To my wonderful husband. Without his endless love and support, none of this would be possible.
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After phosphorous, copper deficiency is the second most widespread mineral deficiency found in grazing cattle. Lack of adequate copper in beef cattle can lead to health complications such as anemia, impaired immune function, and failure to thrive resulting in significant economic losses. Given the severity of these potential complications, the need for a sensitive, convenient copper status indicator is warranted. Recent studies have shown that the levels of copper chaperone for Cu, Zn Superoxide Dismutase (CCS) in erythrocytes are reflective of copper status in mice and rats. The objective of this study was to evaluate erythrocyte CCS as a possible status indicator in the bovine, and to test the response of CCS under conditions of inflammation, as this is a limitation with current copper status indicators. We demonstrate that bovine erythrocyte and liver CCS protein levels significantly increase \((P < 0.05)\) in copper-deficient cattle. Furthermore, CCS protein levels were did not change in a vaccine-induced inflammatory response implying that CCS does not exhibit acute-phase properties. Despite the significant increases in CCS protein concentration to a copper deficiency, the response was not as robust as those observed previously with rodents. CCS may not be sensitive in detecting a moderate copper deficiency in cattle.
CHAPTER 1
INTRODUCTION

After phosphorus, copper deficiency is considered the second most common mineral deficiency in beef cattle (1). The National Animal Health Monitoring and Surveillance program (NAHMS) reports that 43% of beef cattle are classified as copper deficient based on serum copper values. This deficiency persists despite the fact that over 64% of beef cattle operations provide regular copper supplementation well above the National Research Council (NRC) recommendations of 10 ppm (mg/kg) per day on a dry matter basis to maintain copper adequacy (1). Copper deficiency in these animals arises from a secondary condition rather than a lack of sufficient dietary copper.

Secondary copper deficiency results from high levels of copper antagonists in the soil, feed, or water supply. Copper antagonists such as sulfur, iron, molybdenum, and sulfur-containing compounds such as thiomolybdenum, chelate copper in the stomach and rumen and render the molecule unavailable for absorption (2). In the NAHMS Beef 1997 report, forage samples across the US were analyzed for copper content as well as levels of copper antagonistic minerals. In Florida, forage is mainly comprised of Bahiagrass and Bermudagrass, and according to the analysis of Bermudagrass, 64.3% of the forage samples were not only marginally deficient in copper, but 87% of the samples also contained marginal to high levels of copper antagonists (3).

A lack of adequate copper in beef cattle can lead to health complications, even when the animal is only marginally deficient. Complications include anemia due to lack of availability of copper in the ferrooxidase ceruloplasmin (72), loss of hair pigmentation due to the reduced copper-dependent conversion of L-tyrosine to melanin, impaired immune function and failure to thrive (2, 5).
Given the severity and prevalence of a copper deficiency and its adverse effects, a sensitive copper status indicator is needed. Currently, the most accurate form of testing is a liver biopsy (1). The liver is the primary storage site of copper and a hepatic copper concentration of 75-90 ppm (dry matter basis) or below classifies the animal as copper deficient. Although liver samples provide the most accurate indicator of copper status in the system, the procedure is highly invasive to the animal and requires trained personnel. As a result, serum or plasma copper is often used due to the convenience of sampling (1). The main limitation of serum and plasma copper as status indicators is that more than 80% of plasma copper is bound to ceruloplasmin—an acute-phase protein that increases during periods of inflammation and stress (80). In addition, serum copper concentrations vary by the age, gender, and species of the animal (2). These fluctuations limit the validity of the test.

Another potential status indicator that has been explored is the copper-containing enzyme diamine oxidase (DAO), which catalyzes the deamination of histidine, diamines and diamine derivatives. The activity of the enzyme significantly decreases in copper deficiency, and has been shown to be reflective of copper status in the bovine (6). The main limitation with DAO is that it is highly active in the kidney and intestines, and its activity has been shown to increase independent of copper status in animals with renal or intestinal disease.

The protein Cu, Zn superoxide dismutase (SOD1) has also been investigated as a possible copper status indicator. Several experiments have shown that SOD1 protein levels significantly decrease in the erythrocytes of copper-deficient rats and mice due to the missing catalytic copper cofactor (7). Measurement of SOD1 protein concentrations could serve as a potential copper status indicator, though it may not provide an accurate reflection of copper status as the non-functional apo form of the protein is still detectible. A more accurate measurement therefore
would be to examine SOD1 activity as it has been shown to be more reflective of copper status (8).

Previously, studies have shown that the copper chaperone for Cu, Zn Superoxide Dismutase (CCS) protein levels significantly increases in the liver and erythrocytes of copper-deficient mice and rats (7, 9). The CCS protein could be a viable marker of copper deficiency in the bovine, but its expression, acute-phase characteristics, as well as response to copper status has not yet been assessed in the bovine system. CCS could serve as a sensitive copper-status indicator in the bovine.
CHAPTER 2
LITERATURE REVIEW

Introduction to Copper

Copper is a trace mineral that is essential to life. Copper is a d-block transition element that can assume two distinct valence states: the reduced monovalent cuprous ion (Cu⁺) and the oxidized divalent cupric ion (Cu²⁺). Copper has the ability to switch between valance states and this redox chemistry is the reason why it is a crucial component of many enzymes in the body. Table 2-1 lists various proteins that are associated with copper transport and/or metabolism.

Though copper is an important mineral to sustain life, it can also be detrimental to the body. Copper has the ability to generate hydroxyl free radicals through the Fenton reaction: Cu⁺ + H₂O₂ → Cu²⁺ + ·OH + OH⁻ resulting in irreversible oxidative damage to tissues and cells (14). Due to the potential negative effects of copper, its extracellular as well as intracellular transport and utilization are tightly regulated in the body (15).

Intestinal Copper Absorptive Pathway

In most organic food matter, copper is primarily bound to sulfur-containing amino acids and dissociates from these complexes in the early stages of digestion. Copper is absorbed in the duodenum, though the exact mechanism is currently being investigated. It has been proposed that copper absorption is mediated either exclusively or in combination by either copper transporter 1 (CTR1), divalent metal transporter 1 (DMT1) or by an unknown saturable process. The human copper transporter 1 (hCTR1) was discovered in 1997 through functional complementation analysis with the yeast Ctr1 gene, and was later characterized to mediate the monovalent copper (Cu⁺) uptake in an ATP-independent manner with high specificity and affinity (16, 17). To investigate whether CTR1 mediates the apical absorption of copper, a CTR1 intestinal knockout mouse was generated (17). Ablation of intestinal CTR1 resulted in reduced copper transport.
across the intestinal epithelial tract and copper-dependent enzyme deficiencies. Contrary to expectations, copper absorption within the intestinal epithelial cell (IEC) was not inhibited, and the IEC exhibited massive copper accumulation that was eight to ten times that of the control mice. This suggested that copper still crossed the apical membrane and accumulated in a non-bioavailable pool. Transport to the basolateral membrane for excretion into portal circulation did not occur (17). Though it is unclear as to why copper loads in a biologically unavailable pool in the IEC, these data show that CTR1 is essential for copper absorption into portal circulation. Furthermore, CTR1 is essential for life since the CTR1 null mutation is embryonically lethal (18). CTR1 can only mediate the transport of the monovalent cuprous ion (Cu⁺); however the majority of dietary copper exists in the divalent form (Cu²⁺). Reduction of Cu²⁺ at the apical surface of enterocytes therefore needs to occur before transport by CTR1. The six transmembrane endothelial antigen of the prostate (STEAP) proteins are known to reduce copper and could potentially fulfill this role, particularly the STEAP2 and STEAP3 isoforms (19, 20).

