy retention levels in trout whole bodies at 12 wks (Table 9). An effect of Cu level was observed in that PRE was increased (P= 0.0235) when Cu was supplemented (10 ppm). A similar effect of Cu level on ERE was also observed with ERE highest (P= 0.0093) in trout fed the Cu supplemented diets.

Table 9. Nutrient retention levels (SD) of 12 wk whole bodies in experiment two.

Diet		ERE	PRE
Cu	Zn	(%)	(%)
0	0	26.19 (4.17)	19.84 (7.39)
0	30	43.13 (4.81)	41.47 (8.54)
0	300	41.97 (2.11)	38.50 (7.13)
0	1500	41.88 (3.19)	38.88 (3.28)
10	0	42.36 (2.10)	40.24 (.37)
10	30	36.67 (4.78)	35.50 (3.32)
10	300	45.36 (4.84)	36.34 (2.47)
10	1500	48.34 (3.82)	47.89 (3.42)
Pooled SE		0.0132	0.0177
ANOVA Pr >F		0.0003	0.0009
Cu		0.0093	0.0235
Zn		0.001	0.0029
Cu*Zn		0.0026	0.0046

[1] Standard Deviation of Diets [2] Means of two analysis/ diet
[3] Apparent Protein Retention Efficiency (PRE) = protein gain in fish (g)/ protein intake X 100.
[4] Apparent Energy Retention Efficiency (ERE) = energy gain in fish (g)/ energy intake X 100.

Zinc dietary concentration effected PRE. PRE was higher (P=.0235) when Zn was supplemented in the diet, with no differences (P> 0.05) found when Zn was supplemented $\geq$30 ppm. Similar effects of Zn supplementation on ERE were also observed. Zinc supplementation at $\geq$ 30 ppm increased (P= 0.0010) ERE. A Cu by Zn interaction as observed for both PRE and ERE (See Figure 14 & 15). When no Cu was supplemented in the diet, both ERE and PRE were higher when Zn was supplemented in the diet. Additionally, PRE was significantly increased (P< 0.0045) when Zn was supplemented at 1500 ppm.

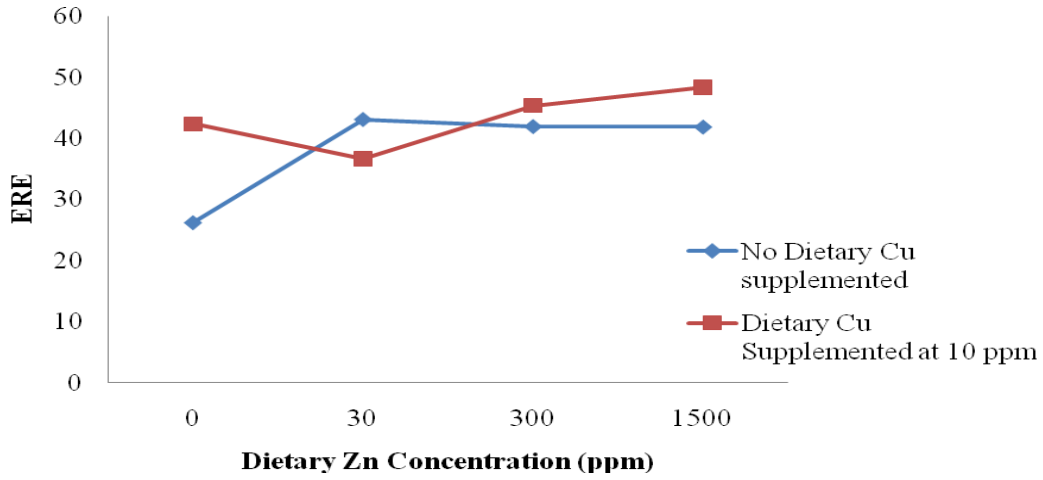

Figure 14. Increasing levels of dietary Zn increased ($P < 0.05$) ERE levels in rainbow trout.