The divalent metal transporter 1 (DMT1) is the other potential candidate for intestinal copper absorption. DMT1 is known to transport iron across the apical membrane, but it can also transport other divalent cations such as Mn²⁺, Cu²⁺, Co²⁺, Ni²⁺, and Pb²⁺ (21, 65). A recent study demonstrated that when Caco-2 cells were treated with the antisense oligonucleotide to DMT1 in order to reduce DMT1 protein expression, iron as well as copper transport were significantly reduced. Furthermore, in cellular uptake studies, Fe and copper could inhibit the other’s uptake suggesting that both metals share a common transporter. This inhibition however was only observed in the presence of ascorbate suggesting that DMT1 transports monovalent (Cu⁺), the predominant form in acidic conditions (82). DMT1 could possibly play a role in non-specific intestinal copper absorption especially if copper levels are high.
Once in the enterocyte, copper is exported into portal circulation by the Menkes protein ATP7A. The ATP7A copper exporter is localized to the trans-golgi network under basal copper concentrations and imports copper to the Golgi apparatus for incorporation into metalloproteins. As copper accumulates, the protein translocates to the basolateral membrane of the enterocyte where it actively exports copper into portal circulation using the energy from ATP hydrolysis (22). Defects in the ATP7A transporter results in Menkes disease, a recessive, X-linked inherited disorder that results in chronic copper loading in the enterocyte due to the dysfunctional copper exporter (23). This leads to a systemic copper deficiency because the copper cannot be released from the enterocyte for utilization in the periphery. Complications from the disease include abnormal hair structure, commonly termed “kinky hair”, mental retardation, connective tissue defects, and severe anemia, which are all symptoms attributed to the dysfunction of copper-dependent enzymes (24, 25).

Once in portal circulation, copper travels to the liver bound to transcuprein (10), albumin (26, 27) or other soluble peptides and amino acids. The liver is the main storage site for copper and is an important regulator in copper homeostasis. In copper excess, the biliary secretory pathway becomes activated and much of the copper becomes incorporated into bile for secretion back into the small intestine (15). Bile is the major excretory pathway of copper and while it is true that bile is actively reabsorbed, much of the copper is lost. Under conditions of copper adequacy, copper is transported to the Golgi apparatus in the liver through the Wilson protein ATP7B for incorporation in the multicopper ferroxidase ceruloplasmin. ATP7B is a transmembrane copper transporter that is complimentary in structure and function to ATP7A, but is predominantly expressed in the liver (62). Missense mutations of the metal-binding domains of the ATP7B copper exporter protein results in Wilson’s disease, an autosomal recessively
inherited disorder of copper metabolism (28). The disease results in severe copper loading in the brain, liver and kidney and leads to hepatoentriculcular degeneration as a result of the impaired ability to export copper to the secretory pathway for biliary excretion or for incorporation into ceruloplasmin. Chronic copper loading increases DNA damage, enzyme inactivation, and lipid peroxidation (29).

Once copper is incorporated into ceruloplasmin, the protein is released into the circulation through the secretory pathway of the Golgi network. Ceruloplasmin accounts for over 80% of the copper found in circulation and though it plays an essential role in iron metabolism, its role in copper metabolism has yet to be elucidated. Ceruloplasmin has long been proposed to function as a copper transporter since it binds the majority of serum copper (15). This hypothesis however, has recently been challenged in light of the Cp knockout mouse that exhibits normal copper metabolism in the periphery indicating that ceruloplasmin is not needed for copper transport (63). Further research is needed to identify the protein responsible for copper delivery to the peripheral tissues.

Peripheral tissues import copper through CTR1 (64) and copper becomes bound to soluble intracellular receptors known as metallochaperones. The metallochaperones cytochrome C oxidase assembly protein (COX17), human ATX1 homologue (HAH1) and copper chaperone for Cu,Zn Superoxide Dismutase (CCS) function to deliver copper to specific target proteins in the cell (15). COX17 delivers copper to the enzyme cytochrome C oxidase located in the terminal region of the electron transport chain in the mitochondrion (30). The transport of copper to the secretory pathways of the cell is mediated by HAH1 (31). CCS transports copper to the SOD1 protein, which functions to protect the cell from free radical damage (Figure 2-1) (32).
Copper Intake

In the US, the recommended daily allowance for copper is 0.9 mg/day for adult humans, with the average adult consuming approximately 0.6-1.6 mg of dietary copper per day (33). Copper is present in a multitude of plant and animal sources. It is abundantly rich in shellfish and organ meats such as liver, and in seeds, grains, and nuts.

Copper deficiency in humans is not a common occurrence, unless a genetic disorder is present. In grazing cattle however, this is a significant issue. The National Research Council recommends a dietary copper intake of 10 ppm (mg/kg) on a dry matter basis to maintain adequate copper status in beef cattle, which is normally attained through supplementation (1). The United States Department of Agriculture (USDA) states in their 1997 NAHMS report that 64% of the beef cattle operations provided regular copper supplementation in the form of free choice minerals in which the cattle consumes mineral mixtures ad libitum (1). Nonetheless, many grazing cattle remain copper-deficient. (34). Approximately 43% of the beef cattle in the US are classified, by serum copper values as having moderate (0.25 ppm-0.65 ppm) to severe (less than 0.25 ppm) copper deficiency (Table 2-2) (1).

Copper deficiency usually persists despite adequate supplementation because a deficiency can result as a primary or a secondary problem. Primary copper deficiency occurs when there is inadequate copper in the diet, which is not usually a problem in the US where as mentioned previously, copper supplementation is common. Most cattle suffer from copper deficiency as a result of a secondary antagonistic condition.

Secondary Copper Deficiency in the Ruminant Digestive System

Intestinal copper absorption is similar in both the monogastric and polygastric digestive systems in that primary absorption takes place in the proximal region of the small intestine. The transit route, however, as well as the bioavailability of copper, is markedly different. The major
unique characteristic of the polygastric digestive system is the four-compartment stomach known as the ruminant. It is composed of the rumen, reticulum, omasum, and the abomasum (Figure 2) (35). Ruminant digestion is orchestrated through the elegant symbiotic relationship that exists between the host organism and bacterial microbes that populate the rumen.

Microbial organisms ferment the cellulose matter from the ingested feed and as a result, they create volatile fatty acids such as acetate, propionate, and butyrate as a waste by-product. The host organism absorbs and metabolizes these volatile fatty acids into energy. This symbiotic relationship between the microbes and the host organism also promotes copper antagonistic activity. The rumen maintains a reducing environment necessary to maintain microbial growth.

Dietary sulfur consumed from the feed, soil, fertilizer, or water is thereby reduced to the sulfide form under these conditions. Free sulfide can either chelate copper directly forming the insoluble copper sulfide (CuS), or it can combine with dietary molybdenum to create substances known as thiomolybdates (36). Thiomolybdates can have up to four degrees of sulfur binding and exhibit extreme copper antagonistic effects (Figure 3). The higher the degree of sulfur binding, the greater the antagonistic activity (36). Thiomolybdates can inhibit copper availability in two ways: by binding the copper in the gastrointestinal system or by interacting with copper once in the systemic circulation. In one study, investigators showed that absorbed thiomolybdates induced increased biliary copper losses from liver stores, reduced copper transport capacity, and dissociated copper from metalloenzymes (37). Spears et al. (37) demonstrated that thiomolybdate formation is a function of the amount of dietary sulfur consumed. When dietary sulfur levels were low, molybdenum did not inhibit copper availability; however, as the concentration of sulfur increased, thiomolybdates formed more readily and decreased copper availability. Iron has also been shown to antagonize copper absorption although the exact mechanism is currently
unknown. It has been suggested that Fe$^{2+}$ can combine with sulfur (S$^{2-}$) to form ferrous sulfide (FeS) complexes in the rumen. These complexes later solublize in the abomasum, and dissociates allowing the free sulfide to chelate copper (CuS) (66). Multiple studies have confirmed that high levels of dietary iron reduce plasma and liver copper to levels indicative of deficiency (67). The National Research Council (NRC) has established critical levels of copper-antagonistic metals in forage that would lead to a copper deficiency (Table 2-3) (3).

In Florida, beef cattle forage consists mainly of Bahiagrass and Bermudagrass. According to the NAHMS 1997 Beef Study analysis of Bermudagrass, 64.3% of the forage samples were marginally deficient in copper and 87% of the samples contained marginal to high levels of copper antagonists (3).

**Effects of Copper Deficiency in Cattle**

The most notable effect of a copper deficiency is anemia. In 1928, the first studies were conducted that shed light on the relationship between copper and iron in rats leading investigators to propose a possible role for copper in hemoglobin formation (38, 39). This hypothesis was confirmed in classical studies using swine that demonstrated that diets deficient in copper but adequate in all other nutrients produced the same anemia as was observed with an iron deficiency, but such that supplemental iron was unable to correct. Copper-deficient animals exhibited a reduction in mean corpuscular volume (MCV), as well as marked microcytosis and hypochromia due to the reduction in hemoglobin formation (40). The pigs became lethargic and if not rescued with copper supplementation, would die from tissue anoxia within 2 months (40). Similar observations have been reported in copper-deficient cattle (74). Studies have shown that in severe and prolonged copper deficiency, cattle develop anemia, though it is not without some degree of variability. Calves have greater hemoglobin content and red cell counts than adult cattle, and sexually mature bulls are reported to have higher levels of erythrocytes than females
of the same age (76). Despite the age and gender differences, anemia is clearly observed in copper-deficient cattle. In a recent study, copper deficiency was induced by feeding calves a copper-deficient diet containing 1.3 mg/kg copper, which was further supplemented with 4 mg/kg molybdenum and 3g/kg sulfur. The onset of copper deficiency occurred at approximately 15 ± 3.2 weeks, and by the end of the study (30 weeks), copper concentrations in the liver (16 µg/g) and plasma (0.1-0.2 mg/L) dropped well below the NRC established levels for copper deficiency. The mean hemoglobin value at this time point was 6.7 g/dL, indicative of anemia (72). Table 2-4 lists the ranges of hematological measurements for the bovine. The anemia that results from a copper deficiency is due to the reduction in both ceruloplasmin and hephaestin activity. Both proteins utilize the redox potential of copper to catalyze the oxidation of ferrous iron (Fe²⁺) to ferric iron (Fe³⁺), which is necessary for the incorporation of iron into the plasma transport protein, transferrin (41). Without the function of these two copper-dependent proteins, iron cannot be efficiently loaded onto transferrin, which transports iron to the bone marrow for hemoglobin synthesis. Inadequate iron delivery to the erythron results in anemia.

In addition to the lethargy caused by the anemia, a copper deficiency can be detected when animals begin to lose hair pigmentation. This is the visual clinical sign of an overt copper deficiency in cattle and first appears around the eyes typically giving the animal a “spectacled” appearance (75). Melanin, the protein attributed to pigmentation is formed by the hydroxylation of tyrosine to L-3,4 dihydroxyphenylalanine (DOPA) followed by the subsequent oxidation to dopaquinone by the copper-dependent enzyme tyrosinase. Tyrosinase activity is credited to the redox potential of two copper cofactors and without sufficient copper due to a deficiency, the enzyme loses its catalytic activity and the synthesis of melanin does not occur (42). The classic “graying” of the hair results due to lack of proper pigmentation.
While anemia and pigmentation abnormalities present a significant problem, especially in the beef industry where animal performance and appearance are critical for economic value, the detrimental effects of copper deficiency arise primarily from immune complications. Immune dysfunction in animals has been linked to a deficiency in copper in both *in vivo* and *in vitro* systems. It is well established that copper deficiency leads to neutropenia reducing the amount of neutrophils available to fight infection thereby placing the organism at increased susceptibility for infection (43). Copper-deficient rats and mice, when exposed to an adjuvant challenge, have been shown to have significantly fewer antibody-producing cells and lower antibody titers compared to controls (44, 45). Furthermore, splenic mononuclear cells (MNCs) extracted from copper-deficient rats exhibited reduced IL-2 activity when exposed to the phytohemagglutinin (PHA) adjuvant at a level of 40-50% that of controls. Although these effects have been observed with various cell lines and rodent models, the effect of a copper deficiency on the immune system in the bovine is less-well characterized. Studies of the *in vivo* bovine response to copper deficiency are inconsistent. A recent study examined the effects of a secondary copper deficiency induced by either iron or molybdenum on the immune function of calves. Calves were injected with porcine erythrocytes (PRBC) at two time points throughout the study to induce an inflammatory response. Blood samples were routinely taken to assess humoral immune function by measuring the plasma concentrations of PRBC antibody. *In vitro* lymphocyte blastogenic response was also assessed. Although antibody titers were lower and blastogenic responses were higher in the molybdenum-induced copper-deficient group compared to both the iron-induced copper deficient and control calves, the results were largely inconsistent and varied between trials (69). In a similar study, bovine immune function was evaluated by administering live herpes virus to control and molybdenum-induced copper-deficient calves. Lymphocyte
proliferation responses in the copper-deficient group varied depending on the mitogen stimulus. The findings of the study did demonstrate significant changes in the acute-phase response, which could play a role in altered immune function (68). Alternatively, the increased susceptibility to infection could result from reduced antioxidant activity in lymphocytes. Indeed a recent study reported that activities of copper-dependent proteins cytochrome C oxidase and Cu,Zn Superoxide Dismutase are significantly lower in peripheral blood lymphocytes, neutrophils and macrophages relative to controls (81).

The combination of severe anemia and a tendency for increased susceptibility to infection contributes to an animal’s overall failure to thrive. Indeed, copper-deficient calves have been shown to grow 30% slower than copper-adequate animals despite no differences in food consumption (72). This results in severe economic losses especially in the beef industry where not only primary, but secondary copper deficiency poses a significant problem.

**Current Copper Status Indicators**

Given the severe complications seen with a copper deficiency, the need for an accurate copper status indicator is warranted, especially in the cattle population where copper deficiency is a problem. Currently, liver biopsies represent the most accurate form of testing because the liver is the primary storage site of copper (1). However, the procedure is highly invasive to the animal, time consuming, and requires trained personnel. Instead, serum copper is often used due to the convenience of sampling (1). In bovines with adequate liver copper stores, plasma copper concentrations will remain within normal ranges. The liver will exhaust its copper stores in order to maintain the level of plasma copper in the blood. Accordingly, when liver stores become depleted below 40 ppm, plasma copper levels fall (71). Low plasma copper levels suggest copper deficiency, but by the time they are detected, the animal could already be suffering from the adverse effects (75). A study compared serum and plasma copper levels since the two indicators
are interconvertibly used to assess copper status. Serum copper levels were found to be consistently lower (approximately 67%) to that of plasma values suggesting that a significant amount of copper is lost to the clotting process (79). Furthermore, approximately 34.5% of the animals were diagnosed according to their serum values as being marginally deficient in copper, which was not the case when compared to their plasma copper concentrations (77). Plasma copper provides a more accurate reflection of the copper status of the system. Both indicators however are limited in that both plasma and serum copper concentrations fluctuate with age and gender of the animal, thus limiting the validity of the test (72, 73). Additionally, more than 80% of copper in serum and plasma is bound to the ferroxidase ceruloplasmin, an acute-phase protein that increases under periods of inflammation and stress (80). Inflammation increases plasma copper concentrations and could mask a copper deficiency.

The copper chaperone for Cu, Zn Superoxide Dismutase (CCS) has been shown to be highly responsive to the copper status in mice and rats (7). In copper deficiency, the expression of the intracellular metallochaperone CCS increases in liver and erythrocytes (7, 9). CCS has not yet been characterized in the bovine and a response to inflammatory changes has also not been assessed. The copper chaperone for Cu, Zn superoxide dismutase could serve as a status indicator for copper in the bovine.