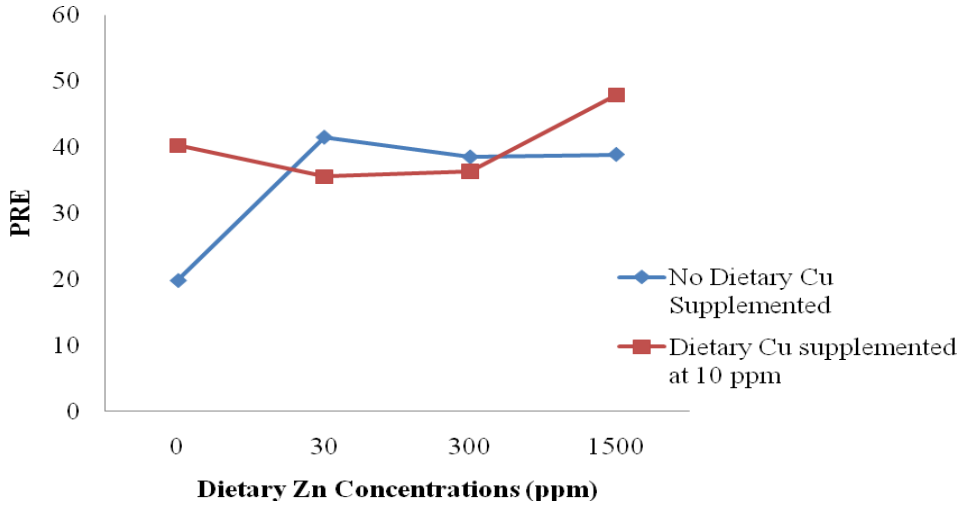

Figure 15. Effect ($P < 0.05$) of increasing levels of dietary Zn on PRE. Dietary Zn enhanced PRE levels.

Whole Body Mineral Retention

Table 10. 12 wk whole body composition (ppm) of Cu and Zn in experiment two.

Diet			
Cu	Zn	Cu	Zn
0	0	0.896614656	7.261740083
0	30	1.13980721	9.895283854
0	300	1.200027627	21.62031863
0	1500	1.062847509	50.53605859
10	0	1.37713038	11.90360127
10	30	1.48066609	9.104348024
10	300	1.421083461	20.8434145
10	1500	1.334587433	51.7314295
Pooled SE		0.098043667	0.971545
ANOVA Pr < F		0.187	<.0001
Cu		0.094	0.9503
Zn		0.0954	<.0001
Cu*Zn		0.806	0.9072

1 Means of 3 replicates/ treatment

Differences were measured in tissue levels of Cu and Zn at 6 and 12 wks (Table 10). At 6 wks, Cu whole body levels were higher (P=0.002) in diets where Cu was supplemented at 10 ppm (whole body Cu level of 0.59 ppm) than in trout fed diets with no Cu supplemented (0.45 ppm Cu). Dietary Zn levels also had an effect on whole body Cu levels where whole body Cu levels were highest in diets supplemented 1500ppm Zn (0.70 ppm Cu), with no differences seen in Cu whole body tissue levels due to the other levels of Zn (See Figure 16). At 12 wks, no differences (P > 0.05) were measured in Cu whole body tissues due to Cu levels, Zn levels or a Zn by Cu interaction.