**Copper Chaperone for Cu, Zn Superoxide Dismutase**

The copper chaperone for Cu, Zn superoxide dismutase was first discovered in *S. cerevisiae* in 1995 through molecular cloning techniques (46). The initial name was LYS7 as it was proposed to be involved in the conversion of homocitrate → homoaconitate in the lysine metabolism pathway. The LYS7 gene predicted a 249 amino acid protein that shared no significant homology to other known proteins. A LYS7Δ mutant was created in yeast to test the
function of the protein and it was discovered that the LYS7Δ strain exhibited pleiotrophic phenotypes that were not exclusive to a defect in lysine metabolism. This suggested that the function of LYS7 was not limited to lysine biosynthesis (46).

Two years later, in 1997, a hypothesis was proposed that LYS7 was functionally linked to a protein known as Cu, Zn Superoxide Dismutase (SOD1) (32). SOD1 is a cytosolic protein that is abundantly and ubiquitously expressed (47). It exists as a homodimer in the body and each monomer requires both a single zinc atom for structural stability, as well as the redox potential of a copper atom for catalytic function. SOD1 catalyzes the disproportionation of the superoxide ion to form the more stable products molecular oxygen and hydrogen peroxide (47). This reaction is crucial for cellular antioxidant defense to prevent oxidative damage to proteins, lipids, and nucleic acids (48). In the absence of the copper cofactor, SOD1 lacks functional activity. It was proposed that LYS7 served as a metallochaperone to deliver copper to this target protein. The SOD1Δ and LYS7Δ null mutants were both auxotrophic to methionine and lysine when grown in aerobic conditions and lacked SOD1 activity when exposed to the oxidant nitro blue tetrazolium (NBT). In addition, immunofluorescence indicates that both proteins colocalize in the cytosol (32).

Multiple human expressed sequence tag (EST) homologues to LYS7 were discovered, and through sequencing, a 274 amino acid human orthologue protein that shared 28% homology to LYS7 was identified. The human orthologue, when transformed into the mutant LYS7Δ and SOD1Δ yeast strains, failed to restore SOD1 activity in SOD1Δ mutants, but was able to fully restore the activity in LYS7Δ mutants. This demonstrated that the human protein was indeed an orthologue to LYS7. Thus a name more appropriate to its function was given: copper chaperone for Cu, Zn superoxide dismutase (CCS) (32). Was CCS specific to only SOD1, or could another
well characterized chaperone performing the same function? Yeast mutants were transformed with another known metallochaperone, ATOX1, to evaluate the restoration of SOD1 activity. ATOX1 was unable to restore SOD1 function in both the LYS7Δ and SOD1Δ mutants, indicating that CCS was a specific and essential chaperone for SOD1 (32).

The discovery of CCS as metallochaperone to SOD1 led to the hypothesis that CCS could play a role in the fatal neurodegenerative disease familial amyotrophic lateral sclerosis (FALS). Amyotrophic lateral sclerosis (ALS) is characterized by the selective loss of motor neurons located in the spinal cord, brain stem and motor cortex (49). FALS represents 10% of ALS cases, and approximately 25% of these result from positional mutations in SOD1 that confer a toxic “gain-of-function” activity (48, 50). Mutations in SOD1 result in loose folding properties and when CCS loads copper to SOD1, the copper molecule is no longer buried within the protein. Exposure of the copper atom to the cellular environment promotes the interaction of copper with hydrogen peroxide and peroxynitrite—catalyzing the formation of free radicals (50). CCS is ubiquitously expressed and colocalizes with SOD1 in multiple regions in the human central nervous system, particularly in motor neurons and astrocytes (51). The hypothesis was proposed that if the source of copper could be blocked—CCS to the FALS-SOD1 mutant—then the neurodegenerative process could be abrogated. In order for this to be a viable hypothesis, it had to be proven that SOD1 as well as the FALS-SOD1 could bind copper in vivo (50).

This led to a series of investigations to determine the mechanism of copper incorporation into the target protein SOD1. The sequence of human CCS was analyzed and compared to the target protein SOD1 (52). Sequence alignment revealed a 47% homology between CCS and SOD1 from amino acids 86-234 identified as domain II. The first 85 amino acids—(domain I), contained an M XCXXC metal binding motif (where X represents any amino acid) that was also
common and essential in the copper ATPases and the ATOX1/HAH1 metallochaperones. The final domain, domain III, demonstrated no known homology to other proteins, but did contain a putative peroxisomal localization sequence (AHL) at the carboxyl terminus (52). SOD1 exists as a homodimer \textit{in vivo}, but there was increasing evidence that suggested that a heterodimer complex could potentially exist between CCS and SOD1 to coordinate the copper transfer process due to the homologous sequences found in domain II. To test this hypothesis, a series of column binding assays were performed using complete or abrogated CCS constructs (domain I or domain II/III) that were linked to glutathione S-transferase (GST) or to the amino acid histidine. The linked CCS molecules were immobilized on target-specific agarose beads and immersed in a solution containing COS-1 cell lysates that were transiently transfected with the SOD1 gene. Western analysis revealed that the SOD1 protein bound to the full-length CCS protein as well as to the CCS constructs that contained domains II/III. SOD1 did not bind to CCS constructs that only contained domain I, suggesting that the MXCXXC motif was not necessary for SOD1 binding. These results indicated that copper incorporation of SOD1 from CCS involved direct protein-to-protein interaction of a heterodimer complex.

The model of the heterodimer complex was further supported by crystal structure analyses. Lamb et al. (53) solved the initial crystal structure for apo-CCS and revealed that the protein actually presented as a dimer \textit{in vivo}. A detailed investigation of domain II revealed that the domain was similar to SOD1 but lacked the catalytic elements of copper binding (48). The dimer interface amino acid residues of the second domain of CCS shared a 64% homology with the residues that are located on the dimer interface of SOD1 and are highly conserved both structurally and sequentially. Furthermore, the mean change in accessible surface area ($\Delta$ASA) in
CCS was similar to the ΔASA of SOD1, which gave strong evidence that a heterodimer complex can be formed without steric interference between the involved residues (48, 53).

The function of the individual protein domains on CCS was assessed by expressing physiological levels of truncated forms of the yCCS protein (yCCS-dI/II, yCCS-dII/III and yCCS-dIII) into LYS7Δ strains and monitoring SOD1 activity by growing yeast under aerobic conditions (54). As stated previously, yeast lacking SOD1 activity are auxotrophic to methionine and lysine in aerobic conditions. Contrary to expectations, the yCCS-dI/II construct did restore SOD1 activity whereas the yCCS-dII/III construct did, though slightly weaker than the wild type protein. This was a pivotal point in the CCS studies because prior to this knowledge the third domain was regarded as a minor player in the copper delivery process to SOD1. The primary focus was placed on domain I because it contained the MXCXXC copper-binding motif, and on domain II due to the structural similarities to the target protein SOD1. The surprising activity that was observed with the isolated yCCS dII/III constructs led to the hypothesis that the third domain may contain a functional copper-binding site. Sequence analysis of the third domain revealed a putative CXC copper-binding motif that was perfectly conserved in the CCS family between mammals, fungi and insects. Treatment of a peptide representing this domain (Alanine^{216} – Lysine^{249}) with Cu(I) tested the functionality of the CXC motif. Domain III possessed the ability to bind copper, but was it essential for CCS function? A simple experiment proved that mutations of the key cysteine residues in the third domain resulted in a complete abrogation of SOD1 activity. Domain III was absolutely critical for CCS function. Taken together a model was proposed with domains I and III acting in tandem to deliver copper to the target protein SOD1 since they both contained copper-binding motifs (54).
Additional crystal structures studies proved that a heterodimer complex existed between CCS and SOD1 that was stabilized through four strong main-chain hydrogen bonds and that domain III was the factor that delivered copper to the active site of SOD1. The CXC motif of domain III was located adjacent to the active site of the SOD1 complex. This was in contrast to domain I, which was located ~35Å from the SOD1 active site where it would not be in a position to deliver copper to the active site (55).

Compiling all the results from multiple studies led to an intricate copper delivery mechanism model between CCS and SOD1 (Figure 4). The model was based on experiments tested in yeast, but has been extrapolated to eukaryotic systems. The model is a 4-step process that involves first the heterodimeric binding between CCS and SOD1, and through the interaction of distinct cysteine amino acid residues of domain I and III, the copper molecule is delivered to the catalytic site of SOD1 post-translationally through the oxidation and reduction of disulfide bonds (32, 56).