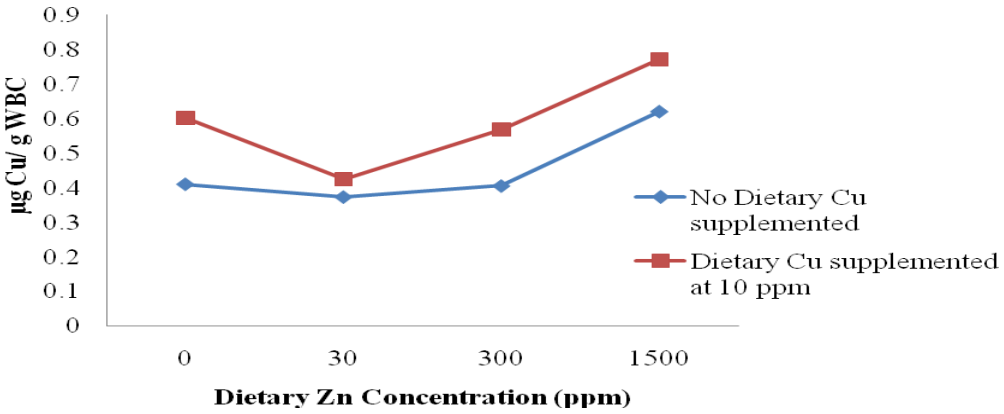

Figure 16. Copper whole body concentrations at 6 wks were highest (P<0.05) when dietary Zn was supplemented at 1500 ppm. Whole body Cu concentrations were also higher in trout fed diets with dietary Cu supplemented.

Zn whole body levels were higher in fish fed increasing levels of dietary Zn. Whole body Zn levels at both 6 and 12 wks were highest (P< 0.0001) at 1500 ppm dietary Zn, with intermediate whole body Zn levels at 300 ppm, and lowest levels at 0 and 30 ppm dietary Zn (See Figure 17). A Cu by Zn interaction (P= 0.0263) was found at 6 wks with dietary Zn having a significant effect on whole body Zn levels at both dietary Cu levels.

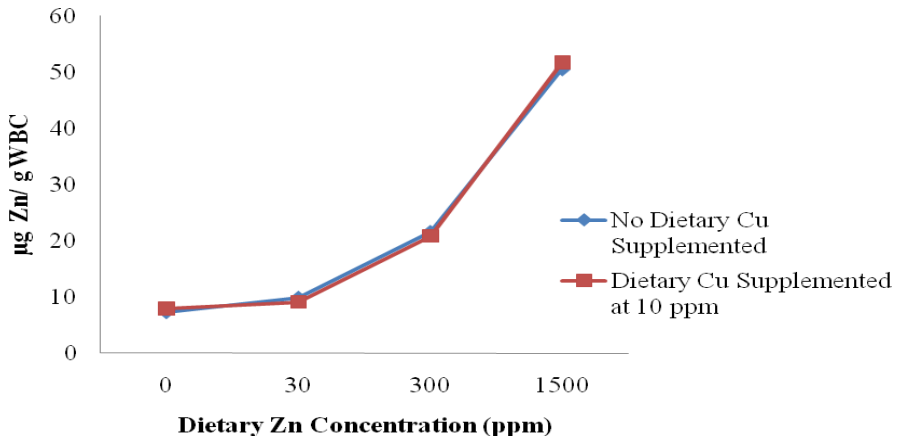

Figure 17. At 12 wks increasing levels of dietary Zn resulted in increasing ($P < 0.05$) Zn whole body levels.

Pathology

Figure18. Caudal fin erosion, a primary sign of a Zn deficiency, seen in trout fed the Cu and Zn deficient diet.