Remarkable progress had been made with CCS but several questions remained. It has been observed that SOD1 has the ability to acquire copper and self-activate in vitro independent of CCS, however under in vivo conditions, this self-activation is not seen. Why is CCS required for in vivo but not in vitro activation? In an elegant study, apo-SOD1 was treated with either Cu(I)-CCS, Cu(I)-GSH, or CuSO4 (57). All three donors had the ability to activate the apo-SOD1 protein, but when the potent copper chelator bathocuproine disulfonate (BCS) was added to solution reducing copper availability to only $10^{-17}$ M of free copper, only the Cu(I)-CCS donor retained the ability to activate the apo-SOD1 protein. The results supported the hypothesis that in vivo, the cellular milieu has an over chelation of copper such that the concentration of
intracellular copper is virtually undetectable giving rise for the necessity for metallochaperones to deliver copper to target proteins.

The compounding evidence shed no doubt that CCS was essential for the activation of SOD1 in yeast, but was it essential in mammals? A CCS⁻/⁻ mouse was created using homologous recombination techniques to inactivate the CCS gene (58). The CCS⁻/⁻ had dramatic effects on SOD1 activity. SOD1 gel activity assays demonstrated a pronounced reduction of SOD1 activity that was observed in all tissues. Metabolic labeling with ⁶⁴Cu proved that the reduction in activity was attributed to the missing catalytic component. However, despite the reduction of activity, there was still retention of 10-20% of wild type SOD1 activity with the exception of liver, which was able to retain 30% activity. Nevertheless, the reduction was severe enough to conclude that CCS was essential for in vivo copper incorporation for efficient activation of the SOD1 enzyme.

A FALS-linked SOD1-mutant mouse model was later created that lacked the CCS gene to test the original hypothesis that if the source of copper could be blocked, the symptoms associated with FALS would be resolved (49). Metabolic labeling with ⁶⁴Cu in the FALS-SOD1 mutants demonstrated that copper was not being incorporated into SOD1. However, contrary to what was expected, mutant FALS-SOD1 retained the ability to induce motor neuron cell death independent of CCS (49). The cure for FALS did not lie in CCS inhibition in an attempt to abrogate copper delivery.

**Copper Chaperone for Superoxide Dismutase as a Potential Copper Status Indicator**

Shortly after the discovery that inhibition of CCS did not prevent disease progression in FALS, a novel function for the protein was proposed. There had been a growing interest in finding a reliable copper status indicator that would reflect accurate measurements of copper concentration since the current copper status indicators were inadequate (8). The focus was
placed on CCS as a potential copper status indicator given that it was essential to activate SOD1. In a pilot study, rats were fed diets with varying copper concentrations. CCS protein expression in erythrocyte and liver samples of rats fed adequate amounts of copper was relatively low. However, in rats fed a copper deficient diet, the CCS protein levels were markedly up-regulated in a dose-responsive manner related to the severity of copper restriction (8). The increase in the abundance of CCS was likely a regulatory mechanism to improve the efficiency of copper scavenging to allow the SOD1 antioxidant capacity to be maintained (9). Copper-deficient rats exhibited a 9-fold increase in CCS protein levels in liver compared to the control group. No changes, however, were observed in mRNA levels between groups suggesting that the increase in protein levels was from a decrease in degradation (9). CCS is regulated by a posttranslational mechanism involving copper. Subsequent studies showed that copper regulates CCS expression by promoting its degradation. The proposed mechanism is that holo-CCS, which would be abundant in a copper-adequate environment, adopts a conformation that is not as stable as the apo-CCS form, which is more predominantly found in a copper-deficient state. The instability of the holo-CCS reduces the lifespan of the protein and it is targeted for degradation by the 26S proteosome (9).

Species-specific differences have been observed in the copper-dependent regulation of CCS. In a study performed by Bertinato et al. (9), CCS protein levels were examined in two liver cell lines maintained in a copper-deficient environment: one from rat and the other from human. The CCS protein levels from the rat cell line gave a more robust 6-fold increase in protein concentration compared to that of the human liver cell line, where a more modest 3-fold increase was observed. The species-specific variations in CCS response to copper status should be taken into consideration.
Although CCS protein levels have been shown to increase in liver samples in a copper deficiency, for a status indicator to be applicable to bovines, a more convenient sampling source is necessary. Two studies were performed to examine CCS erythrocyte expression in mice and rats. Both studies concluded that CCS expression in erythrocytes is responsive to changes in copper status (7, 8). CCS protein levels were significantly higher in the copper-deficient groups compared to controls and expression in erythrocytes was similar to that which was observed in previous studies examining liver cells. The current project proposes to investigate the use of erythrocyte CCS as an indicator of copper status in the bovine.

**Specific Aims**

**Aim 1:** Establish a method to measure bovine erythrocyte CCS levels: To use CCS as a potential status indicator for copper in the bovine, a convenient sampling source is desirable. Red blood cells are readily available and erythrocyte CCS protein has been shown to be reflective of copper status in rats and mice (7).

**Aim 2:** Determine if bovine erythrocyte CCS levels are responsive to copper status: To determine if bovine CCS protein exhibit similar increases in concentration in experimentally induced copper-deficient cattle.

**Aim 3:** Determine if CCS levels are modulated by inflammation: To determine if CCS protein levels are altered under conditions of inflammation as it would limit the validity and use of CCS as a copper status indicator.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcuprein</td>
<td>-high affinity Cu2+ carrier that transports copper to the liver (10)</td>
</tr>
<tr>
<td>Albumin</td>
<td>-Serum protein that acts as a Cu2+ carrier for transport to the liver (11)</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>-Serum ferroxidase that oxidizes ferrous iron to ferric iron, the form of iron bound by transferrin.</td>
</tr>
<tr>
<td>Hephaestin</td>
<td>-Membrane protein that utilizes the redox potential of copper to oxidize ferrous iron to the ferric form for binding to transferring</td>
</tr>
<tr>
<td>Cytochrome C Oxidase</td>
<td>-Terminal enzyme in the electron transport chain that utilizes copper for electron transfer needed for ATP synthesis.</td>
</tr>
<tr>
<td>Cu, Zn Superoxide Dismutase (SOD1)</td>
<td>-Cytosolic protein that functions in antioxidant defense. SOD1 utilizes copper as a cofactor to catalyze the dismutation of the superoxide ion into hydrogen peroxide and molecular oxygen.</td>
</tr>
<tr>
<td>Lysyl Oxidase</td>
<td>-Copper-dependent amine oxidase that catalyzes the post-translational modifications that are critical for the biogenesis of connective tissue (12).</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>-Contains two copper cofactors that catalyze the first enzymatic step in melanin pathway—converting tyrosine to dihydroxyphenylalanine (DOPA), then subsequently to dopaquinone (13).</td>
</tr>
</tbody>
</table>
Table 2-2. NAHMS classification of copper adequacy in the bovine (3)

<table>
<thead>
<tr>
<th></th>
<th>Adequate</th>
<th>Marginal Deficiency</th>
<th>Severe Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Copper (ppm)</td>
<td>&gt;0.65</td>
<td>0.25-0.65</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Liver Copper (ppm on DM basis)</td>
<td>&gt;90</td>
<td>75-90</td>
<td>&lt;75</td>
</tr>
<tr>
<td>Dietary copper (mg/kg)</td>
<td>&gt;10</td>
<td>4.0-9.9</td>
<td>&lt;4.0</td>
</tr>
</tbody>
</table>

Table 2-3. NRC forage analysis of minerals that can lead to copper antagonistic effects (3).

<table>
<thead>
<tr>
<th>Copper Antagonists</th>
<th>Trace Element</th>
<th>Minimal (ppm)</th>
<th>Marginal (1-3)</th>
<th>High (&gt;3)</th>
<th>MTC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molybdenum</td>
<td>&lt;1</td>
<td>1-3</td>
<td>&gt;3</td>
<td>5+</td>
<td></td>
</tr>
<tr>
<td>Sulfur (%DM)</td>
<td>&lt;0.1</td>
<td>0.15-0.20</td>
<td>&gt;0.20-0.30</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Iron (ppm)</td>
<td>50-200</td>
<td>&gt;200-400</td>
<td>&gt;400</td>
<td>1000</td>
<td></td>
</tr>
</tbody>
</table>

*Maximum Tolerable Concentration

Table 2-4. Bovine blood parameters in the classification of anemia (76)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Mild-Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/100ml)</td>
<td>8-12</td>
<td>7.9-4.1</td>
<td>&lt; 4.0</td>
</tr>
<tr>
<td>Erythrocyte count (10^6/mm³)</td>
<td>5.0-8.0</td>
<td>4.9-2.6</td>
<td>&lt; 2.5</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>30-40</td>
<td>29-26</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>
Figure 2-1. Copper transport. 1) Copper absorption through the duodenal enterocyte is mediated either exclusively by CTR1 or in combination with DMT1. 2) Copper import into secretory pathways or export into portal circulation occurs through the Menkes protein ATP7A. 3) In portal circulation, copper is transported to the liver bound to albumin, transcuprein or other small peptides. 4) Once in the liver, copper is transported into the Golgi apparatus by the Wilson Protein ATP7B. In the Golgi, copper is either incorporated into ceruloplasmin in copper adequacy, or is excreted in the bile in conditions of copper excess. 5) Ceruloplasmin accounts for over 80% of the copper present in the plasma. Copper is imported into peripheral cells through CTR1 and is transported to target proteins by the intracellular metallochaperones CCS, HAH1 and COX17.
Figure 2-2. Bovine anatomy of the polygastric system. Food enters the rumen via the esophagus which is the primary site for microbial fermentation. Once adequate fermentation has taken place, food then passes to the reticulum—a temporary holding site before entering the omasum where fluid absorption takes place. Food then continues to the abomasum which is considered the “true stomach”. The digestive enzymes in the abomasum break apart the food molecules before entering the small intestines for absorption.
Figure 2-3. Thiomolybdates sequestering copper. Molybdenum can combine with sulfur to form substances known as thiomolybdates. Thiomolybdates can have up to four degrees of sulfur binding. The higher the degree of sulfur binding, the higher the copper-antagonistic effect.

Figure 2-4. Proposed mechanism for CCS-mediated copper insertion into SOD1. 1) Domain I and III acquire copper from the cellular milieu which induces conformational changes that cause the CCS dimer to dissociate. 2) The CCS monomer then combines with a SOD1 monomer to form a heterodimeric complex through domain II of CCS. The third domain delivers copper to the active site of the SOD1 molecule through oxidation and reduction of disulfide bonds thereby activating the SOD1 protein. 3) The complex then dissociates and CCS scavenger activity is recycled. The activated SOD1 monomer combines with another of its kind to form the active SOD1 dimer.
CHAPTER 3
MATERIALS AND METHODS

Study Designs for Specific Aims I, II and III

For specific aims I and II, bovine blood and liver samples used for this study were obtained from Dr. Jerry Spears (Department of Animal Science, North Carolina State University). Whole blood and liver samples were extracted at harvest (day 490) from weanling heifers and steers that were randomized to one of three diet treatments for 27 weeks: Copper deficient (Cu⁻; n=8), copper adequate (Cu⁺; n = 6), and copper deficient + supplemented with high dietary manganese (Cu⁺+Mn ; n = 7). The composition of the diets met or exceeded the NRC requirements in all nutrients with the exception of copper, which was omitted from the Cu⁻ and Cu⁺+Mn diet treatments. Copper deficiency was further induced by the administration of 2 mg/kg of molybdenum—a potent copper antagonist to the diet in the Cu⁻ and Cu⁺+Mn treated groups. Full diet protocol and experimental design are referenced as follows (S. Hansen, Department of Animal Science, North Carolina State University). For specific aim III, blood and liver samples from 11 heifers were collected at day 0 to establish a baseline measurement of CCS and liver copper before the subcutaneous administration of 2 mL of the Mannheimia haemolytica vaccine. After vaccine administration, daily blood samples (5 mL from jugular vein) were collected in heparinized tubes and placed immediately on ice for storage until analysis. Liver samples were obtained via liver biopsy on day 2 and 4 of the study and were frozen on dry ice and stored -80°C.

Erythrocyte Processing

The erythrocyte processing protocol was modified from Prohaska et al (7). This protocol was applied to whole blood samples for all three sources of blood tested: rat, human and bovine.
The rat and human samples were used for the preliminary detection and optimization studies of CCS. Once an optimized method was established, the bovine samples were analyzed.

Fresh whole blood samples were collected in heparinized tubes and stored at 4°C. One mL of blood was centrifuged at 1000 x g for 5 min at 4°C to pellet the red blood cells. The resultant supernatant was aspirated to remove the plasma and buffy coat; the estimated final red cell volume was recorded. Erythrocytes were re-suspended by gently mixing in 1 mL of Alsever’s solution (Sigma-Aldrich). Cells were centrifuged and washed as above a total of three times. After the final washing, a final red cell volume of ~200 μL remained. Cells were lysed by adding 200 μL of lysis buffer [10mM Tris pH 7.2 and 20 μL of 7X complete mini protease inhibitor cocktail (Roche Diagnostics)]. Cell lysates were centrifuged at 13,000 x g for 10 min at 4°C to remove residual red cell debris. The supernatant was stored at -80°C until analysis.

**Liver Tissue Processing**

Liver samples (0.5-0.85 g) were homogenized in 1 mL of lysis buffer by using a Polytron at setting 3 for 20 s. Liver homogenates were stored at –80°C until analysis.

**Western Blot Analysis of Blood and Liver Cell Lysates**

Proteins were quantified by using the RC DC protein assay (Bio-Rad). Proteins (80 μg) were mixed with Laemmlil buffer and heated to 95°C for 5 min before loading onto a 12% SDS-PAGE gel. Proteins were electrophoretically size-fractionated and transferred to a 0.45 μm nitrocellulose membrane (Optitran; Schleicher & Schuell). Equivalent protein loading and transfer were verified by Ponceau staining. Nonspecific protein binding to the membrane was blocked by incubating the blot for 1 h in blocking buffer [5% nonfat dried milk suspended in Tris-buffered saline, pH 7.4, and 0.01% Tween-20 (TBST)]. Membranes were incubated with rabbit anti-human CCS (Santa Cruz Biotechnology) for 1 h. After primary antibody incubation, the membranes were washed several times with TBST before incubating with the secondary
antibody (horseradish peroxidase-linked donkey anti-rabbit IgG antibody; Amersham Biosciences UK Limited) for 40 min. Membranes were washed with TBST and next with TBS before the final incubation with the chemiluminescence substrate (SuperSignal WestPico; Pierce) for 5 min. Blots were exposed to x-ray film for imaging. Antibodies were subsequently stripped by incubating blot at low pH (25 mM glycine, pH 2.8, 1% SDS) for 5 min. Blots were re-probed with rabbit anti-actin or tubulin antibodies (Sigma-Aldrich) as an indicator of protein loading. Immunoreactive band intensities were quantified by densitometric analysis (Gene Tools; SynGene).