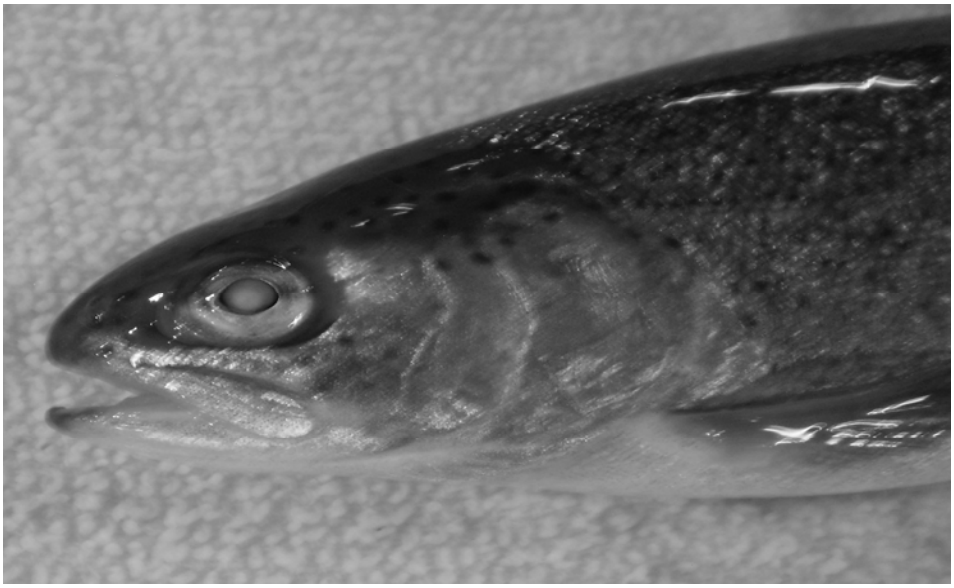

Figure19. Effects of the Cu and Zn deficient diet fed to trout resulting in cataracts.

Mortalities were highest in trout fed the Cu and Zn deficient diet (25%) vs. < 4% average for to all other treatments. Additionally, grossly visible cataracts were observed at 12 wks in 69% of trout fed the Cu and Zn deficient diet compared to an average of 0.01% for all other treatments (Figure 19). Similarly, although not quantified, severe caudal fin erosion (Figure 18) was also observed at a higher incidence in trout fed the deficient diet.

DISCUSSION

Study One

Barrows et al. (2009) demonstrated that fish fed plant-based diets required different mineral supplementation than those fed fishmeal-based diets. Data from experiment one extends that work by also demonstrating that Cu bioavailability is similarly altered by dietary protein source. The presence of high levels of Cu, Mg and S in the whole body tissues of trout fed plant-based diets further suggests increased bioavailability of these respective minerals. These results contrast with Francis et al. (2001) whose review suggested that anti-nutrients contained in plant-based ingredients, could bind minerals digested, resulting in reduced mineral retention. Thus the plant protein used in the present studies did not appear to have negative impact even though previous researchers have indicated problems with some of our ingredients, specifically soybean meal (Gaylord et al., 2010). Alternatively, the endogenous mineral levels within the diet may have been high enough to overcome the anti-nutrient factors present in the plant-based diet.

In contrast, trout fed fishmeal-based primarily as the protein source in the diet, had reduced performance in both body indices and nutrient retention. This was indicated by the higher levels of fat in the body, the higher HSI observed at 6 and 12 wks, as well as lower muscle ratios, all of which suggest an inability to utilize the dietary protein efficiently. Similar studies comparing plant-based and fishmeal based protein sources have observed conflicting results due to protein source. Barrows et al. (2007) found that

rainbow trout fed fish meal free diets with plant meals or plant protein concentrates generally had slightly reduced growth and feed efficiency compared fish fed commercial formulations. Long-term feeding of diets based on plant protein mixtures to rainbow trout also has been shown to reduce growth and affect body and fillet quality traits (de Francesco et al., 2004). Reasons for these discrepancies could include variability in the quality of the fishmeal used or the different formulation targets used for the diets in the respective studies. In the current study, the analyzed mineral levels revealed high levels of Ca in the fishmeal based diet suggestive of high bone content in the raw fishmeal. High ash levels have been used as an indicator of protein quality for rainbow trout because reduced growth has been observed with extended feeding of high ash ingredients. Alternatively, although both the plant and fishmeal based diets were comparable in analyzed crude protein levels, lysine, methionine, threonine, and taurine in addition to some other macro minerals, were supplemented to the plant-based diet. Thus, these supplements may explain the improved growth observed in the trout fed plant-based diets. Further research is required to identify what else may be enhancing plant-based diets on a nutritional basis.

As far as Cu source, whether inorganic or organic, no differences were observed in regards to Cu bioavailability, specifically in growth or hepatic Cu stores. Equivalent accumulation in both hepatic Cu and whole body stores, were similar to findings by Kjoss et al. (2006). One primary reason why organic sources are used is to protect the mineral sources from binding with other minerals (ions), or with anti-nutrients present in the plant-based diet. If such nutrients and minerals were present, it would be more likely

that CuLys would have had higher Cu uptake in comparison to $CuSO_4$ due to its known protective effects. Equivalent growth performance and accumulation of trout fed either Cu source could therefore suggest a lack of anti-nutrients and ions, or that they were not present at high enough levels for supplementation of CuLys to be beneficial. Lending support for this theory is the high performance of the fish fed the plant-based diet.

In contrast, the level of Cu did have a significant effect on Cu bioavailability in the rainbow trout for both growth and hepatic Cu concentrations. Copper concentrations in the liver and whole body stores increased with increasing Cu supplementation. The importance of Cu level was revealed during the early periods of the study where higher feed intakes and FCR and lower growth rates were observed in trout fed the Cu deficient diet. The higher feed intakes observed during this period may suggest that fish attempted to compensate for low dietary Cu levels by consuming more; although there is limited research to support this theory. Increased intake has been observed for rainbow trout suffering from other micronutrient deficiencies. Gaylord et al. (2006) in a study examining the benefit of taurine supplementation in plant-based diets fed to rainbow trout reported increased feed consumption in trout fed plant-based diets where no taurine was supplemented.

The analyzed composition of the non-supplemented plant-based control diet was 4 ppm, which according to NRC, met the 3 ppm Cu requirement for rainbow trout. Nevertheless, increased growth performance of rainbow trout fed plant-based diets was observed when Cu levels greater than the previously reported requirement were supplemented. Barrows et al. (2008, 2009) found similar results with rainbow trout,

where different vitamin premixes and macro minerals were necessary to optimize growth and FCR in plant-based diets compared to traditional fishmeal-based diets. Additional dietary Cu supplementation also improved PRE for both protein sources fed to trout. In order to reach these higher muscle ratios found in trout fed plant-based diets, the higher levels of dietary Cu (15 and 20 ppm) needed to be supplemented. Similar results were reported Gaylord et al. (2006) and Barrows et al. (2008) found addition of nutrients (taurine and vitamin premixes) improved PRE of both plant and fishmeal based diets.

Study Two

Proper mineral supplementation in plant-based diets continued to be of consequence in the second experiment. The necessity of higher levels of Cu supplementation to maintain growth observed for plant-based diet suggested that the presence of other minerals in the plant-based diet that could possibly be blocking Cu uptake. Based on the analyzed dietary Zn levels of the plant-based diet in study one, it was hypothesized that there could be negative effects of high dietary Zn levels on growth of rainbow trout.

However, results from study two did not support that hypothesis. Increasing levels of dietary Zn up to 1500 ppm improved weights gain, feed conversion and increased 6 wk hepatic Cu concentration. Even at the highest level of dietary Zn supplementation, no toxic effects were observed. The higher weight gains, feed intakes and improved FCR due to higher Zn supplementation, could be, in part, be due to a rebound-response of trout fed a Cu and Zn deficient diet for 6 wks prior. However, this benefit of Zn

supplementation was observed throughout the entire length of the study. These results are in agreement with Wekell et al. (1983) who found improved growth rate of rainbow trout when Zn was supplemented at 1700 ppm. Similarly, Knox et al. (1984) reported no differences in growth or feed efficiencies of trout fed 1000 ppm of dietary Zn in comparison to fish fed lower levels of dietary Zn. Dietary Zn supplementation also increased utilization of energy and protein. Similar to studies conducted by Barrows et al., (2008 and 2009) examining the necessity of various nutrients (vitamins and macro-minerals) supplementation to plant-based diets, further support the need for dietary Zn at levels greater than the previously reported requirement (NRC 1993) to sustain growth and PRE in trout fed plant-based diets.