**Statistical Analysis**

All values are expressed as means ± SEM. Statistical analyses were performed using Prism 4.03 (GraphPad) software. Data from Study Aim II were analyzed by unpaired t-test, whereas data from Study Aim III were analyzed by paired t-test. Differences with $P<0.05$ were considered significant.
CHAPTER 4
RESULTS

Detection of CCS in Bovine Erythrocytes

Measurement of bovine CCS by Western blot analysis requires an antibody that cross reacts with the bovine protein. We first tested the immunoreactivity of a commercial antibody (Santa Cruz), raised against full-length human CCS protein. The probability seemed high that this polyclonal antibody would recognize bovine CCS given that bovine and human CCS are 88% orthologous (Figure 4-1). Western analysis of bovine liver lysates revealed a predominant immunoreactive band migrating with an apparent molecular mass of 30-35 kDa (Figure 4-2), which is similar to the calculated molecular mass of bovine CCS. This indicated that the antibody did cross-react with the bovine species. Subsequent western analysis of bovine erythrocytes revealed two bands migrating between 30-35 kDa. Signal intensities increased as protein concentration increased (Figure 4-3). Bovine liver and human erythrocyte lysates were used as positive controls for bovine erythrocyte analysis and revealed bands of similar apparent molecular masses.

Bovine CCS Expression Increases in Copper Deficiency

The experimental diets reduced the copper content in the liver and plasma by 97% and 86%, respectively, compared to initial values. These animals were then classified as being copper deficient according to the NRC guidelines (Table 5-1). Erythrocyte and liver samples were obtained from copper-normal and copper-deficient animals and were evaluated for CCS content through western blot analysis. The Cu⁻ and Cu⁻Mn groups were combined into one copper-deficient group since they did not differ with respect to copper deficiency. Immunoblot analysis revealed that CCS levels were higher in the copper-deficient groups for both erythrocyte (Figure 4-4A) and liver (Figure 4-5A) samples. To verify that the increase in CCS protein levels were
not due to differences in lane loading, the membranes were stripped and reprobed with antibodies against structural proteins. An anti-actin antibody was used for lane loading control in erythrocyte samples, whereas an anti-tubulin antibody was used for liver. Neither actin nor tubulin proteins exhibited changes in copper-deficient samples compared to controls. Quantification by densitometric analysis of erythrocyte and liver CCS protein content revealed a statistically significant 63% (Figure 4-4B) and 44% (Figure 4-5B) increase in the copper-deficient animals compared to their respective controls ($P< 0.05$).

**Bovine CCS Levels do not Change with Inflammation**

Animals were classified as copper normal based on liver copper concentrations prior to the vaccine challenge (Table 5-2). Administration of the *Mannheimia haemolytica* vaccination at baseline (Day 0) induced a positive inflammatory response as indicated by a 75% increase in ceruloplasmin activity on day 4 (Figure 4-6). This increase is similar to the 70-97% increase in ceruloplasmin activity in turpentine-induced inflammation in bovine (80). CCS protein levels were measured in liver and erythrocyte samples taken at baseline and on the final day of treatment (Day 4). Figure 4-7A demonstrates that erythrocyte CCS protein levels do not change from baseline to day 4 under inflammatory conditions. Similar results are observed in the liver (Figure 4-7C). Densitometry and statistical analysis confirmed that CCS protein levels exhibited no significant change in erythrocytes or liver (Figure 4-7B and D, respectively). Similar lane loading was verified through actin and tubulin Western analyses. Taken together the results demonstrate that bovine erythrocyte and liver CCS levels increase in copper deficiency and do not change in a 4-day period after a vaccine-induced inflammatory stimulus.
Figure 4-1. Alignment of human and bovine CCS. Sequence alignment of bovine and human CCS. The human CCS sequence is positioned on the top line (hCCS) while the bovine protein sequence is found below (bCCS). Amino acid markers are listed to the right and represent hCCS. Shaded regions represent identical amino acids and the boxed amino acids represent the conserved motifs MXCXXC and CXC that are required for copper binding. The lines correspond with the protein domains of hCCS and the domain legend is shown below. Sequence BLAST analysis reveals an 88% homology between human and bovine CCS protein sequence. The commercial anti-CCS antibody from Santa Cruz was synthesized against full length human CCS (1-274).
Figure 4-2. Anti-human CCS antibody cross reacts with bovine liver CCS. Proteins from bovine liver lysates were electrophoretically size fractionated on 12% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with rabbit anti-human CCS primary antibody and then subsequently probed with the anti-rabbit HRP-linked secondary antibody. Chemiluminescence revealed a predominant CCS-immunoreactive band around 30-35 kDa.

Figure 4-3. Anti-human CCS detects bovine erythrocyte CCS in a dose-dependent manner. The anti-human CCS antibody detects bovine CCS and is sensitive given the level of protein loaded. Beef liver lysates (BLL) and human blood (HB) were used as positive controls.
Table 4-1. Liver and plasma copper values at harvest (day 490). Liver and plasma samples were collected from animals in each experimental group and analyzed for copper content using flame atomic absorption spectroscopy. Data represent ppm for liver and plasma copper levels ± SEM and were generously obtained from S. Hansen and Dr. Jerry Spears (Department of Animal Science, North Carolina University).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cu⁺ (n=6)</th>
<th>Cu⁻ (n=8)</th>
<th>Cu⁺Mn (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Copper mg/kg DM</td>
<td>206 ± 31.5</td>
<td>6.9 ± 0.05</td>
<td>5.2 ± 0.34</td>
</tr>
<tr>
<td>Plasma copper mg/L</td>
<td>1.25 ± 0.08</td>
<td>0.23 ± 0.005</td>
<td>0.11 ± 0.007</td>
</tr>
</tbody>
</table>

Figure 4-4. Copper deficiency increases CCS expression in bovine erythrocytes. Weanling calves were fed a copper-adequate (Cu⁺) (n = 6) or a copper-deficient diet (Cu⁻) (n = 15) for 27 weeks after weaning. A) Proteins from erythrocyte homogenates were electrophoretically separated by using a 12% SDS-PAGE, transferred to nitrocellulose, and analyzed for CCS by Western blotting. Positions and masses of molecular weight markers (in kDa) are show to the right. To visualize lane loading, the blot was stripped and re-probed for actin. Representative samples from each group are shown. B) Band intensities for erythrocyte CCS were quantified by densitometry for all 21 samples. Data represent the mean ± SEM. Asterisk denotes statistical significance (P<0.05)
Figure 4-5. CCS expression increases in bovine liver in copper deficiency. Weanling calves were fed a copper-adequate (Cu+) (n = 6) or a copper-deficient diet (Cu-) (n = 15) for 27 weeks after the weaning period. A) Proteins from liver homogenates were electrophoretically separated by using a 12% SDS-PAGE, transferred to nitrocellulose, and analyzed for CCS by Western blotting. Positions and masses of molecular weight markers (in kDa) are show to the right. To visualize lane loading, the blot was stripped and re-probed for tubulin. B) Band intensities for liver CCS were quantified by densitometry for all 21 samples. Data represent the mean ± SEM. Asterisk denotes statistical significance (P<0.05)

Table 4-2. Liver concentrations at baseline. Liver samples were collected from each animal at baseline prior to vaccine administration and analyzed for copper content using the inductively coupled plasma mass spectroscopy (ICP-MS).