Zn whole body levels increased with dietary Zn supplementation at both 6 and 12 wks, following a linear trend. This agrees with Gatlin et al. (1989) where higher dietary Zn supplementation (200 ppm) increased Zn bone concentrations. In contrast, Knox et al. (1984) found that Zn liver concentrations were not increased by high dietary Zn supplementation (1000 ppm). Reduced growth, cataracts, caudal fin erosion, and increased mortalities were all observed following 18 wks of being fed the Cu and Zn deficient diet. Such pathologies have previously been reported as indices of a Zn deficiency in rainbow trout (Ketola et al., 1979; Ogino et al., 1979). Supplementation of additional dietary Zn improved growth of fish and prevented cataracts in the Ketola et al. (1979) study. The current study observed similar results, once Zn was supplemented in the diet; Zn deficiency signs were substantially reduced or not grossly detectable. While the current study agrees with the previous literature (Ketola et al., 1979; Ogino et al.,

1979) regarding the necessity of dietary Zn supplementation to prevent mortalities and the occurrence of cataracts, there remains some question in regards to the optimum level of dietary Zn that should be supplemented to a plant-based diet given the fact that growth was maximized at the highest supplementation level.

In contrast to the positive effect of Zn supplementation in study two, no benefit of Cu supplementation on weight gain was observed over the 12 wk period. However, it important to note that the Cu deficient diets analyzed composition was 10 ppm, which from the first study was found to be an adequate level for growth in a plant-based diet. Dietary Cu level did have an effect on Cu whole body stores at 6 wks. When 10 ppm Cu was supplemented to a plant-protein based rainbow trout diet, an increase in Cu whole body stores was observed at 6 wks. By 12 wks however, Cu levels were comparable across Cu levels, regardless of whether or not Cu was supplemented. This could indicate that Cu whole body stores were maximized between 6 and 12 wks. In contrast, Gatlin et al. (1989) reported increased liver Cu concentrations when the highest level of Cu (20 ppm) was supplemented.

Minimum reported requirements for Cu and Zn for maximum growth and optimum health of rainbow trout have been reported as 3 ppm Cu and 15-30 ppm Zn (Ogino and Yang et al., 1980 and 1978, respectively). As analyzed, the Cu (9.4 ppm) and Zn (37.1) ppm deficient diet provided enough dietary minerals to meet the NRC recommendations (1993) for both Cu and Zn. Additionally, in the Zn supplemented diets analyzed Zn levels of >2000 ppm were achieved resulting in Cu: Zn ratios that were higher than those previously shown to have antagonistic effects on liver Cu levels in

rainbow trout (Knox et al., 1984). In that study, Knox et al. (1984) reported reduced liver Cu concentrations when dietary Cu level was 2.5 ppm and Zn was supplemented at a much higher level (1000 pm). In contrast, no antagonistic relationship was observed in the current study. In fact, positive Cu and Zn interactions were observed for growth, protein and Cu retention. These results suggest that increasing dietary Zn not only improved protein utilization but no inhibitory effects were observed when Cu was supplemented in the diet along with Zn. This is in agreement with earlier work conducted by Gatlin et al. (1989) when dietary Zn was supplement at 200 ppm, liver Cu was not reduced by the high level of dietary Zn. It is important to note for the Knox (1984) study that the highest Zn liver concentrations were observed in trout fed the highest Cu (500 ppm) and Zn (1000 ppm) diet suggesting a positive relationship between Cu and Zn on tissue levels, similarly to what was observed in the current study.