<table>
<thead>
<tr>
<th>Animal</th>
<th>Liver Copper [mg/kg DM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>91</td>
</tr>
<tr>
<td>2</td>
<td>173</td>
</tr>
<tr>
<td>3</td>
<td>111</td>
</tr>
<tr>
<td>4</td>
<td>128</td>
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<tr>
<td>7</td>
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<tr>
<td>8</td>
<td>200</td>
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Figure 4-6. Ceruloplasmin activity increases with vaccination. A *Mannheima haemolytica* (One Shot®) vaccination was administered to 11 yearling heifers at baseline (Day 0). Plasma was collected daily through to day 4 and analyzed for ceruloplasmin activity using the ELISA method. Results represent the least square means (n = 10) in mg/dl ± SEM. Data based on PROC MIXED analysis. Asterisks denote significance p < 0.01. Data obtained from Dr. John Arthington (Range Cattle Research and Education Center, University of Florida).
Figure 4-7. Bovine liver and erythrocyte CCS expression does not change in inflammation. A *Mannheima haemolytica* (One Shot®) vaccination was administered to 11 yearling heifers at baseline (Day 0). Liver extracts were collected at baseline, day 2, and day 4 of treatment and erythrocyte extracts were collected for a period of 5 days (baseline to day 4). CCS protein expression was assessed. A) CCS expression was measured in erythrocyte lysates at baseline and day 4 by Western blot analysis (top panel). Representative blots from each group are shown. The membrane was subsequently stripped and re-probed with anti-Actin as a lane loading control (bottom panel). B) Relative intensities of erythrocyte CCS levels at baseline and Day 4 were quantified by densitometry. Data are representative of the mean ± SEM. C) Western analysis of baseline liver CCS protein expression was compared to day 4 CCS levels (top panel). The membrane was then stripped and re-probed with anti-tubulin antibody to verify equal lane loading (bottom panel) D) Graphical representation of densitometric analysis of liver CCS levels at baseline and Day 4. Data represent the mean ± SEM.
CHAPTER 5
DISCUSSION

The increase in bovine erythrocyte and liver CCS levels under conditions of copper deficiency is similar to findings of previous studies of copper-deficient rats and mice (7, 8). However, the 0.63- and 0.44-fold increase in bovine erythrocyte and liver CCS, respectively, was much less than the 7.2- and 9.6-fold increase in CCS protein levels in erythrocytes and liver in copper-deficient rats (8). Given the severity of the copper-deficient state of these animals based on NRC reference standards, the nominal increase in CCS levels suggests that bovine erythrocyte CCS would not be a sensitive copper status indicator in detecting a moderate copper deficiency. In the beef cattle industry, it would be important to detect a copper deficiency as early as possible so that intervention measures could be initiated before problems occur that would lead to an economic loss.

There are several potential explanations as to why the bovine CCS response to copper deficiency is not as robust as with rodents. The first would be the sequence differences. The bovine CCS protein sequence is shorter (216 aa) compared to its orthologues in humans and rodents (274 aa) and does not possess the CXC motif in domain III that is critical for CCS function. Schmidt et al. (54) reported that when minor amino acid mutations were introduced in the CXC motif of the third domain, CCS function was completely abrogated. The fact that bovine CCS can function without this domain suggests that the bovine species employs an alternative mechanism for CCS regulation and stability and may provide an explanation as to why the bovine CCS protein is not as responsive to copper deficiency. The question, however, still remains as to how SOD1 can be activated without the CXC metal binding motif in CCS. Recently, an independent CCS mediated-SOD1 activation mechanism has been proposed based on the evidence demonstrated in CCS -/- mice that retained a reduced, but significant form of...
SOD1 activity (58). The enzyme glutathione peroxidase has been proposed to exhibit compensatory activity to deliver copper to SOD1 when CCS is lacking. This alternative pathway of copper incorporation into SOD1 could play a more prominent role in the bovine and warrants further investigation (59).

Another potential explanation for the reduced bovine CCS response to a copper deficiency could be the cell type examined. Erythrocyte CCS was selected to represent a convenient sampling source. However, mature erythrocytes are quite unique in that they do not possess a nucleus or organelles and are consequently incapable of de novo protein synthesis. Therefore, after the orthochromatic erythroblast stage of erythropoiesis when enucleation occurs, CCS protein levels are presumably fixed (60). Though the nature of the erythrocyte is quite exceptional in that these cells lack the mechanics for protein synthesis, the erythrocyte CCS can still be evaluated as a potential copper status indicator because the regulatory mechanism and stability of CCS is mainly post transcriptional. As previously reported, the increase in CCS protein levels observed in a copper deficiency occurs through a decrease in degradation rather than an increase in protein synthesis. Bertinado et al. (9) proposed that a copper-loaded CCS adopts a conformation that is less stable and increases the rate of degradation. Therefore, in a copper-deficient state, CCS would be mainly in the apo-form increasing the stability of the protein to promote maximal copper scavenger potential. This implies that the inability of de novo protein synthesis of the erythrocyte cell system should not be a primary factor as to why a reduced response was observed with bovine erythrocyte CCS to a copper deficiency. Furthermore, the erythrocyte cell system was also used to evaluate CCS in rodents and potent responses to a copper deficiency were observed (9). In contrast, bovine liver cells do possess a nucleus to support de novo synthesis of CCS; yet liver CCS exhibited similar minor increases in
protein levels. Taken together, the nominal increases in CCS protein levels in response to a copper deficiency is most likely attributed to the protein sequence differences as discussed previously.

In erythrocytes, CCS presented as a double band at the 30-35 kDa marker, whereas in liver this doublet was not present suggesting that bovine erythrocyte CCS is slightly different from the liver form of the protein. It is possible that the erythrocyte form of the bovine CCS protein exhibits post-translational modifications such as glycosylation, phosphorylation or acetylation. Also, it is possible that the lower band could represent a degradation product or splice variant.

The findings of this study demonstrate that CCS protein levels likely do not change under conditions of inflammation. The inflammatory model selected was based on a pilot study that tested the inflammatory response in the bovine system of two commonly used vaccinations in the beef industry: the One Shot© (Mannheimia haemolytica) and UltraBac 7© (multiple Clostridium strains) (Dr. John Arthington, University of Florida, unpublished results). Ceruloplasmin, an acute phase protein, was measured for a period of 21 days to determine the degree of inflammation. The use of acute phase serum proteins as markers for non-specific inflammation are well supported in the literature (12). Animals that were vaccinated with the One Shot© vaccine had greater ceruloplasmin concentrations compared to saline-injected controls and UltraBac7©-treated animals. In addition, the acute-phase response generated by this vaccine gave earlier peak responses (Day 3 and Day 4 of treatment) compared to the UltraBac 7 ©. Based on these results, the One Shot© vaccine was selected to induce an inflammatory response and a 5-day study design was deemed sufficient to observe the maximal acute-phase response in this study. Although this time frame has been proven to produce a potent acute phase response, and inflammation was confirmed by the significant increase in ceruloplasmin activity, consistent
with the pilot study, it is not known whether 5 days is sufficient to evaluate the effect of inflammation on CCS. Inflammation is a major limitation with serum and plasma copper as a status indicator because the majority of serum copper is bound to ceruloplasmin, which increases during inflammation. The increase in ceruloplasmin, as well as other acute phase proteins under inflammatory conditions is quite rapid and usually occurs within 24 h of infection. Therefore a five-day model should be sufficient to capture the acute inflammatory state of the system. Furthermore, another study evaluated the effects of inflammation on erythrocyte copper using ICP-MS and found that though serum copper increased as expected, erythrocyte copper concentrations did not change (61). It is plausible to assume that since there were no changes in erythrocyte copper, that the copper containing enzymes in the erythrocyte such as CCS would also not exhibit any changes. Liver CCS would be more susceptible to changes in protein levels since both transcriptional as well as the post-transcriptional modifications of CCS are possible, yet no changes in protein levels were observed.

Bovine erythrocyte CCS levels increases in copper deficiency and could be used as a copper status indicator as it is represents a convenient sampling source. Moreover CCS levels do not vary with inflammation—a limitation with many current indicators of copper status. The level of response to copper deficiency, however, is not as robust as that seen in rodents, indicating that erythrocyte CCS may not readily detect a moderate copper deficiency in cattle.
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BIOGRAPHICAL SKETCH

Joeva Hepburn was born in Kingston, Jamaica in 1983. Joeva completed her B.S. in food science and human nutrition at the University of Florida in 2005 with a specialization in dietetics and a minor in French. Currently, Joeva is completing her master's degree in food science and human nutrition at the University of Florida through a combined program in which she will also fulfill the Dietetic Internship requirements (MS-DI). Joeva has always had a profound interest in nutrition especially from the research perspective. In 2006, Joeva worked with Dr. Gail Kauwell in the food science and human nutrition department studying folate metabolism using the microbiological assay. Currently, Joeva is working with Dr. John Arthington and Dr. Mitchell Knutson evaluating a copper chaperone protein and its applications to the bovine system. After graduation, Joeva plans to pursue her PhD degree in biochemistry and molecular biology through the College of Medicine at the University of Florida.