Another theory to explain the lack of antagonistic relationship could be the length of the depletion and re-feeding periods in the second study. Kjoss et al. (2006) conducted a depuration and loading phase comparing bioavailability of various sources of Cu and Zn in rainbow trout. In that study, each phase lasted approximately two weeks, with substantial tissue responses shown. Therefore, the 6 wk depuration period in the second study was likely sufficient to deplete high Cu and Zn tissue levels that may have been present. The longer length was chosen for the current study because the trout were previously fed a commercial diet that was supplemented well above the mineral requirements. Another purpose of the 6 wk depuration period was to observe the rate of Cu and Zn repletion and identify any possible relations between Cu and Zn in the rate of

repletion. McDowell (1992) stated, animals who are Cu or Zn deficient have increased Cu and Zn absorptive capacity, compared to animals provided with adequate amounts of both Cu and Zn. However, because of the extended depuration period, re-feeding for 12 wks even at the high Zn levels may not have been sufficient to restore tissue mineral levels to the point that antagonism could be observed.

One theory to explain differences between our studies and those of Knox et al. (1984) could include the fact that in the current experiments routine water samples to monitor waterborne Cu and Zn concentrations. Thus, because the current study was conducting in a re-circulating water system, waterborne sources could have masked dietary treatment effects. However, according to Clearwater et al. (2002), dietary concentrations of both Cu and Zn have greater effects on animal growth and tissue levels than Cu and Zn waterborne concentrations.

Alternatively, because only whole body tissue Cu and Zn levels were examined in the second study, a reduced ability to detect more subtle differences in tissue mineral levels could have resulted. Of note, in the first study, liver and whole body Cu levels displayed similar trends. However, because mineral-specific tissue pools were not investigated, it is impossible to say that antagonistic effects at specific tissue locations are not present. Examination of both minerals in the primary tissue pools for Cu and Zn, the liver (McDowell 1992) and the eye (Ketola et al., 1979) could provide further clarification of these relationships in rainbow trout.

CONCLUSIONS

Proper mineral supplementation in diets where alternative protein sources are provided to rainbow trout is essential for proper growth and health of the trout. The focus of the current research was to identify the effects of different protein sources, the type of Cu sources provided, and the presence of varying levels of dietary Cu and Zn in the diets, on Cu bioavailability of these minerals is necessary. From the two experiments conducted it was found that both protein source and dietary levels of Cu and Zn had a significant effect on Cu bioavailability in rainbow trout.

Results of experiment one demonstrate that plant-based diets supplemented with Cu at levels higher than NRC (1993) recommended levels can perform as well or better than trout fed fish meal based diets. Additionally, liver Cu levels were higher in trout fed plant-based diets compared to trout fed fishmeal-based diets. No differences in growth rates or liver Cu concentration levels were observed due to $CuSO_4$ or Cu-Lysine supplementation. In order to increase growth in trout fed plant-based diets, Cu supplementation between 5 and 10 ppm from either Cu source is recommended.

Results of the second study indicate rainbow trout fed plant-based diets also require Zn supplementation at levels higher than NRC (1993) recommended levels to obtain maximum growth ($\geq$ 300 ppm) and to prevent incidence of cataracts ($\geq$ 30 ppm). In contrast to the first study, no effect of supplemental Cu was observed for weight gain. Of note, the analyzed levels of the non-Cu supplemented diet in the second study were 9 ppm vs. the 4 ppm Cu level analyzed for the same diet in the first study.

From the two experiments conducted, there is a need for further research in regards to proper Cu and Zn supplementation in a plant-based diet. First, the dietary Zn requirement for rainbow trout fed a plant-based diet needs to be re-examined. In the second study, the highest weight gains occurred at 300 and 1500 ppm, thus, perhaps examining additional supplementation levels between 30 and 1500 ppm would provide clarification. Other studies could also be conducted to examine whether other minerals present in the plant-based diets could be responsible for the reduced Cu uptake, specifically, Fe or S.

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